G. S. Gupta

Animal Lectins: Form, Function and Clinical Applications

Volume 1

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Foreword I

Lectins are typically carbohydrate-binding proteins that are widely distributed in Nature. With the growing interest in the field of glycobiology, the body of research related to animal lectins has grown at an explosive rate, particularly in the past 25 years. However, the Lectinology field is still relatively young, nascent, and evolving. "**Animal Lectins: Form, Function and Clinical Applications**" presents the most up-to-date analysis of these carbohydrate - binding, and potentially lifesaving proteins in two comprehensive volumes. Lectionology is an exciting area of research that has helped immensely in our understanding of host-pathogen interactions. Importantly, C-type lectins can act as pattern-recognition receptors (PRR) that sense invading pathogens.

The interactions between lectins and carbohydrates have been shown to be involved in diverse activities such as opsonization of microbes, cell adhesion and migration, cell activation and differentiation, and apoptosis. Developments in the area of lectin research has opened a new aspect in studying the immune system, and at the same time, provided new therapeutic routes for the treatment and prevention of diseases.

This present book on animal lectins discusses the biochemical and biophysical properties of animal lectins at length along with their functions in health and diseases. Importantly, the potential interrelationships between lectins of the innate immune system and latent viruses that reside within host cells, sometimes integrated into the genome have been beautifully highlighted. These interactions help to explain autoimmune diseases and shed light on the development of cancer diagnostics. The present book on animal lectins presents new insights into the biological roles of most animal lectins, including their role in prevention of infections through innate immunity. The contents of "**Animal Lectins: Form, Function and Clinical Applications**" provide functional explanation for the enormous diversity of glycan structures found on animal cells. There are still several other areas wherein lectins and their specificities are not well defined and the biological functions of the interactions remain elusive, thereby underscoring the need for further research. The book offers novel ideas for students of Immunology, Microbiology, as well as young researchers in the area of Biochemistry. I congratulate the authors in completing this truly enormous task.

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Foreword II

Lectins are proteins that bind to soluble carbohydrates as well as to the functional groups of carbohydrate chains that are part of a glycoprotein or glycolipid found on the surfaces of cells. Lectins are known to be widespread in nature and play a role in interactions and communication between cells typically for recognition. Carbohydrates on the surface of one cell bind to the binding sites of lectins on the surface of another cell. For example, some bacteria use lectins to attach themselves to the cells of the host organism during infection. Binding results from numerous weak interactions which come together to form a strong attraction. A lectin usually contains two or more binding sites for carbohydrate units. In addition, the carbohydrate-binding specificity of a certain lectin is determined by the amino acid residues that bind to the carbohydrates. Most of the lectins are essentially nonenzymic in action and nonimmune in origin. They typically agglutinate certain animal cells and/or precipitate glycoconjugates. Plant lectins are highly resistant to breakdown from heat or digestion. They provide a defense for plants against bacteria, viruses, and other invaders, but can create problems for humans. In animals, lectins regulate the cell adhesion to glycoprotein synthesis, control protein levels in blood, and bind soluble extracellular and intracellular glycoproteins. Also, in the immune system, lectins recognize carbohydrates found specifically on pathogens or those that are not recognizable on host cells. Embryos are attached to the endometrium of the uterus through L-selectin. This activates a signal to allow for implantation. *E. coli* are able to reside in the gastrointestinal tract by lectins that recognize carbohydrates in the intestines. The influenza virus contains hemagglutinin, which recognizes sialic acid residues on the glycoproteins located on the surface of the host cell. This allows the virus to attach and gain entry into the host cell. Clinically, purified lectins can be used to identify glycolipids and glycoproteins on an individual's red blood cells for blood typing.

The journey of *Animal Lectins: Form, Function, and Clinical Applications*, essentially the Encyclopedia of Animal Lectins, starts with an introductory chapter on lectin families, in general, followed by specific animal lectin families, such as intracellular sugar-binding ER chaperones, calnexin and calreticulin; the P-type lectins working in the endocytic pathway, the lectins of ERAD pathway, and the complex mannose-binding ERGIC-53 protein and its orthologs circulating between the ER, ERGIC, and the Golgi apparatus; and the fairly small galectins that are synthesized in the cytosol but may be found at many locations. Chapters on R-type lectin families, pentraxins, siglecs, C-type lectins, regenerating gene family, tetranectin group of lectins, ficolins, F-type lectins, and chi-lectins have covered the up-to-date literature on the subject and contain illustrations of their structures, functions, and clinical applications. The emerging group of annexins as lectins has been given a place as a separate family.

The C-type lectins comprising 17 subfamilies such as collectins, selectins, NK cell lectin receptors, latest discoveries of lectins on dendritic cells and others form the backbone of Volume 2. The chapters are well written, although there is variability on how they are focused. Most of the chapters focus on lectin structures, functions, their ligands, and their medical

relevance in terms of diagnosis and therapy. The journey ends with five reviews on clinical applications of lectins with a survey of literature on endogenous lectins as drug targets. This may reflect state of the art in each area or the interests of the author(s).

In post-genomic years, with the human genome sequence at hand, a complete overview of many human lectin genes has become available, as illustrated by C-type lectin domain 3D structures having as little as 30% amino acid sequence similarity. The major effort in the future will be on elaboration of similar studies on other families of lectins with carbohydrate specificities and in vivo lectin–ligand interactions. In this respect, further writings will bring the reader to the forefront of knowledge in the field. Thus, on thirst of learning about animal recognition systems, *Animal Lectins: Form, Function, and Clinical Applications* is an excellent reference book for those studying biochemistry, biotechnology, and biophysics with a specialization in the areas of immunology, lectinology, and glycobiology as well as for pharmacy students involved in drug discoveries through lectin–carbohydrate interactions. Both novice and advanced researchers in biomedical, analytical, and pharmaceutical fields need to understand animal lectins.

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Preface

Lectins are phylogenetically ancient proteins that have specific recognition and binding functions for complex carbohydrates of glycoconjugates, that is, of glycoproteins, proteoglycans/glycosaminoglycans, and glycolipids. They occur ubiquitously in nature and typically agglutinate certain animal cells and/or precipitate glycoconjugates without affecting their covalent linkages. Lectins mediate a variety of biological processes, such as cell–cell and host–pathogen interactions, serum–glycoprotein turnover, and innate immune responses. Although originally isolated from plant seeds, they are now known to be ubiquitously distributed in nature. The successful completion of several genome projects has made amino acid sequences of several lectins available. Their tertiary structures provide a good framework upon which all other data can be integrated, enabling the pursuit of the ultimate goal of understanding these molecules at the atomic level. With growing interest in the field of glycobiology, the function–structure relations of animal lectins have increased at an explosive rate, particularly in the last 20 years. Since lectins mediate important processes of adhesion and communication both inside and outside the cells in association with their ligands and associated co-receptor proteins, there is a need of a reference book that can describe emerging applications on the principles of structural biology of animal lectins at one point. No book has ever described in a coordinated fashion structures, functions, and clinical applications of 15 families of animal lectins presently known. Therefore, with the increasing information on animal lectins in biomedical research and their therapeutic applications, writing of a comprehensive document on animal lectins and associated proteins in the form of *Animal Lectins: Form, Function, and Clinical Applications* has been the main objective of the present work. The entire manuscript has been distributed into two volumes in order to produce an easily readable work with easy portability. Volume 1 comprises most of the superfamilies (Chaps. 1–21) of lectins, excluding C-type lectins or C-type-lectin-like domain. In volume 2, we have mainly focused on C-type lectins, which have been extensively studied in vertebrates with wider clinical applications (Chaps. 22–46).

Animal Lectins: Form, Function, and Clinical Applications reviews the current knowledge of animal lectins, their ligands, and associated proteins with a focus on their structures and functions, biochemistry and patho-biochemistry (protein defects as a result of disease), cell biology (exocytosis and endocytosis, apoptosis, cell adhesion, and malignant transformation), clinical applications, and their intervention for therapeutic purposes. The book emphasizes on the effector functions of animal lectins in innate immunity and provides reviews/chapters on extracellular animal lectins, such as C-type lectins, R-type lectins, siglecs, and galectins, and intracellular lectins, such as calnexin family (M-type, L-type, and P-type), recently discovered F-box lectins, ficolins, chitinase-like lectins, F-type lectins, and intelectins, mainly in vertebrates. The clinical significance of lectin–glycoconjugate interactions has been exemplified by inflammatory diseases, defects of immune defense, autoimmunity, infectious diseases, and tumorigenesis/metastasis, along with therapeutic perspectives of novel drugs that interfere with lectin–carbohydrate interactions.

Based on the information gathered on animal lectins in this book, a variety of medical and other applications are in the offing. Foremost among these is the lectin-replacement therapy for patients suffering from lectin deficiency defects. We have pointed out the advancements of

such studies where such progress has been made. Other uses in different stages of development are antibacterial drugs; multivalent hydrophobic carbohydrates for anti-adhesion therapy of microbial diseases; highly effective inhibitors of the selectins for treatment of leucocyte-mediated pathogenic conditions, such as asthma, septic shock, stroke, and myocardial infarction; inhibitors of the galectins and other lectins involved in metastasis; and application of lectins for facile and improved disease diagnosis. Recent advances in the discovery of M6PR-homologous protein family (Chap. 5), lectins of ERAD pathway, F-box proteins and M-type lectins (Chap. 6), and mannose receptor–targeted drugs and vaccines (Chaps. 8 and 46) can form the basis of cutting-edge technology in drug delivery devices.

Based on the structures of animal lectins, classified into at least 15 superfamilies, C-type lectins and galectins are the classical major families. Galectins are known to be associated with carcinogenesis and metastasis. Galectin-3 is a pleiotropic carbohydrate-binding protein, involved in a variety of normal and pathological biological processes. Its carbohydrate-binding properties constitute the basis for cell–cell and cell–matrix interactions (Chap. 12) and cancer progression (Chap. 13). Studies lead to the recognition of galectin-3 as a diagnostic/prognostic marker for specific cancer types, such as thyroid and prostate. In interfering with galectin–carbohydrate interactions during tumor progression, a current challenge is the design of specific galectin inhibitors for therapeutic purposes. Anti-galectin agents can restrict the levels of migration of several types of cancer cells and should, therefore, be used in association with cytotoxic drugs to combat metastatic cancer (Chap. 13). The properties of siglecs that make them attractive for cell-targeted therapies have been reviewed in Chaps. 16, 17, and 46.

F-type domains are found in proteins from a range of organisms from bacteria to vertebrates, but exhibit patchy distribution across different phylogenetic taxa, suggesting that F-type lectin genes have been selectively lost even between closely related lineages, thus making it difficult to trace the ancestry of the F-type domain. The F-type domain has clearly gained functional value in fish, whereas the fate of F-type domains in higher vertebrates is not clear, rather it has become defunct. Two genes encoding three-domain F-type proteins are predicted in the genome of the opossum (*Monodelphis domestica*), an early-branching mammal. There is plenty of scope to discover F-type lectins in mammalian vertebrates (Chap. 20). Since the reports on the C-reactive protein (CRP) as a cardiovascular marker (Chap. 8), novel biomarkers in cardiovascular and other inflammatory diseases have emerged in recent years. The substantial knowledge on CRP is now being complemented by new markers such as YKL-40, a member of chi-lectins group of CTLD (Chap. 19). The YKL-40 (chitinase-3-like protein 1, or human cartilage glycoprotein-39) displays a typical fold of family 18 glycosyl hydrolases and is expressed and secreted by several types of solid tumors, including glioblastoma, colon cancer, breast cancer, and malignant melanoma. Chitinase-3-like protein 1 was recently introduced into clinical practice; yet its application is still restricted.

In volume 2, we have mainly focused on C-type lectins, which have been extensively studied in vertebrates. A C-type lectin is a type of carbohydrate-binding protein domain that requires calcium for binding interactions in general. Drickamer et al. classified C-type lectins into seven subgroups (I to VII) based on the order of the various protein domains in each protein. This classification was subsequently updated in 2002, leading to seven additional groups (VIII to XIV). A further three subgroups (XV to XVII) were added recently. The C-type lectins share structural homology in their high-affinity carbohydrate recognition domains (CRDs) and constitute a large and diverse group of extracellular proteins that have been extensively studied. Their activities have been implicated as indispensable players in carbohydrate recognition, suggesting their possible application in discrimination of various correlative microbes and developing biochemical tools. The C-type lectins, structurally characterized by a double loop composed of two highly conserved disulfide bridges located at the bases of the loops, are believed to mediate pathogen recognition and play important roles in the innate immunity of both vertebrates and invertebrates. A large number of these proteins

have been characterized and more than 80 have been sequenced. Recent data on the primary sequences and 3D structures of C-type lectins have enabled us to analyze their molecular evolution. Statistical analysis of their cDNA sequences shows that C-type-lectin-like proteins, with some exceptions, have evolved in an accelerated manner to acquire their diverse functions.

The C-type lectin family includes the monocyte mannose receptor (MMR), mannose-binding lectin (MBL), lung surfactant proteins, ficolins, selectins, and others, which are active in immune functions and pathogen recognition (Chaps. 23–34 and 41–45). Several C-type lectins and lectin-like receptors have been characterized that are expressed abundantly on the surface of professional antigen-presenting cells (APCs). Dendritic cells (DCs) are equipped with varying sets of C-type lectin receptors that help them with the uptake of pathogens. Important examples are langerin, DC-SIGN, DC-SIGNR, DCAR, DCIR, dectins, DEC-205, and DLEC (Chaps. 34–36). DCs are key regulators in directing the immune responses and, therefore, are under extensive research for the induction of antitumor immunity. They scan their surroundings for recognition and uptake of pathogens. Intracellular routing of antigens through C-type lectins enhances loading and presentation of antigens through MHC class I and II, inducing antigen-specific $CD4^+$ and $CD8^+$ T-cell proliferation and skewing T-helper cells. These characteristics make C-type lectins interesting targets for DC-based immunotherapy. Extensive research has been performed on targeting specific tumor antigens to C-type lectins, using either antibodies or natural ligands such as glycan structures. In Chaps. 34–36, we have presented the current knowledge of DC receptors to exploit them for antitumor activity and drug targeting in the near future (Chap. 46).

The monocyte mannose receptor (MMR) or the mannose receptor (MR) (CD206) is a member of the Group VI C-type lectins along with ENDO180, DEC205, and the phospholipase A2 receptor. Expressed on a broad range of cell types, including tissue macrophages and various epithelial cells, the MMR is active in endocytosis and phagocytosis. It is also thought to be involved in innate immunity, though its exact role remains unclear. Structurally, MMR is a complex molecule, which has been reviewed as an R-type lectin in volume 1 (Chap. 15) and as a C-type lectin in volume 2 (Chap. 35). Further research is required to fully understand the function of MMR. In addition, the Reg family constitutes an interesting subset of the C-type lectin family. The Reg family members are small, secreted proteins, which have been implicated in a range of physiological processes such as acute phase reactants and survival/growth factors for insulin-producing pancreatic β-cells, neural cells, and epithelial cells of the digestive system (Chap. 39). The C-type lectin DC-SIGN is unique in the regulation of adhesion processes, such as DC trafficking and T-cell synapse formation, besides its well-studied function in antigen capture. In particular, the DC-SIGN and associated homologues contribute to the potency of DC to control immunity (Chaps. 36 and 46). There is always significant interest in the development of drug and antigen delivery systems via the oral route due to patient compliance and acceptability. The presence of DCs with knowledge of associated receptors in the gastrointestinal tract offers principles of methodology for the development of oral vaccines (Chap. 46).

The search of the database of NCBI revealed that the C-type lectins attract much more attention, which resulted in recent discoveries of novel groups of lectins (Groups XV–XVII; Chap. 40). The clinical applications of C-type lectins have been exemplified in Chaps. 42–46. Although a variety of lectins have enabled greater insight into the diversity and complexity of lectin repertoires in vertebrates in two volumes, the nature of the protein–carbohydrate interactions and the potential mechanisms of different functions for invertebrate lectins are under intense investigation. Future progress will elucidate the contribution of different lectin families and their cross talk with each other or with other molecules with respect to mounting protective immune responses in invertebrates and vertebrates.

MBL as a reconstitution therapy in genetically determined MBL deficiency has advanced significantly. Since the genetically determined MBL deficiency is very common and can be

associated with increased susceptibility to a variety of infections, the potential benefits of MBL reconstitution therapy still need to be evaluated. In a phase I trial on MBL-deficient healthy adult volunteers, MBL did not show adverse clinical effects (Chap. 23). SP-A and SP-D have been recently categorized as "Secretory Pathogen Recognition Receptors." Treatment with a recombinant fragment of human SP-D consisting of a short collagen-like stalk (but not the entire collagen-like domain of native SP-D), neck, and CRD inhibited development of emphysema-like pathology in SP-D-deficient mice (Chaps. 24, 25, and 43). Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited nephropathy, affecting over 1:1000 of the population worldwide. It is a systemic condition with frequent hepatic and cardiovascular manifestations in addition to the progressive development of fluid-filled renal cysts that eventually result in loss of renal function in the majority of affected individuals. The cysts that grow in the kidneys of the majority of ADPKD patients are the result of mutations within the genes *PKD1* and *PKD2* that code for polycystin-1 (PC-1) and PC-2, respectively (Chap. 45). The annexins or lipocortins are a multigene family of proteins that bind to acidic phospholipids and biological membranes. Some of the annexins bind to glycosaminoglycans (GAGs) in a Ca^{2+}-dependent manner and function as recognition elements for GAGs in extracellular space. The emerging groups of C-type lectins include layilin, tetranectin, and chondrolectin (Group VIII of CTLD) (Chap. 40) and CTLD-containing protein - CBCP in Group XVII. Fras1, QBRICK/Frem1, Frem2, and Frem3 form the family of Group XVII (Chap. 41).

There is plenty of scope to discover lectins in invertebrates and amphibians which offer novel biomaterials useful in therapeutics, with a hope that the list of native lectins as well as genetically modified derivatives will grow with time. Thus, understanding animal lectins and the associated network of proteins is of high academic value for those working in the field of protein chemistry and designing new drugs on the principle of protein–carbohydrate or protein–protein interactions. Refined information on the sites of interactions on glycoproteins in toto with lectins is the subject of future study. Efforts are being made to develop an integrated knowledge-based animal lectins database together with appropriate analytical tools. Thus, *Animal Lectins: Form, Function, and Clinical Applications*, the Encyclopedia of Vertebrate Lectins, is unique in its scope and differs from earlier publications on animal lectins. It is more than lectinology and is suitable to the students and researchers working in the areas of biochemistry, glycobiology, biotechnology, biophysics, microbiology and immunology, pharmaceutical chemistry, biomedicine, and animal sciences in general.

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Abbreviation

AA4	murine homologue of human C1qRp
AAA	*Anguilla Anguilla* Agglutinin
AAA	abdominal aortic aneurysm
AAL	aleuria aurantia lectin
ABPA	allergic bronchopulmonary aspergillosis
ACS	acute coronary syndrome
Ad/AD	Alzheimer's disease
ADAMTS	a disintegrin and metalloproteinase with thrombo-spondin motifs
ADPKD	autosomal dominant polycystic kidney disease
AF	atrial fibrillation
AFP	antifreeze polypeptide
AGE	advanced glycation end product
AICL	activation-induced C-type lectin
ALI	acute lung injury
AM	alveolar macrophage
AM	adrenomedullin
AML	acute myeloid leukemia
AMφ	alveolar macrophage
AP	adaptor protein
APC	antigen-presenting cell
APLEC	antigen presenting lectin-like receptor complex
APR	acute-phase response
ARDS	acute-RDS
ARDS	adult respiratory distress syndrome
ASGPR	asialoglycoprotein receptor
ATM	ataxia telangiectasia, mutated
ATRA	all-trans retinoic acid
ATRNL	attractin-like protein
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BBB	blood-brain barrier
BDCA-1	blood dendritic cell antigen 1
BDNF	brain derived neurotrophic factor
BEHAB	brain enriched hyaluronan binding
BG	Birbeck Granule
BiP	immunoglobulin binding protein
BOS	bronchiolitis obliterans syndrome
BPD	bronchopulmonary dysplasia
BRAL-1	brain link protein-1
BRAP	BRCA1-associated protein
C1q	complement C1q module
C1r	complement C1r module

C4S	chondroitin-4-sulfate
C6S	chondroitin-6-sulfate
CAMs	cell adhesion molecules
CARD9	caspase activating recruitment domain 9
CBM	carbohydrate binding module
CC16	clara cell 16
CCSP	clara cell specific protein
CCV	clathrin coated vesicles
CD	Celiac disease
CD	Crohn's disease
CDH	congenital diaphragmatic hernia
CD-MPR	cation dependent M6P receptor
CEA	carcinoembryonic antigen (CEA)
CEACAM1	carcinoembryonic antigen (CEA)-related cell adhesion molecule 1
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CGN	cis-Golgi network
CHODL	chondrolectin
CI-MPR	cation-independent mannose 6-phosphate receptor
CLECSF2	activation-induced C-type lectin
CLL-1	C-type lectin-like molecule-1
CLRD	C-type lectin domain
CNS	central nervous system
Cnx/CNX	calnexin
COAD	chronic obstructive airways disease
COPD	chronic obstructive pulmonary disease
COPI	coatomer protein complex I
COPII	coatomer protein complex II
CR	complement protein regulatory repeat/ complement receptor
CRC	colorectal cancer
CRD	carbohydrate recognition domain
CREB	cAMP responsive element binding protein
CRP	complement regulatory protein domain
CRP	C-Reactive Protein
Crt/CRT	calreticulin
CS	chondroitin sulfate
CS-GAGs	chondroitin sulfate glycosaminoglycans
CSPG	chondroitin sulfate proteoglycan
CSPG2	chondroitin sulfate proteoglycan 2 or PG-M or versican
CTLD	C-type lectin-like domain
DC	dendritic cell
DC-SIGN	dendritic cell-specific ICAM-3-grabbing nonintegrin
DDR	discoidin domain receptor
Dex	dexamethasone
dGal-1	dimeric galectin-1
DN	down's syndrome
DOPG	dioleylphosphatidylglycerol
DPPC	dipalmitoylphosphatidyl choline
DSPC	disaturated phosphatidyl choline
EAE	experimental autoimmune encephalomyelitis
EBM	Epstein-Barr virus
ECM	extracellular matrix
Ed	embryonic day

EDEM	ER degradation enhancing α-mannosidase-like protein
EE	early endosomes
EGF	epidermal growth factor
ELAM-1	endothelial-leukocyte adhesion molecule 1
LECAM2	leukocyte-endothelial cell adhesion molecule 2
EMBP	eosinophil major basic protein
EMSA	electrophoretic mobility shift assay
eNOS	endothelial cell NO synthase
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERK	extracellular signal-regulated kinase
ERT	enzyme replacement therapy
ESP	early secretoty pathway
FA5	blood coagulation factor 5
FA8	blood coagulation factor 8
FAK	focal adhesion kinase
FBG	fibrinogen-like (FBG)-domains
FDl	fibrinogen-like domain 1
FGF	fibroblast growth factor
FN	fibronectin
FN2	fibronectin type II module
FN3	fibronectin type III module
Fuc	fucose
FV	blood coagulation factor V
FVIII	blood coagulation factor VIII
GAGs	glycosaminoglycans
Gal	galactose
Gal-1/Gal-3	galectin-1/galectin-3
Gb3	globotriaosylceramide
Gb4	globotetraosylceramide
GGA	Golgi-localizing, gamma-adaptin ear homology domain, ARF binding
GlyCAM-1	glycosylation-dependent cell adhesion molecule 1
GM1	ganglioside 1
GM2	ganglioside 2
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP-140	granule membrane protein 140
Grifin	galectin-related inter-fiber protein
GVHD	graft versus host disease
HAPLN2	hyaluronan and proteoglycan link protein 2
HBV	hepatitis B virus
HCMV	human cytomegalo virus
HCV	hepatitis C virus
HEV	high endothelial venule
HHL	human hepatic lectin
HHL1	human hepatic lectin subunit-1
HHL2	human hepatic lectin subunit-2
HNSCC	head and neck squamous cell carcinoma
HSPG	heparan sulfate proteoglycan
HT	Hashimoto's thyroiditis
HUVEC	human umbilical vein endothelial cell
i.m	intramuscular
i.p	intraperitoneal
i.v.	intravascular

IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IGF-1	insulin-like growth factor, type 1
IGF2/MPR	insulin-like growth factor 2 receptor (IGF2R)
IGF2R	insulin-like growth factor 2 receptor
IGF-II/CIMPR	insulin-like growth factor-II or cation-independent mannose 6-phosphate receptor
IgSF	immunoglobulin superfamily
IL	interleukin
ILT	immunoglobulin-like transcripts
INTL	intelectin
IPCD	interstitial pneumonia with collagen vascular diseases
IPF	idiopathic pulmonary fibrosis
iRNA/RNAi	RNA interference
ITAM	immunoreceptor tyrosine-based activatory motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer cell Ig-like receptors
LAMP-1/Lamp1	lysosome-associated membrane protein 1
LDL	low density lipoprotein
Le^a	Lewis A
Le^b	Lewis B
Le^x	lewis X
LFA-2	leukocyte function antigen-2
LFA-3	leukocyte function antigen-3
LFR	lactoferrin (LF) receptor (R)
LL	dileucine
LN	lymph node
LOX-1	lectin-type oxidized LDL receptor 1
LPS	lipopolysaccharide
M-6-P	mannose-6-phosphate
M6PR/MPR	Mannose-6-phosphate receptor
MAdCAM-1	mucosal addressin cell adhesion molecule-1 (or addressin)
ManLAMs	mannose-capped lipoarabinomannan
MAPK	mitogen-activated protein kinase
MASP	MBL-associated serine protease
MBL	mannan-binding lectin
MBP	mannose binding protein
MCP-1	monocyte chemoattractant protein 1
MHC	major histocompatibility complex
MICA/MIC-A	MHC class I polypeptide-related sequence A
MICB/MIC-B	MHC class I polypeptide-related sequence B
MMP	matrix metalloproteinases
MMR	macrophage mannose receptor
MPS	mucopolysaccharidosis
MR/ManR	mannose receptor
NCAM	neural cell adhesion molecule
NG2	neuronglia antigen 2
NK cell	natural killer cell
NK	natural killer
NKC	natural killer gene complex

n-LDL	native LDL
NO	nitric oxide
NOD mice	non-obese diabetic mice
NPI	neuronal pentraxin I
NTP	neuronal thread protein
OLR1	oxidized low-density lipoprotein receptor 1
Ox-LDL	oxidized low density lipoprotein
PAMPs	pathogen-associated molecular patterns
PAP	pulmonary alveolar proteinosis
PB	peripheral blood
PC	polycystin
PD	Parkinson disease
pDC/PDC	plasmacytoid dendritic cell
PDI	protein disulfide isomerase
PECAM-1	platelet-endothelial cell adhesion molecule-1
PG	phosphatidylglycerol
PGN	peptidoglycan
PI3-kinase	phosphatidyl-inositol (PI)3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLN	peripheral lymph node
PNN	perineuronal nets
PPARγ	peroxisome proliferator-activated receptor γ
PRR	pattern-recognition receptor
PSA	prostate-specific antigen
PSGL-1	P-selectin glycoprotein ligand1
PTX	pentraxins
R	review
RAE-1/Rae-1	retinoic acid inducible gene-1
RB	retinoblastoma protein
RCC	renal cell carcinoma
RCMV	rat cytomegalovirus
RDS	respiratory distress syndrome
Reg	regenerating genes
RAET	retinoic acid early (RAE) transcript
rER	rough ER
RHL	rat hepatic lectin
RNAi	RNA interference
ROS	reactive oxygen species
RSV	respiratory syncytial virus/ respiratory syndrome virus
SAA	serum amyloid A
SAP	serum amyloid P component
SARS	severe acute respiratory syndrome
SC	scavenger receptor module
SCLC	small cell lung carcinoma
SCR	short consensus repeat
s-diLe^x	sulfated polysaccharide ligands
Siglec	sialic-acid-binding immunoglobulin-like lectin
Sjs	Sjogren syndrome
SH1/2	Src homology 1/2
SHP-1/2	Src homology 1/2 containing phosphatase
SLAM	signaling lymphocyte activated molecule

SLE	systemic lupus erythematosus
sLe^a/s-Le^a	Sialyl Lewis A
sLe^x/s-Le^x	Sialyl Lewis X
Sn	sialoadhesin
SNP	single-nucleotide polymorphism
snRNPs	small nuclear ribonucleoproteins
SOCS3	suppressor of cytokine signaling 3
SP-A	surfactant protein A
SP-D	surfactant protein D
ST	sialyl transferase
STAT	signal transducers and activators of transcription
STAT1	signal transducer and activator of transcription 1
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TGF-β	transforming growth factorβ
TGN	trans-Golgi network
TIM	triosephosphate isomerase
TLR	toll-like receptor
TNF	tumor necrosis factor
Ub	ubiquitin
UDPG	uridine diphosphate (UDP)-glucose
UL16	unique long 16
ULBP	UL16 binding protein
uPAR	uPA receptor or urokinase receptor
uPARAP	urokinase receptor-associated protein
UPR	unfolded protein response
VCAM-1	vascular adhesion molecule-1
VEGF	vascular endothelial growth factor
VLA	very late antigen
VN	vitronectin
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor
β2-m	β2-microglobulin

Part I

Introduction

Lectins: An Overview

1

G.S. Gupta

1.1 Lectins: Characteristics and Diversity

1.1.1 Characteristics

Glycan-recognizing proteins can be broadly classified into two groups: lectins [which typically contain an evolutionarily conserved carbohydrate-recognition domain (CRD)] and sulfated glycosaminoglycan (SGAG)-binding proteins (which appear to have evolved by convergent evolution). The term "lectin" is derived from the Latin word legere, meaning, among other things, "to select". Lectins are phylogenetically ancient and have specific recognition and binding functions for complex carbohydrates of glycoconjugates, i.e., glycoproteins, proteoglycans/glycosaminoglycans and glycolipids. Although lectins were first discovered more than 100 years ago in plants, they are now known to be present throughout nature and found in most of the organisms, ranging from viruses and bacteria to plants and animals. It is generally believed that the earliest description of a lectin was given by Peter Hermann Stillmark in his doctoral thesis in 1888 to the University of Dorpat. Stillmark isolated ricin, an extremely toxic hemagglutinin, from seeds of the castor plant (Ricinus communis) (Stillmark 1988). The animal hemagglutinins were noted quite early, almost in all invertebrates or lower vertebrates, but until the middle of the 1970s, only three of these (of eel, snail, and horseshoe crab) were isolated and characterized. The first of the animal lectins shown to be specific for a sugar (L-fucose) was from eel (Watkins and Morgan 1952). The isolation of the first mammalian lectin, the galactose-specific hepatic asialoglycoprotein receptor, was achieved by Gilbert Ashwell at the NIH and by Anatol G. Morell at the Albert Einstein Medical School (New York) in 1974 (Hudgin et al. 1974; Stockert et al. 1974). At the same time, Teichberg et al. 1975 isolated a β-galactose-specific lectin from electric eel that was designated galectins (Barondes et al. 1994), of which 15 members so far have been characterized. Since the beginning of 1980s, the number of purified animal lectins started to grow quickly, largely thanks to the advent of recombinant techniques.

Presently, lectins are known as a heterogeneous group of carbohydrate binding proteins of non-immune origin, which agglutinate cells and/or precipitate glycoconjugates without affecting their covalent linkages (Goldstein et al. 1980). This definition implied that each lectin molecule has two or more carbohydrate binding sites to allow cross-linking between cells and between sugar containing macromolecules. During last few decades, however, interest in lectins has been greatly intensified after realization that they act as mediators of cell recognition in biological system (Sharon 2006). Though, lectins are similar to antibodies in their ability to agglutinate red blood cells, they are not the product of immune system. Lectins not only distinguish between different monosaccharides, but also specifically bind to oligosaccharides, detecting subtle differences in complex carbohydrate structure. They are distinct also from carbohydrate modifying enzymes, as they do not carry out glycosidase or glycosyl transferase reactions. All foods of plant origin contain specific lectins (Goldstein et al. 1980) and other anti-nutritional factors. These lectins when consumed in raw form, both in food and feed, may have serious and deleterious effects (Liener 1986).

Lectins are capable of specific binding to oligosaccharide structures present on cell surfaces, the extracellular matrix, and secreted glycoproteins. They are involved in intra- and intercellular glycan routing using oligosaccharides as postal-code equivalents and acting as defense molecules homing in on foreign or aberrant glycosignatures, as crosslinking agent in biosignaling and as coordinator of transient or firm cell-cell/cell-matrix contacts. By delineating the driving forces toward complex formation, knowledge about the causes for specificity can be turned into design of custom-made high-affinity ligands for clinical application, e.g. in anti-adhesion therapy, drug targeting or diagnostic histopathology (Kaltner and Gabius 2001). Galectins and collectins (mannose-binding lectins and surfactant proteins) illustrate the ability of endogenous glycan-binding proteins to act as cytokines, chemokines or growth factors, and thereby modulating innate and adaptive immune responses under physiological or pathological conditions.

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Understanding the pathophysiologic relevance of endogenous lectins in vivo will reveal novel targets for immunointervention during chronic infection, autoimmunity, transplantation and cancer (Toscano et al. 2007). Lectins by the ability to bind specific carbohydrate structures mediate cell-cell and cell-pathogen interactions. Since, the biological function of lectins is their ability to recognize and bind to specific carbohydrate structures involving cells and proteins, some viruses use lectins to attach themselves to the cells of the host organism during infection. Cyanobacterial and algal lectins have become prominent in recent years due to their unique biophysical traits, such as exhibiting novel protein folds and unusually high carbohydrate affinity, and ability to potently inhibit human immuno-deficiency virus (HIV-1) entry through high affinity carbohydrate-mediated interactions with the HIV envelope glycoprotein gp120. The antiviralcyanobacterial lectin *Microcystis viridis* lectin (MVL), which contains two high affinity oligomannose binding sites, is one such example. However, studies representing the demonstration of dual catalytic activity and carbohydrate recognition for discrete oligosaccharides at the same carbohydrate-binding site in a lectin have started emerging (Bourgeois et al. 2010; Shahzad-ul-Hussan et al. 2009). A large variety of lectins are expressed in the human organism and found in a variety of sizes and shapes, but can be grouped in families with similar structural features. Several reviews have focussed on current knowledge of human lectins with a focus on biochemistry and pathobiochemistry (principles of protein glycosylation and defects of glycosylation as a basis of disease) and cell biology (protein sorting, exocytosis and endocytosis, apoptosis, cell adhesion, cell differentiation, and malignant transformation). The clinical significance of lectin-glycoconjugate interactions is described by example of inflammatory diseases, defects of immune defense, autoimmunity, infectious diseases, and tumor invasion/metastasis. Moreover, therapeutic perspectives of novel drugs that interfere with lectin-carbohydrate interactions have appeared in literatue.

The combining sites of lectins are diverse, although they are similar in the same family. Specificities of lectins are determined by the exact shape of the binding sites and the nature of the amino acid residues to which the carbohydrate is linked. Small changes in the structure of the sites, such as the substitution of only one or two amino acids, may result in marked changes in specificity. The carbohydrate is linked to the protein mainly through hydrogen bonds, with added contributions from van der Waals contacts and hydrophobic interactions. Coordination with metal ions may occasionally play a role too. Surface lectins of viruses, bacteria and protozoa serve as a means of adhesion to host cells: a prerequisite for the initiation of infection. Blocking the adhesion by carbohydrates that mimic those to which the lectins bind prevents infection by these organisms. The way is thus open for the development of anti-adhesive therapy against microbial diseases.

1.1.2 Lectins from Plants

Although lectins were first discovered more than 100 years ago in plants, the first lectin to be purified, in 1960s, on a large scale was Concanavalin A (ConA), which is now the most-used lectin for characterization and purification of sugar-containing molecules and cellular structures. Plant lectins with potent biological activity occur in foods like cereals and vegetables. The legume lectins are classified under one family since they have high sequence similarities, very similar tertiary structure and biophysical properties. Furthermore, most of these legume lectins exist as oligomers in nature, and all of them require metal ions for their carbohydrate-binding activities. Foods with high concentrations of lectins may be harmful if consumed in excess in uncooked or improperly uncooked form. Adverse effects may include nutritional deficiencies, and immune (allergic) reactions. Possibly, most effects of lectins are due to gastrointestinal distress through interaction of the lectins with the gut epithelial cells. The toxicity of plant lectins can lead to diarrhoea, nausea, bloating, and vomiting. Soybean is the most important grain legume crop, the seeds of which contain high activity of soybean lectins (soybean agglutinin or SBA). The SBA is able to disrupt small intestinal metabolism and damage small intestinal villi via the ability of lectins to bind with brush border surfaces in the distal part of small intestine.

Many plant lectins possess anticancer properties in vitro, in vivo, and in human trial studies. The plant lectins represent a well-defined class of anti-HIV (microbicidal) drugs with a novel HIV drug resistance profile different from those of other existing anti-HIV drugs. The function of lectins in plants is still uncertain. It is hypothesized that they may serve as mediators of the symbiosis between nitrogen fixing microorganisms, primarily rhizobium, and leguminous plants, a process of immense importance in both the nitrogen cycle of terrestrial life and in agriculture (Sharon and Lis 2003; Shanmugham et al. 2006). The large concentration of lectins in plant seeds decreases with growth, and suggests a role in plant germination and perhaps in the seed's survival itself. Many legume seeds have been proven to contain high lectin activity, termed as hemagglutinating activity. Lectins from legume plants, such as PHA or Concanavalin A, have been widely used as model systems to understand the molecular basis of how proteins recognize carbohydrates, because they are relatively easy to obtain and have a wide variety of sugar specificities. The structures of many plant lectins have been deduced. For the most part, leguminous lectins assemble into a compact β-barrel configuration devoid of α helices and dominated by two antiparallel pleated sheets. Interestingly, the organization of the antiparallel β sheets and the overall tertiary structure of the leguminous plant lectins are very similar to that seen for

animal galectins, despite the fact that leguminous lectins and galectins share no sequence homology and galectins do not require metals for activity. In leguminous lectins, the metal-binding sites are located on a single long loop.

Saccharide-binding specificity of ConA from seeds of the jack bean *Canavalia ensifomist* is shown toward the α–D-mannopyranoside or α–D-glucopyranoside ring with unmodified hydroxyl groups at the 3, 4 and 6 positions. ConA activates T cells in polyclonal activation and regulates Ca^{2+} entry in human neutrophils. It specifically recognizes the pentasaccharide core [β-GlcNAc-(1–2)-α-Man-(1–3)-(β-GlcNAc-(1–2)-α-Man-(1–6)]-Man) of N-linked oligosaccharides (Bouckaert et al. 1999). The crystal structures of several legume lectins have led to a detailed insight of the atomic interactions between carbohydrates and proteins. Several plant and animal Lecins are used as biochemical tool in affinity chromatography for purifying proteins. In general, proteins may be characterized with respect to glycoforms and carbohydrate structure by means of affinity chromatography, blotting, affinity electrophoresis and affinity immunoelectrophoreis with lectins. Legume lectins have both calcium and manganese binding sites. The calcium ion puts several conserved residues in positions that are critical for carbohydrate binding (Brinda et al. 2005).

1.1.3 Lectins in Microorganisms

Numerous bacterial strains and viruses produce surface lectins. Adhesion of microorganisms to host tissues is the pre-requisite for the initiation of majority of infectious diseases. Microorganisms have evolved along with lectins that interact with glycoproteins, proteoglycans, and glycolipids. Adhesion of infectious organism is mediated by lectins present on the surface of organim that binds to complementary carbohydrates on the surface of the host tissues. Until early 1980s, only bacteria specific for mannose were identified, namely type 1 fimbriated strains *E. coli*. Since then, *E. coli* strains with diverse specificities were discovered (Sharon 2006). These include urinary strains carrying P fimbriae that are specific for galabiose (Galα4Gal), and neural S fimbriated strains specific for NeuAc(α2–3) Galβ3GalNAc. In addition, bacteria with affinities for other sugars have been described, e.g., *Neisseria gonorrhoea*, a genital pathogen, which recognizes Nacetyllactosamine (Galβ4GlcNAc, LacNAc). *Helicobacter pylori*, the causative agent of peptic ulcer expresses a number of distinct binding specificities (Sharon 2006). Several of these lectins recognize NeuAc (α2–3) Galβ4Glc (Sia3′Lac) and its N-acetylglucosamine analog (Sia3′LacNAc) while others are specific for the Leb determinant Fucα2Galβ3(Fucα4) GlcNAc (R: Gupta et al. 2009). A broad range of proteins bind high-mannose carbohydrates found on the surface of the envelope protein gp120 of the HIV and thus interfere with the viral life cycle, providing a potential new way of controlling HIV infection. These proteins interact with the carbohydrate moieties in different ways (Bourgeois et al. 2010). Among bacterial lectins, best characterized are type 1 (mannose specific) fimbrial lectins of *E. coli* that consist almost exclusively of one class of subunit with a molecular mass of 17 kDa. The various bacterial surface lectins appear to function primarily in the initiation of infection by mediating bacterial adherence to epithelial cells, e.g. in the urinary and gastrointestinal tracts (Sharon 2006). Further studies of these systems may lead to a deeper understanding of the molecular basis of infectious diseases, and to new approaches for their prevention.

1.1.4 Animal Lectins

During last few decades, interest in animal lectins has been greatly intensified after realization that they act as mediators of cell recognition in biological systems. Lectin activities specific for different monosaccharides or glycans (fucose, galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylneuraminic acid, fucose and heparin) have been identified. Most of them show a cellular specificity and developmental regulation. But some of them seem to be involved in signaling events both intracellularly (nuclear lectins) or at the cell surface by autocrine and paracrine mechanisms. As early as 1988, most animal lectins were thought to belong to one of two primary structural families, the C-type and S-type (presently known as galectins) lectins. However, it is now clear that animal lectin activity is found in association with an astonishing diversity of primary structures. At least 15 structural families are known to exist at present (Table 1.1), while many other lectins have structures apparently unique amongst carbohydrate-binding proteins, although some of those "orphans" belong to recognised protein families that are otherwise not associated with sugar recognition. Furthermore, many animal lectins also bind structures other than carbohydrates via protein–protein, protein–lipid or protein–nucleic acid interactions.

1.2 The Animal Lectin Families

1.2.1 Structural Classification of Lectins

Lectins occur in plants, animals, bacteria and viruses. Initially described for their carbohydrate-binding activity (Sharon and Lis 2001), they are now recognised as a more diverse group of proteins, some of which are involved in protein-protein, protein-lipid or protein-nucleic acid interactions (Kilpatrick 2002). The lectin superfamily as classified

Table 1.1 Summary of some lectin families

S. no	Lectin family	Typical saccharide ligands	Subcellular location	Functions
1	Calnexin and calreticulin	Glc_1Man_9	ER	Protein sorting and as molecular chaperones in ER (Ireland et al. 2006).
2	M-type lectins related to the α-mannosidases	Man_8	ER	Degradation of glycolproteins (Molinari et al. 2003).
3	L-type lectins (ERGIC-53 and VIP-36, Pentraxins)	Various	ER, ERGIC, Golgi	Protein sorting in ER (Arar et al. 1995; Bottazzi et al. 2006).
4	P-type lectins	Man6-phosphate, phosphomannosyl receptors	Secretory pathway	Protein sorting post-Golgi, glycoprotein trafficking, enzyme targeting (Dahms and Hancock 2002).
5	C-type lectins (collectins, selectins, lecticans, others Volume 2)	Mannosides, galactosides, sialic acids, others	Cell membrane, extracellular	Cell adhesion/selectins, glycoprotein clearance, innate immunity (Zelensky and Gready 2005).
6	S-type lectins (galectins)	β-Galactosides	Cytoplasm, extracellular	Glycan crosslinking in the extracellular matrix (Elola et al. 2007).
7	I-type lectins (siglec-1, -2, -3, others)	Sialic acid and other glycosaminoglycans	Cell membrane	Cell adhesion (Angata and Brinkman-Van der Linden 2002).
8	R-type lectins (macrophage mannose receptor; discoidins)	Various	Golgi, Cell membrane	Enzyme targeting, glycoprotein hormone turnover (Lord et al. 2004; Pluddemann et al. 2006).
9	F-box lectins (Fbs1, Fbs2, Fbg3, others)	$GlcNAc_2$	Cytoplasm	Degradation of misfolded glycoproteins (Ho et al. 2006).
10	Fibrinogen-type lectins (ficolins,tachy-lectins 5A and 5B)	GlcNAc, GalNAc	Cell membrane, extracellular	Innate immunity. (Matsushita and Fujita 2001; Mali et al. 2006).
11	Chi-lectins (CHI3L1, CHI3L4, Ym1, Ym2)	Chito-oligosaccharides	Extracellular	Collagen metabolism (Tharanathan and Kittur 2003).
12	F-type lectins (Eel aggutinins/ fucolectins)	Fuc-terminating oligosaccharides	Extracellular	Innate immunity (Odom and Vasta 2006).
13	Intelectins (Endothelial lectin HL-1; Eglectin)	Gal, galactofuranose, pentoses	Extracellular/ cell membrane	Innate immunity, fertilization, embryogenesis (Tsuji et al. 2001).
14	Annexins (Annexin IV, V, VI)	Glycosaminoglycans, heparin and heparan sulfate	Cell membrane	Cell-adhesion, formation of apical-secretory vesicles (Moss and Morgan 2004; Turnay et al. 2005).

by SCOP (structural classification of proteins) (Lo Conte et al. 2002) comprises 15 families that include the legume lectins, β-glucanases, endoglucanases, sialidases, galectins, pentraxins and calnexin/calreticulin, among others. All of them belong to all-β class and have ConA (concanavalin-A-like) jelly-roll fold that can be seen. The jelly-roll motif consists of three sets of anti-parallel β-sheets, as can also be seen from the legume lectins shown in Fig. 1.1. There is a six-stranded flat 'back' sheet, a curved seven-stranded 'front' sheet and a short five-member sheet at the 'top' of the molecule. The sheets are connected by several loops of various lengths. The legume lectins have high sequence similarities, very similar tertiary structure and biophysical properties and hence are classified under one family. Since last decade, new information has been acquired from structural analysis of proteins belonging to the legume lectin family. Studies indicate that legume lectins belong to an interesting family of proteins with very similar tertiary structures but varied quaternary structures. Presently legume lectins offer good models to study the role of primary structures in finding the modes of quaternary association. Most of the legume lectins exist as oligomers in nature, and all of them require metal ions for their carbohydrate-binding activities. The metal-binding and carbohydrate-binding sites in various legume lectins have overlapping structures. However, these lectins do differ widely in their carbohydrate specificities and in their quaternary associations. Other lectins in this superfamily viz, galectins, pentraxins, calnexin and calreticulin have less sequence similarity with the legume lectins. Despite this disimilarities, they do share the same jelly-roll tertiary structure and show different quaternary associations. The unique three-dimensional structure of both monomeric and oligomeric proteins is encoded in their sequence. The biological functions of proteins are dependent on their tertiary and quaternary structures, and hence it is important to understand the determinants of quaternary association in proteins. Although a large number of investigations have been carried out in this direction, the underlying principles of protein oligomerization are yet to be completely understood. Brinda et al. (2005) reviewed the results of a legume lectins and on animal lectins, namely galectins, pentraxins, calnexin, calreticulin and rhesus rotavirus Vp4

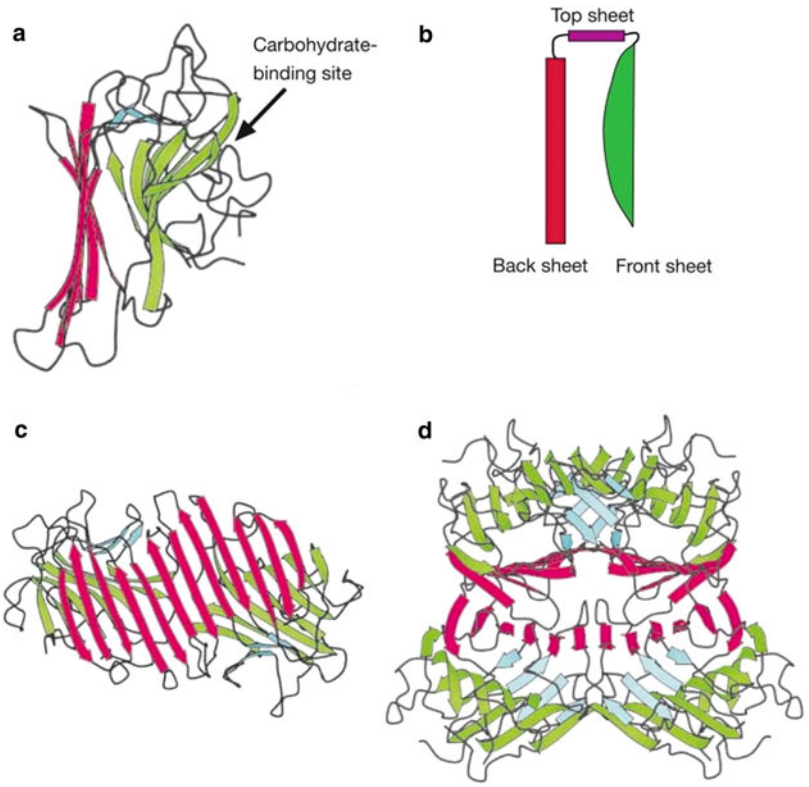

Fig. 1.1 Structure of concanavalin A (ConA). (**a**) The tertiary structure of the monomer is best described as a "jelly-roll fold." (**b**) This fold consists of a flat six-stranded antiparallel "back" β-sheet (*red*), a curved seven-stranded "front" β-sheet (*green*), and a five-stranded "top" sheet (*pink*) linked by loops of various lengths. (**c**) Dimerization of ConA involves antiparallel side-by-side alignment of the flat six-stranded "back" sheets, giving rise to the formation of a contiguous 12-stranded sheet (**d**) The tetramerization of ConA occurs by a back-to-back association of two dimers (Reprinted by permission from Srinivas et al. 2001© Elsevier)

sialic-acid-binding domain. The study has provided the signature sequence motifs for different kinds of quaternary association seen in lectins. The network association of lectin oligomers has enabled to detect the residues which make extensive interactions ('hubs') across the oligomeric interfaces that can be targetted for interface-destabilizing mutations. Brinda et al. (2005) illustrated the potential of such a representation in elucidating the structural determinants of protein-protein association in general and their significance to protein chemists and structural biologists in particular.

Animal lectins that recognize endogenous ligands appear to play a role in fertilization and development, and their function often involves cell-to-cell or cell-to-matrix interaction. Lectins that recognize exogenous ligands probably evolved for non-self discrimination, and they may be soluble or surface bound. The 15 families of animal lectins, defined in structural terms, participate in intra- and intercellular glycan routing using oligosaccharides as postal-code equivalents and acting as defense molecules homing in on foreign or aberrant glycosignatures, as crosslinking agent in biosignaling and as coordinator of transient or firm cell-cell/cell-matrix contacts. Ver tebrate lectins possess functionally diverse group of protein domains which can bind specific CRDs/oligosaccharide structures present on cell surfaces, the extracellular matrix, and secreted glycoproteins (Drickamer 1999). Of the eight well-established CRD groups, four contain lectins that are predominantly intracellular and four contain lectins that generally function outside the cell (Table 1.1). The intracellular lectins—calnexin family, M-type, L-type and P-type are located in luminal compartments of the secretory pathway and function in the trafficking, sorting and targeting of maturing glycoproteins. The extracellular lectins—C-type (collectins, selectins, mannose receptor, and others), R-type, siglecs, and S-type (galectins)—are either secreted into the extracellular matrix or body fluids, or localized to the plasma membrane, and mediate a range of functions including cell adhesion, cell signaling, glycoprotein clearance and pathogen recognition. Collagenous lectins such as mannan-binding protein (MBPs), ficolins, surfactant proteins (collectins), conglutinin, and related ruminant lectins are multimeric proteins with CRD aligned in a manner that facilitates binding to microbial surface polysaccharides. In soluble collectins such as MBL, the subunits possessing a single CRD associate to form a "bouquet"-like oligomer with all CRDs facing a potential target

surface (Irache et al. 2008). However, in "cruciform" organization of conglutinin subunits, the single CRDs are arranged in a manner that can also cross-link multiple targets. Fibrinogen-type lectins include ficolins, tachylectins 5A and 5B, and Limax flavus (Spotted garden slug) agglutinin. These proteins have clear distinctions from one another, but they share a homologous fibrinogen-like domain used for carbohydrate binding. A less frequent organizational plan is the presence of tandem CRDs encoded within a polypeptide, such as observed in macrophage mannose receptor, the immulectins, and tandem-type galectins.

Recent findings point to the existence of additional new groups of animal lectins—F-box lectins, chitinase-like lectins (Chi-lectins), F-type lectins, intelectins—some of which have roles complementary to those of the well-established lectin families (Table 1.1). Some of the unclassified orphan lectins group includes: amphoterin, Cel-II, complement factor H, thrombospondin, sailic acid-binding lectins, adherence lectin, and cytokins (such as tumor-necrosis factor, and annexin and several interleukins). More detailed information on the structure, sugar-binding activity, biological function and evolution of proteins (Cambi and Figdor 2003, 2009) in each of the lectin families as well as annotated sequence alignments can be obtained in relevant chapters of this book.

1.3 C-Type Lectins (CLEC)

1.3.1 Identification of CLEC

A C-type lectin (CLEC) or a C-type lectin receptor (CLR) is a type of carbohydrate-binding protein domain known as a lectin. The C-type designation is from their requirement for calcium for binding. The C-type lectin superfamily is a large group of proteins which is characterized as having at least one carbohydrate recognition domain (CRD) (Drickamer 1999). The C-type lectin fold has been found in more than 1,000 proteins, and it represents a ligand-binding motif that is not necessarily restricted to binding sugars. Proteins that contain C-type lectin domains have a diverse range of functions including cell-cell adhesion, immune response to pathogens and apoptosis. However, many C-type lectins actually lack calcium- and carbohydrate-binding elements and thereby have been termed C-type lectin-like proteins. Therefore, CLR or CLEC broadly denotes proteins with a C-type lectin domain, regardless of their ability to bind sugars.

1.3.2 C-Type Lectin Like Domain (CTLD/CLRD)

Use of terms of "C-type lectin", "C-type lectin domain" (CLRD), "C-type lectin-like domain" (also abbreviated as CLRD), often used interchangeably and use of CRD in the literature, have been clarified by Zelensky and Gready (2005). With the large number of CLR sequences and structures now available, studies indicate that the implications of the CRD domain are broad and vary widely in function. In metazoans, most proteins with a CLR are not lectins. Moreover, proteins use the C-type lectin fold to bind other proteins, lipids, inorganic molecules (e.g., Ca_2CO_3), or even ice (e.g., the antifreeze glycoproteins). An increasing number of studies show that "atypical" C-type lectin-like proteins are involved in regulatory processes pertaining to various aspects of the immune system. Examples include the NK cell inhibitory receptor Ly49A, a C-type lectin-like protein, which is shown to complex with the MHC class I ligand (Correa and Raulet 1995), and the C-type lectin-like protein mast cell function-associated antigen which is involved in the inhibition of IgE-Fc γRI mediated degranulation of mast cell granules (Guthmann et al. 1995). Yet glycan binding by the C-type lectins is always Ca^{2+}-dependent because of specific amino acid residues that coordinate Ca^{2+} and bind the hydroxyl groups of sugars (Cummings and McEver 2009). To resolve the contradiction, a more general term C-type lectin like domain (CLRD) was introduced to distinguish a group of Ca^{2+}-independent carbohydrate-binding animal proteins from the Ca^{2+}-dependent C-type of animal lectins (CLR/CLEC). The usage of this term is however, somewhat ambiguous, as it is used both as a general name for the group of domains with sequence similarity to C-type lectin CRDs (regardless of the carbohydrate-binding properties), and as a name of the subset of such domains that do not bind carbohydrates, with the subset that does bind carbohydrates being called C-type CRDs. Also both 'C-type CRD' and 'C-type lectin domain' terms are still being used in relation to the C-type lectin homologues that do not bind carbohydrate, and the group of proteins containing the domain is still often called the 'C-type lectin family' or 'C-type lectins', although most of them are not in fact lectins. The abbreviation CRD is used in a more general meaning of 'carbohydrate-recognition domain', which encompasses domains from different lectin groups. Occasionally CRD is also used to designate the short amino-acid motifs (i.e. amino-acid domain) within CLRDs that direCLRy interact with Ca^{2+} and carbohydrate (Zelensky and Gready 2005). In this book authors use the term C-type lectin domain or C-type lectin-like domain (CLRD) interchangeably in its broadest definition to refer to protein domains that are homologous to the CRDs of the C-type lectins, or which have structure resembling the structure of the prototypic C-type lectin CRD or as used by different researchers in their work. More over, due to contradictions (Zelensky and Gready 2005) and uncertainties which may arise in future studies our sequence of chapters is not based on structure databases as in the SCOP; instead chapters are

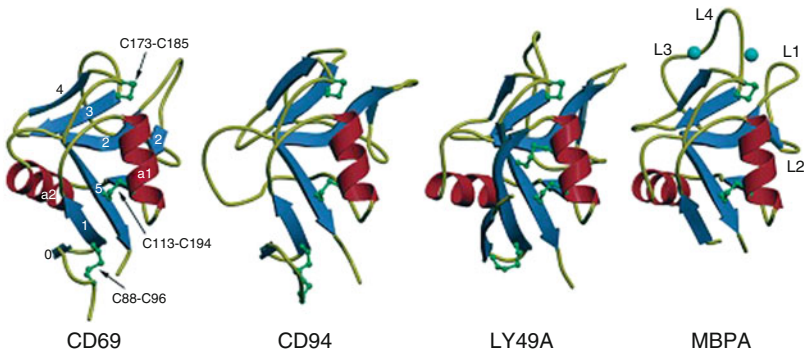

Fig. 1.2 CLRD Structures: Ribbon diagrams of the natural killer domains (NKDs) from human CD69 (Llera et al. 2001), a CTLD, divergent from true C-type lectins, human CD94 (PDB ID: 1B6E), mouse Ly49A (PDB ID: 1QO3), and the CRD of rat MBP-A (PDB ID: 1YTT) are shown in a common orientation obtained by pairwise superpositions. Ca^{2+} ions bound to MBP-A are shown as light blue spheres, whereas its loop regions without regular secondary structure, three of them involved in Ca^{2+} coordination, are labeled L1 to L4. Secondary structure elements in CD69 have been labeled following the numbering for MBP-A, prototype of the family. Therefore, the first β strand, which is absent in MBP-A and is characteristic of the long-form CTLDs, has been labeled as β0, whereas the strand that forms a β-hairpin with strand β2 has been named β2'. Differences in the orientation of helix α2, which is absent in CD94, are evident (Adapted from Llera et al. 2001)

linked more to cell functions or cell biology. Thus the C-type lectin fold is an evolutionarily ancient structure that is adaptable for many uses.

1.3.3 Classification of CLRD-Containing Proteins

The C-type lectin superfamily is a large group of proteins that are characterised by the presence of one or more CLRDs. The superfamily is divided into 17 groups based on their phylogeny and domain organisation (Drickamer and Fadden 2002; Zelensky and Gready 2005). Despite the presence of a highly conserved domain, C-type lectins are functionally diverse and have been implicated in various processes including cell adhesion, tissue integration and remodelling, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis.

C-type lectins can be divided into seventeen subgroups based on additional non-lectin domains and gene structure: The CLRD-containing proteins have been sorted based on two independent sets of criteria. The two approaches give essentially the same results, indicating that members of each group are derived from a common ancestor, which had already acquired the domain architecture that is characteristic of the group. Initially classified into seven subgroups (I to VII) based on the order of various protein domains in each protein: (I) hyalectans, (II) asialoglycoprotein receptors, (III) collectins, (IV) selectins, (V) NK cell receptors, (VI) Endocytic receptors, and (VII) simple (single domain) lectins (Drickamer 1993; McGreal et al. 2004). This classification was subsequently updated in 2002, leading to seven additional groups (VIII to XIV) (Drickamer and Fadden 2002). A further three subgroups were added (XV to XVII) by Zelensky and Gready (2005). The section of animal lectins genomics resource includes information on the structure and function of proteins in each group, as well as annotated sequence alignments, and a comprehensive database for all human and mouse CLRD-containing proteins (Chaps. 22–40).

1.3.4 The CLRD Fold

The CLRD fold has a double-loop structure (Fig. 1.2). The overall domain is a loop, with its N- and C-terminal β strands (β1, β5) coming close together to form an antiparallel β-sheet. The second loop, which is called the long loop region, lies within the domain; it enters and exits the core domain at the same location. Four cysteines (C1-C4), which are the most conserved CLRD residues, form disulfide bridges at the bases of the loops: C1 and C4 link β5 and α1 (the whole domain loop) and C2 and C3 link β3 and β5 (the long loop region). The rest of the chain forms two flanking α helices (α1 and α2) and the second ('top') β-sheet, formed by strands β2, β3 and β1. The long loop region is involved in Ca^{2+}-dependent carbohydrate binding, and in domain-swapping dimerization of some CLRDs (Fig. 1.2), which occurs via a unique mechanism (Mizuno et al. 1997; Feinberg et al. 2000; Mizuno et al. 2001; Hirotsu et al. 2001; Liu and Eisenberg 2002). For conserved positions involved in CLRD fold maintenance and their structural roles readers are referred elsewhere (Zelensky and Gready 2003). In addition to four conserved cysteines, one other sequence feature and the highly conserved 'WIGL' motif is located on the β2 strand and serves as a useful landmark for sequence analysis (Chap. 22).

1.3.4.1 Ca^{2+}-Binding Sites in CLRDs

Four Ca^{2+}-binding sites in the CLRD domain recur in CLRD structures from different groups. The site occupancy depends on the particular CLRD sequence and on the crystallization conditions; in different known structures zero, one, two or three sites are occupied. Sites 1, 2 and 3 are located in the upper lobe of the structure, while site 4 is involved in salt bridge formation between α2 and the β1/β5 sheet. Sites 1 and 2 were observed in the structure of rat MBP-A complexed with holmium, which was the first CLRD structure determined. Site 3 was first observed in the MBP-A complex with Ca^{2+} and oligomannose asparaginyl-oligosaccharide. It is located very close to site 1 and all the side chains coordinating Ca^{2+} in site 3 are involved in site 1 formation. As biochemical data indicate that MBP-A binds only two calcium atoms (Loeb and Drickamer 1988), Ca^{2+}-binding site 3 is considered a crystallographic artifact (Zelensky and Gready 2005).

1.4 Disulfide Bonds in Lectins and Secondary Structure

1.4.1 Disulfide Bond

Disulfide bond is a covalent bond, usually derived by the coupling of two thiol groups. The disulfide bond is a strong typical bond with dissociation energy being 60 kcal/mole. Disulfide bonds play an important role in the folding and stability of proteins, usually proteins which are secreted in to extracellular medium. Since most cellular compartments are reducing environments, disulfide bonds are generally unstable in the cytosol, with some exceptions. In proteins, disulfide bonds are formed between the thiol groups of cysteine amino acids. The methionine, cannot form disulfide bonds. A disulfide bond is typically denoted by hyphenating the abbreviations for cysteine, e.g., when referring to Ribonuclease A the "Cys26-Cys84 disulfide bond", or the "26–84 disulfide bond", or most simply as "C26-C84" where the disulfide bond is understood and does not need to be mentioned. The prototype of a protein disulfide bond is the two-amino-acid peptide, cystine, which is composed of two cysteine amino acids joined by a disulfide bond.The disulfide bond stabilizes the folded form of a protein in several ways: (1) It holds two portions of the protein together, biasing the protein towards the folded topology. Stated differently, the disulfide bond destabilizes the unfolded form of the protein by lowering its entropy. (2) The disulfide bond may form the nucleus of a hydrophobic core of the folded protein, i.e., local hydrophobic residues may condense around the disulfide bond and onto each other through hydrophobic interactions. (3) Related to #1 and #2, the disulfide bond link two segments of the protein chain, the disulfide bond increases the effective local concentration of protein residues and lowers the effective local concentration of water molecules. Since water molecules attack amide-amide hydrogen bonds and break up secondary structure, a disulfide bond stabilizes secondary structure in its vicinity. For example, researchers have identified several pairs of peptides that are unstructured in isolation, but adopt stable secondary and tertiary structure upon forming a disulfide bond between them.

1.4.2 Pathway for Disulfide Bond Formation in the ER of Eukaryotic Cells

The folding of many secretory proteins depends upon the formation of disulfide bonds. The formation of intra- and interchain disulfide bonds constitutes an integral part of the maturation of most secretory and membrane-bound proteins in the endoplasmic reticulum. Evidence indicates that members of the protein disulfide isomerase (PDI) superfamily are part of the machinery needed for proper oxidation and isomerization of disulfide bonds. Recent advances in genetics and cell biology have outlined a core pathway for disulfide bond formation in the ER of eukaryotic cells. In this pathway, oxidizing equivalents flow from the ER membrane protein Ero1p to secretory proteins via protein disulfide isomerase (PDI) (Frand et al. 2000; Ellgaard and Rudock 2005). Models based on in vitro studies predict that the formation of mixed disulfide bonds between oxidoreductase and substrate is intermediate in the generation of the native intrachain disulfide bond in the substrate polypeptide (Gruber et al. 2006). The ER-resident oxidoreductases PDI, together with the lectins calnexin and calreticulin, are central in glycoprotein folding in the endoplasmic reticulum of mammalian cells (Molinari and Helenius 1999). Studies suggest that the calnexin cycle has evolved with a specialized oxidoreductase to facilitate native disulfide formation in complex glycoproteins (Jessop et al. 2009).

1.4.3 Arrangement of Disulfide Bonds in CLRDs

Drickamer and Dodd (1999) summarized positions of six different disulfide bonds in CLRDs (Fig. 1.3). Chemical evidence for the presence of each of these bonds, except number 4, has been provided in at least one CLRD (Fuhlendorff et al. 1987; Usami et al. 1993). The positions of disulfide bonds designated 1, 2, and 3 have been demonstrated by X-ray crystallography as well (Weis et al. 1991; Nielsen et al. 1991), while homology modeling of CLRDs containing disulfide bonds 5 and 7 shows that they

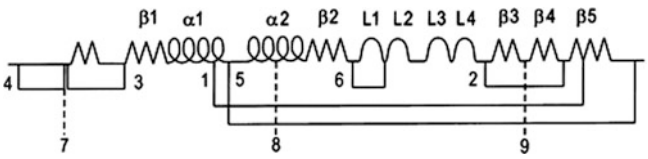

Fig. 1.3 Disulfide bonds in CLRDs. Secondary structure shared by most CLRDs is summarized, with coils representing α-helices, jagged lines denoting β-strands and loops shown as curved segments. The number of these elements corresponds to the secondary structure organisation of rat serum mannose-binding protein (Weis et al. 1991). Potential disulfide bonds within the CLRD are numbered 1 through 6 and cysteines that participate in interchain disulfide bonds are numbered 7 through 9 (Adapted by permission from Drickamer and Dodd 1999 © Oxford University Press)

could readily be accommodated into the C-type lectin fold. The patterns of cysteine residues in the CLRDs from *C.elegans* are consistent with the presence of disulfide bonds in each of the arrangements shown in Fig. 1.3 except for bond type 4. No additional pairs of cysteine residues within the CLRDs are consistently evident for any of the subgroups, indicating that the cysteine residues are mostly involved in disulfide bonds of the types already characterized in vertebrate homologues. CLRDs lacking one of a pair of cysteine residues almost invariably also lack the cysteine side chain to which the first residue would be linked. Like the CLRDs from other organisms, those from *C.elegans* each contain a subset of the possible disulfide bonds. CLRDs in a given subgroup generally show the same disulfide bonds, although a few domains contain extra unique pairs of cysteine residues that might form disulfides. CEL-I from the sea cucumber, Cucumaria echinata is composed of two identical subunits held by a single disulfide bond. A subunit of CEL-I is composed of 140 amino acid residues. Two intrachain (Cys3-Cys14 and Cys31-Cys135) and one interchain (Cys36) disulfide bonds were also identified from an analysis of the cystine-containing peptides obtained from the intact protein (Yamanishi et al. 2007). Thus, the similarity in disulfide bond structure in each subgroup reflects the overall similarity in sequence of the CLRDs (Drickamer and Dodd 1999).

1.4.4 Functional Role of Disulfides in CLRD of Vertebrates

Single cysteine residues appear in several of these groups at positions 7 and 8 as well as at a unique position 9. The turn between β-strands 3 and 4 is exposed on the surface of the domain, so it is expected that cysteine residues at position 9 would be accessible for formation of disulfide bonds. It is possible that such bonds could form with other cysteine residues within the same polypeptide but outside the CLRDs. However, no likely pairing partner is evident for any of these residues, suggesting that they are more likely to form interchain disulfide bonds. Homo- and hetero-dimer formation through cysteine residues at positions 7 and 8 has been particularly well documented in snake venom proteins containing CLRDs (Atoda et al. 1991; Usami et al. 1993). The botrocetin, which promotes platelet agglutination in the presence of von Willebrand factor, from venom of the snake Bothrops jararaca is a heterodimer composed of the α subunit and the β subunit held together by a disulfide bond. Seven disulfide bonds link half-cystine residues 2–13, 30–128, and 103–120 of the α subunit; 2–13, 30–121, and 98–113 of the β subunit; and 80 of the α subunit to 75 of the β subunit. In terms of amino acid sequence and disulfide bond location, two-chain botrocetin is homologous to echinoidin (a sea urchin lectin) and other C-type lectins (Usami et al. 1993). The disulfide bond pattern of Trimeresurus stejnegeri lectin (TSL), a CTLD, showed four intrachain disulfide bonds: Cys3-Cys14, Cys31-Cys131, Cys38-Cys133 and Cys106-Cys123, and two interchain linkages, Cys2-Cys2 and Cys86-Cys86 (Zeng et al. 2001). The antifreeze polypeptide (AFP) from the sea raven, Hemitripterus americanus, a member of the cystine-rich class of blood antifreeze proteins, contains 129 residues with 10 half-cystine residues and all 10 half-cystine residues appeared to be involved in disulfide bond formation. The disulfide bonds are linked at Cys7 to Cys18, Cys35 to Cys125, and Cys89 to Cys117. Similarities in covalent structure suggested that sea raven AFP, pancreatic stone protein, and several lectin-binding proteins may possess a common fold (Ng and Hew 1992).

Functional rat or human asialoglycoprotein receptors (ASGP-Rs), the galactose-specific C-type lectins, are hetero-oligomeric integral membrane glycoproteins. Rat ASGP-R contains three subunits, designated rat hepatic lectins (RHL) 1, 2, and 3; human ASGP-R contains two subunits, HHL1 and HHL2. Both receptors are covalently modified by fatty acylation (1996). Unfolded forms of the HHL2 subunit of the human ASGP-Rs are degraded in the ER, whereas folded forms of the protein can mature to the cell surface (Wikström and Lodish 1993). Deacylation ofASGP-Rs with hydroxylamine results in the spontaneous formation of dimers through reversible disulfide bonds, indicating that deacylation concomitantly generates free thiol groups. Results also show that Cys57 within the transmembrane domain of HHL1 is not normally palmitoylated. Thus, Cys35 in RHL1, Cys54 in RHL2 and RHL3, and Cys36 in HHL1 are fatty acylated, where as Cys57 in HHL1 and probably Cys56 in RHL1 are not palmitoylated (Zeng and Weigel 1996). Eosinophil granule major basic protein 2 (MBP2 or major basic protein homolog) is a paralog of major basic protein (MBP1) and, similar to MBP1, is cytotoxic and cytostimulatory in vitro. MBP2, a small protein of 13.4 kDa molecular weight, contains 10 cysteine residues. Mass spectrometry shows two cystine disulfide linkages (Cys20-Cys115 and Cys92-Cys107) and 6 cysteine residues

with free sulfhydryl groups (Cys2, Cys23, Cys42, Cys43, Cys68, and Cys96). MBP2, similar to MBP1, has conserved motifs in common with C-type lectins. The disulfide bond locations are conserved among human MBP1, MBP2 and C-type lectins (Wagner et al. 2007; Swaminathan et al. 2001).

The biological functions of rat surfactant protein-A (SP-A), an oligomer composed of 18 polypeptide subunits are dependent on intact disulfide bonds. Reducible and collagenase-reversible covalent linkages of as many as six or more subunits in the molecule indicate the presence of at least two NH2-terminal interchain disulfide bonds. However, the reported primary structure of rat SP-A predicts that only Cys6 in this region is available for interchain disulfide formation. Direct evidence for a second disulfide bridge was obtained by analyses of a set of three mutant SP-As with telescoping deletions from the reported NH2-terminus. Two of the truncated recombinant proteins formed reducible dimers despite deletion of the domain containing Cys6. A novel post translational modification results in naturally occurring cysteinyl isoforms of rat SP-A which are essential for multimer formation (Elhalwagi et al. 1997). Pulmonary SP-D is assembled predominantly as dodecamers consisting of four homotrimeric subunits each. Association of these subunits is stabilized by interchain disulfide bonds involving two conserved amino-terminal cysteine residues (Cys15 and Cys20). Mutant recombinant rat SP-D lacking these residues (RrSP-Dser15/20) is secreted in cell culture as trimeric subunits rather than as dodecamers. Disulfide cross-linked SP-D oligomers are required for the regulation of surfactant phospholipid homeostasis and the prevention of emphysema and foamy macrophages in vivo (Zhang et al. 2001).

A knowledge-based approach and energy minimization suggest that three-dimensional structures of each of the lectin domains of P-selectin, E-selectin, and L-selectin contains 118 amino acids. The structures thus found for P-, L-, and E-selectin lectin domains share a common feature, i.e., they all contain two α-helices, and two antiparallel β-sheets of which one is formed by two strands (strands 1 and 5) and the other by three strands (strands 2, 3, and 4). Besides, they all possess two intact disulfide bonds formed by the pair of Cys19-Cys117, and the pair of Cys90-Cys109. A notable feature is the convergence-divergence duality of the 77–107 polypeptide in the three domains; i.e., part of the peptide is folded into a closely similar conformation, and part of it into a highly different one (Chou 1996).

1.4.5 Disulfide Bonds in Ca^{2+}-Independent Lectins

The lectin from Japanese frog (Rana japonica) eggs, which specifically agglutinates transformed cells, is a single-chain protein consisting of 111 residues, with a pyroglutamyl residue at the amino terminus. Four disulfide bonds link half-cystinyl residue 19–72, 34–82, 52–97, and 94–111. The sequence and the location of the disulfide bonds are highly homologous to those of bull frog (Rana catesbeiana) egg S-lectin. They are also homologous to human angiogenin, a tumor angiogenesis factor, and a family of pancreatic ribonucleases (Kamiya et al. 1990). The positions of three disulfide bonds of Selenocosmia huwena lectin-I (SHL-I) from the venom of the Chinese bird spider S. huwena could be assigned as Cys2-Cys14, Cys7-Cys19, Cys13-Cys26 (Li and Liang 1999). Galactose-specific lectin SEL 24 K from the egg of Chinook salmon Oncorhynchus tshawytscha contains seven disulfide bonds and one pair of free cysteines. After proteolysis, peptides containing one or two disulfide bonds were identified by reduction and mass spectral comparison (Yu et al. 2007).

ERGIC-53 is present exclusively as a hexameric complex in cells. However, the hexamers exist in two forms, one as a disulfide-linked, SDS-resistant complex, and the other as a SDS-sensitive complex made up of three disulfide-linked dimers that are likely to interact through the coiled-coil domains present in the luminal part of the protein (Neve et al. 2005). Recombinant human galectin-1 secretory protein exists as an oxidized form containing three intramolecular disulfide bonds (Cys2-Cys130, Cys16-Cys88 and Cys42-Cys60). Galectin-1 promotes axonal regeneration only in the oxidized form containing three intramolecular disulfide bonds, not in the reduced form which exhibits lectin activity (Inagaki et al. 2000). Ficolins form trimer-based multimers that are N-terminally linked by disulfide bonds (Ohashi and Erickson 2004; Hummelshoj et al. 2008). Retinoschisin or RS1 is a discoidin domain-containing protein encoded by the gene responsible for X-linked retinoschisis. The RS1 functions as a cell adhesion protein to maintain the cellular organization and synaptic structure of the retina. Cys residues involved in intramolecular and intermolecular disulfide bonds are essential for protein folding and subunit assembly (Kiedzierska et al. 2007; Wu and Molday 2003).

1.5 Functions of Lectins

Interactions between cells or between cell and substratum involve specific receptors and their ligands. Lectin-like receptors are involved in signal transduction in a great variety of ways; at the molecular level; they mimic in most of the cases the function of growth factor receptor either coupled to tyrosine kinase activity or to heterotrimeric G protein. They lead to a multiplicity of cellular events following their activation depending on factors such as cellular type, species and/or tissue. Nevertheless the potential of surface lectins as transducers is emphasized by the observation

that in a few cases lectin-like receptors induce either novel signal transduction mechanism or new intracellular events with regards to what it has been observed as a consequence of growth factor receptor activation. This observation brings the idea that lectins may offer, as cell surface transducers, an alternative or additional signaling potential to cell. Lectins have no single function, and their relative abundance is not necessarily related to the importance of their function. Immune responses are mediated mainly by protein/protein interactions. In addition, protein/carbohydrate (sugar) interactions through specific lectin and chi-lectin are also involved in several immune and biological responses under not only the state of health but also inflammatory conditions. Interestingly, recent studies have identified unexpected roles of animal lectins and chi-lectin in intestinal inflammation. During fertilization, the sea urchin sperm acrosome reaction (AR), an ion channel-regulated event, is triggered by glycoproteins in egg jelly (EJ). During last two decades crystal structures of several lectins have been solved. These structures provide important insights into how these transmembrane-spanning receptors function (Rini and Lobsanov 1999).

1.5.1 Lectins in Immune System

The immune system consists of various types of cells and molecules that specifically interact with each other to initiate the host defense mechanism. Among cell surface receptors, the lectins are of peculiar interest because glycolipids, glycoproteins and proteoglycans have been shown to interact with lectins on the surface of animal cells. To initiate immune responses against infection, antigen presenting cells (APC) must recognize and react to microbes. Recognition is achieved by interaction of particular surface receptors on APC with corresponding surface molecules on infectious agents. The three types of professional APC are: (1) mature dendritic cells, found in lymphoid tissues and derived from immature tissue dendritic cells that interact with many distinct types of pathogens (Jenner et al. 2006), (2) macrophages, specialized to internalize extracellular pathogens, especially after they have been coated with antibody, and to present their antigens, and (3) B cells, which have antigen-specific receptors that enable them to internalize large amounts of specific antigen, process it, and present it naïve T cell for activation. Dendritic cells and Langerhans cells are specialized for the recognition of pathogens and have a pivotal role in the control of immunity. They are present in essentially every organ and tissue, where they operate at the interface of innate and acquired immunity. Recently, several C-type lectin and lectin-like receptors have been characterized that are expressed abundantly on the surface of these professional antigen-presenting cells. It is now becoming clear that lectin receptors not only serve as antigen receptors but also regulate the migration of dendritic cells and their interaction with lymphocytes. By contrast, pattern recognition receptors (PRR) recognize and interact with pathogens direCLRy. In addition to scavenger receptors and toll-like receptors, the PRR include the C-type lectin-like receptors (CLR) that bind carbohydrate moieties of many pathogens (Weis et al. 1998). The CLR include the following: (1) mannose receptors for mannose or its polymers (2) mannose-binding lectins for encapsulated group B or C meningococci, (3) DC-SIGN and structurally related receptors (DC-SIGNR) for mannose on HIV, *Leishmania*, and *Mycobacteria*, and (4) dectin-1 and dectin-2 for β-glucan on yeasts and fungi (Sato et al. 2006, 2009).

Collectins are a family of collagenous calcium-dependent defense lectins in animals. Their polypeptide chains consist of four regions: a cysteine-rich N-terminal domain, a collagen-like region, an alpha-helical coiled-coil neck domain and a C-terminal lectin or carbohydrate-recognition domain. These polypeptide chains form trimers that may assemble into larger oligomers. The best studied family members are the mannan-binding lectin, which is secreted into the blood by the liver, and the surfactant proteins A and D, which are secreted into the pulmonary alveolar and airway lining fluid. The collectins represent an important group of pattern recognition molecules, which bind to oligosaccharide structures and/or lipid moities on the surface of microorganisms. They bind preferentially to monosaccharide units of the mannose type, which present two vicinal hydroxyl groups in an equatorial position. High-affinity interactions between collectins and microorganisms depend, on the one hand, on the high density of the carbohydrate ligands on the microbial surface, and on the other, on the degree of oligomerization of the collectin. Apart from binding to microorganisms, the collectins can interact with receptors on host cells. Binding of collectins to microorganisms may facilitate microbial clearance through aggregation, complement activation, opsonization and activation of phagocytosis, and inhibition of microbial growth. In addition, the collectins can modulate inflammatory and allergic responses, affect apoptotic cell clearance and modulate the adaptive immune system (van de Wetering et al. 2004; Kerrigan and Brown 2009).

Lung surfactant proteins A and D bind essential carbohydrate and lipid antigens found on the surface of microorganisms via low affinity C-type lectin domains and regulate the host's response by binding to immune cell surface receptors. SP-A and SP-D contribute to host defense against respiratory viral infection. The most extensive body of evidence relates to influenza A viruses (IAV), and evidence from gene-deleted mice also indicate a role for surfactant collectins in defense against respiratory syncytial virus (RSV) and adenovirus. Despite extensive structural similarity, the two proteins show many functional differences and considerable divergence in their interactions

with microbial surface components, surfactant lipids, and other ligands. Recent data have also highlighted their involvement in clearance of apoptotic cells, hypersensitivity and a number of lung diseases. The relative importance of antiviral versus anti-inflammatory effects of SP-A and SP-D in viral infections and the potential use of these collectins as therapeutics for viral infections are under investigation. Current research suggests that structural biology approaches will help to elucidate the molecular basis of pulmonary collectin-ligand recognition and facilitate development of new therapeutics based upon SP-A and SP-D (Palaniyar et al. 2002; Seaton et al. 2010; Waters et al. 2009).

Soluble mediators, including complement components and the MBL make an important contribution to innate immune protection and work along with epithelial barriers, cellular defenses such as phagocytosis, and pattern-recognition receptors that trigger pro-inflammatory signaling cascades. These four aspects of the innate immune system act in concert to protect from pathogen invasion. Mannan-binding lectin (MBL), L-ficolin, M-ficolin and H-ficolin are all complement activating soluble pattern recognition molecules with recognition domains linked to collagen-like regions. All four may form complexes with four structurally related proteins, the three MBL-associated serine proteases (MASPs), MASP-1, MASP-2, and MASP-3, and a smaller MBL-associated protein (MAp19). The four recognition molecules recognize patterns of carbohydrate or acetyl-group containing ligands. After binding to the relevant targets all four are able to activate the complement system. We thus have a system where four different and/or overlapping patterns of microbial origin or patterns of altered-self may be recognized, but in all cases the signaling molecules, the MASPs, are shared. The clinical impact of deficiencies of MBL and MASPs in humans have been reported (Thiel 2007; Ip et al. 2009). A similar lectin-based complement system, consisting of the lectin-protease complex and C3, is present in ascidians, our closest invertebrate relatives, and functions in an opsonic manner (Fujita et al. 2004).

Ficolins are soluble oligomeric proteins composed of trimeric collagen-like regions linked to fibrinogen-related domains (FReDs) that have the ability to sense molecular patterns on both pathogens and apoptotic cell surfaces and activate the complement system. The ficolins have acetyl-binding properties, which have been localized to different binding sites in the FReD-region (Thomsen et al. 2011). From a structural point of view, ficolins are assembled from basal trimeric subunits comprising a collagen-like triple helix and a globular domain composed of 3 fibrinogen-like domains. The globular domains are responsible for sensing danger signals whereas the collagen-like stalks provide a link with immune effectors. The structure and recognition properties of the **3** human ficolins have been studied in recent years by crystallographic analysis. The ligand binding sites have been identified in the 3 ficolins and their recognition mechanisms have been characterized at the atomic level (Garlatti et al. 2009).

The mammalian natural killer gene complex (NKC) contains several families of type II transmembrane C-type lectin-like receptors (CLRs) that are best known for their involvement in the detection of virally infected or transformed cells, through the recognition of endogenous (or self) proteinacious ligands. However, certain CLR families within the NKC, particularly those expressed by myeloid cells, recognize structurally diverse ligands and perform a variety of other immune and homoeostatic functions. One such family is the 'Dectin-1 cluster' of CLRs, which includes MICL, CLEC-2, CLEC12B, CLEC9A, CLEC-1, Dectin-1 and LOX-1. We reviewed each of these CLRs, exploring our current understanding of their ligands and functions and highlighting where they have provided new insights into the underlying mechanisms of immunity and homeostasis (Huysamen and Brown 2009).

The Dectin-2 family of C-type lectins includes Dectin-2, BDCA-2, DCIR, DCAR, Clecsf8 and Mincle whose genes are clustered in the telomeric region of the NK-gene cluster on mouse chromosome 6 and human chromosome 12. These type II receptors are expressed on myeloid and non-myeloid cells and contain a single extracellular carbohydrate recognition domain and have diverse functions in both immunity and homeostasis. DCIR is the only member of the family which contains a cytoplasmic signaling motif and has been shown to act as an inhibitory receptor, while BDCA-2, Dectin-2, DCAR and Mincle all associate with FcRγ chain to induce cellular activation, including phagocytosis and cytokine production. Dectin-2 and Mincle have been shown to act as pattern recognition receptors for fungi, while DCIR acts as an attachment factor for HIV. In addition to pathogen recognition, DCIR has been shown to be pivotal in preventing autoimmune disease by controlling DC proliferation, whereas Mincle recognizes a nuclear protein released by necrotic cells (Graham and Brown 2009). Binding of fungal PAMPs to PRRs triggers the activation of innate effector cells. Recent findings underscore the role of DCs in relaying PAMP information through their PRRs to stimulate the adaptive response. In agreement, deficiencies in certain PRRs strongly impair survival to Candida infections in mice and are associated with enhanced susceptibility to mucocutaneous fungal infections in humans. Understanding the complex signaling networks protecting the host against fungal pathogens is a challenging problem (Bourgeois et al. 2010).

Macrophage lectins contribute to host defence by a variety of mechanisms. The best characterised, mannose receptor (MR) and complement receptor three (CR3), are both able to mediate phagocytosis of pathogenic microbes and induce intracellular killing mechanisms. MR is a C-type

lectin primarily expressed by macrophages and dendritic cells. Its three distinct extracellular binding sites recognise a wide range of both endogenous and exogenous ligands. The MR has been implicated in both homeostatic processes and pathogen recognition. However, the function of MR in host defence is not yet clearly understood as MR-deficient animals do not display enhanced susceptibility to pathogens bearing MR ligands. The regulation of the effector functions induced via MR is complex, and may involve both host and microbial factors (Gazi and Martinez-Pomares 2009; Linehan et al. 2000).

1.5.2 Lectins in Nervous Tissue

There is increasing evidence that lectins are widely distributed in mammalian tissues, including the nervous tissue. Based on histochemical techniques using neoglycoproteins, lectin activities specific for different monosaccharides or glycans have been identified (fucose, galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylneuraminic acid and heparin). Most of them showed a cellular specificity and developmental regulation in the CNS. Several lectins isolated from the nervous tissue seem to play an essential role during ontogenetic processes, especially as far as cell adhesion and cell recognition mechanisms are concerned (axonal growth and fasciculation, neuron migration, synaptogenesis, myelination). But some of them seem to be involved in signaling events both intracellularly (nuclear lectins) or at the cell surface by autocrine and paracrine mechanisms (R: Zanetta 1998). Siglecs (sialic acid-binding Ig-like lectins) are mainly expressed in the immune system. Sn (sialoadhesin) (siglec-1), CD22 (siglec-2) and siglec-15 are well conserved, whereas the CD33-related siglecs are undergoing rapid evolution, as reflected in their repertoires among the different mammals studied. The CD33-related siglecs are both inhibitory and activating forms of receptors. Alzheimer's disease (AD) is a progressive neurodegenerative disease. Recent progress PRRs of monocytes and macrophages has revealed that the Siglec family of receptors is an important recognition receptor for sialylated glycoproteins and glycolipids. Recent studies have revealed that microglial cells contain only one type of Siglec receptors, Siglec-11, which mediates immunosuppressive signals and thus inhibits the function of other microglial pattern recognition receptors, such as TLRs, NLRs, and RAGE receptors. Recent studies clearly indicate that aggregating amyloid plaques are masked in AD by sialylated glycoproteins and gangliosides (Lopez and Schnaar 2009). This kind of immune evasion can prevent the microglial cleansing process of aggregating amyloid plaques in AD (Crocker and Redelinghuys 2008; Salminen and Kaarniranta 2009).

Endogenous Lectins in Tumors in CNS: The analysis of endogenous sugar receptors, as part of an intercellular information code system, may represent a way of studying the mechanism of tumor differentiation and its propagation. The carbohydrate part of cellular glycoconjugates -glycoproteins, glycolipids and proteoglycans—and specific endogenous sugar receptors, i.e. lectins, can establish a system of biological recognition based on protein-sugar interactions on the cellular and subcellular levels. Presence of sugar receptors has been noted in different types of meningiomas, glioblastomas, gangliocytomas, anaplastic and well-differentiated oligodendrogliomas and ependymomas as well as in neurinomas and neurofibromas of peripheral nerves. In comparison to well-differentiated ependymomas, the anaplastic form of this tumor shows generally a higher capacity to specifically bind the neoglycoproteins, containing α- or β-glucosides. Inverse intensity of glycohistochemical reaction is observed with galactose-6-phosphate-, galactose-β(1.3)-N-acetylglucosamine-N-acetyl-D-glucosamine- and mannose-(BSA-biotin), respectively, in anaplastic and differentiated oligodendrogliomas. Dedifferentiated-tumorous neurons, i.e. gangliocytomas, and distinct subtypes of meningiomas show an altered spectrum of endogenous sugar receptors in comparison to neurons of normal counterparts. Receptors for N-acetyl-D-galactosamine were present only in the anaplastic form, while glucuronic acid-specific receptors were found only in the meningotheliomatous meningiomas. Analysis of the spectrum of endogenous sugar receptors can serve to distinguish between different cell populations composing a given tumor, as shown in neurofibromas in the cases of Schwann cells and fibroblastoid cells stained with N-acetyl-D-glucosamine (Bardosi et al. 1991).

1.6 The Sugar Code and the Lectins as Recepors in System Biology

In order to understand intra- and intercellular processes, the parameters based on protein sequences are not sufficient to explain molecular events in the cellular processes such as cell adhesion or cell communication. Carbohydrates play a major role in such processes. Carbohydrates are often referred to as the third molecular chain of life, after DNA and proteins. Cabohydrate-protein interactions are responsible for important biological functions such as inter-cellular communication particularly in the immune system. Carbohydrates are uniquely suited for this role in molecular recognition, as they possess the capacity to generate an array of structurally diverse moieties from a relatively small number of monosaccharide units. This could be attributed to the fact, that unlike the components of nucleic acids, carbohydrates can link together in multiple, nonlinear ways because each building block has about four functional groups for linkage. They can

even form branched chains. Hence, the number of possible polysaccharides is enormous. Since carbohydrates assume a large variety of configurations, many carbohydrate-binding proteins are being considered as targets for new medicines. The accurate *in silico* identification of carbohydrate-binding sites is a key issue in genome annotation and drug targeting. Different aspects of protein carbohydrate recognition have also been extensively studied.

Sugars in the form of monosaccharides, oligosaccharides, polysaccharides and glycoconjugates (glycoproteins, glycolipids) are vital components of pathogens and host cells, and are involved in cell signaling associated with modulation of inflammation in all integumental structures. Infact, sugars are the molecules most commonly involved in cell recognition and communication. In skin, they are essential to epidermal development and homeostasis. They play important roles in microbial adherence, colonization and biofilm formation, and in virulence. Thus, it shows that biological information is not only stored in protein sequences but also in the structure of the glycan part of the glycoconjugates. For example, in immune system, the spatially accessible carbohydrate structures that contribute to the cell's glycome are decoded by recognition systems in order to maintain the immune homeostasis of an organism. Hence the term 'Sugar code' has been introduced to bridge the gap between cell's communication and adhesion. Sugar code like genetic code yet to be defined in the form of monosaccharide alphabets, possesses more information and may have more storage capacity than other molecular units. As suggested (Gabius 2000) oligosaccharides surpass peptides by more than seven orders of magnitude in the theoretical ability to build isomers, when the total of conceivable hexamers is calculated.

1.6.1 Host-Pathogen Interactions

Study on protein-carbohydrate interactions is an exciting area of research with huge potential for development and exploration. This is particularly true for host-pathogen interactions that lead to infectious diseases; as the surfaces of cells and pathogens display complex carbohydrate structures and carbohydrate binding proteins on their surface. Most of the infectious diseases occur due to protein-carbohydrate interactions on the surface of host and disease causing pathogen (Malik and Ahmad 2010). As a result, considerable efforts have been directed toward understanding and mimicking the recognition processes and developing effective agents to develop carbohydrate-based therapeutics, targeting of drugs to specific disease cells via carbohydrate–lectin interactions; and carbohydrate based anti-thrombotic agents. Although, many human pathogens, including the influenza virus, possess surface proteins that complex with specific membrane-bound oligosaccharides on human cells (Sonnenburg et al. 2004; Takahashi et al. 2004; Varki et al. 2009), the cell surface glycans continually undergo structural variations during disease progression. Hence the variety of carbohydrate structures that occur on diseased cells gives rise to highly complex carbohydrate–lectin interactions and signaling processes. Emerging roles of carbohydrates and glycomimetics in anticancer drug design are being recognized. The information from these studies is useful for designing tailored compounds that inhibit a glycan-binding site.

Pathogens Are Recognized by Different Lectin Receptors: Innate immunity is the earliest response to invading microbes and acts to contain infection in the first minutes to hours of challenge. Unlike adaptive immunity that relies upon clonal expansion of cells that emerge days after antigenic challenge, the innate immune response is immediate. The glycocalyx is a glycan layer found on the surfaces of host cells as well as microorganisms and enveloped virus. Its thickness may easily exceed 50 nm. The glycocalyx does not only serve as a physical protective barrier but also contains various structurally different glycans, which provide cell- or microorganism-specific 'glycoinformation'. This information is decoded by host glycan-binding proteins, lectins. Pathogen recognition is central to the induction of adaptive immunity. Dendritic cells (DCs) express different pattern recognition receptors (PRRs), such as Toll-like receptors and C-type lectins that sense invading pathogens. Pathogens trigger a specific set of PRRs, leading to activation of intracellular signaling processes that shapes the adaptive immunity. It is becoming clear that cross talk between these signaling routes is crucial for pathogen-tailored immune responses. The DC-SIGN interacts with different mannose-expressing pathogens such as Mycobacterium tuberculosis and HIV-1. Mycobacterium tuberculosis (Mtb) is recognized by pattern recognition receptors on macrophages and DCs, thereby triggering phagocytosis, antigen presentation to T cells and cytokine secretion. The DC-SIGN has specificity for mannose-containing glycoconjugates and fucose-containing Lewis antigens. Mannosylated moieties of the mycobacterial cell wall were shown to bind to DC-SIGN on immature dendritic cells and macrophage subpopulations. This interaction reportedly impaired dendritic cell maturation, modulated cytokine secretion by phagocytes and DCs and was postulated to cause suppression of protective immunity to TB. However, experimental Mtb infections in mice transgenic for human DC-SIGN revealed that, instead of favoring immune evasion of mycobacteria, DC-SIGN may promote host protection by limiting tissue pathology. The dominant Mtb-derived ligands for DC-SIGN are presently uncertain, and a major role of DC-SIGN in the immune response to Mtb infection may lie in its capacity to maintain a balanced inflammatory state during chronic TB. For several pathogens that interact with DC-SIGN, including Mycobacterium tuberculosis and HIV-1,

Raf-1 activation leads to acetylation of NF-kB subunit p65, which induces specific gene transcription profiles. In addition, other DC-SIGN-ligands induce different signaling pathways downstream of Raf-1, indicating that DC-SIGN-signaling is tailored to the pathogen (Dunnen et al. 2009; Ehlers 2010; Gringhuis and Geijtenbeek 2010). Many of these C-type lectin receptors (CLRs), coupled to Syk kinase, signal via CARD9 leading to NF-κB activation, which in turn contributes to the induction of both innate and adaptive immunity. Dectin-1, Dectin-2 and Mincle are all CLRs that share this common signaling mechanism and have been shown to play key roles in antifungal immunity (Drummond et al. 2011). Thus, interaction of CLRs and TLRs with PAMPs initiates a cascade of events leading to production of reactive oxygen intermediates, cytokines and chemokines, and promotes inflammation. Thus sugars may provide valuable adjunctive anti-inflammatory and/or antimicrobial treatment (Geijtenbeek and Gringhuis 2009; Lloyd et al. 2007). Microbial PAMPs are recogniized by CLRs such as MBL, the tandem-repeat-type macrophage mannose receptor, DC-SIGN or dectin-1 of dendritic cells, certain TLRS or the TCR of NKT cells. Galectins, the key sensors reading the high-density sugar code, exert regulatory functions on activated T cells, among other activities. Autoimmune diseases are being associated with defined changes of glycosylation. This correlation deserves to be thoroughly studied on the levels of structural mimicry and dysregulation as well as effector molecules to devise innovative anti-inflammatory strategies (Buzás et al. 2006).

Pathogen recognition by dendritic cells (DCs) is central to the induction of adaptive immunity. PRRs on DCs interact with pathogens, leading to signaling events that dictate adaptive immune responses. It is becoming clear that C-type lectins are important PRRs that recognize carbohydrate structures. Triggering of C-type lectins induces signaling cascades that initiate or modulate specific cytokine responses and therefore tailor T cell polarization to the pathogens. Under steady state conditions DCs continuously sample antigens, leading to tolerance, whereas inflammatory conditions activate DCs, inducing immune activation. DCs express CLRs for antigen capture and presentation, whereas TLRs are involved in pathogen recognition and DC activation. Recent reports demonstrate that communication between TLRs and CLRs can affect the direction of immune responses. Several pathogens specifically target CLRs to subvert this communication to escape immune surveillance, either by inducing tolerance or skewing the protective immune responses (Geijtenbeek et al. 2004; van Kooyk et al. 2004). Langerhans cells (LC) are the first DCs to encounter pathogens entering the body via mucosa or skin. Equipped with PRRs, LCs are able to detect and respond to pathogens. Langarin is an important CLR, exclusively expressed by LC in humans. Langerin forms a protective barrier against HIV-1 infection by binding and degradation of this virus. In addition, antigens targeted to Langerin are presented to T cells to induce an adaptive immune response. How its functions and how Langerin polymorphisms influence the function of Langerhans cells, has been reviewed (van der Vlist and Geijtenbeek 2010). However, evidence is accumulating that many CLRs are also able to recognize endogenous 'self' ligands and that this recognition event often plays an important role in immune homeostasis. Endogenous ligands for human and mouse CLRs have been described. Special attention has been drawn to the signaling events initiated upon recognition of the self ligand and the regulation of glycosylation as a switch modulating CLR recognition, and finally in immune homeostasis (García-Vallejo and van Kooyk 2009).

Known biological function//targets of carbohydrates and combinatorial synthesis, structural analysis of natural//non-natural carbohydrates and further insights into the functional consequences of the sugar code's translation are thus expected to have notable repercussions for diagnostic and therapeutic procedures (Gabius 2011). To fill the need for expression analysis of glycogenes, Comelli et al. (2006) employed the Affymetrix technology to develop a focused and highly annotated glycogene-chip representing human and murine glycogenes, including glycosyltransferases, nucleotide sugar transporters, glycosidases, proteoglycans, and glycan-binding proteins. Comparison of gene expression profiles with MALDI-TOF profiling of N-linked oligosaccharides suggested that the α1-3 fucosyltransferase 9 is the enzyme responsible for terminal fucosylation in kidney and brain- a finding validated by analysis of fucosyltransferase 9 knockout mice. Two families of lectins, C-type lectins and Siglecs, are predominately expressed in the immune tissues, consistent with the emerging functions in both innate and acquired immunity.

1.6.2 Altered Glycosylation in Cancer Cells

Altered glycosylation is a universal feature of cancer cells, and certain glycan structures are well-known markers for tumor progression. Some glycan biosynthetic pathways are frequently altered in cancer cells. Reports suggest that the expression of branched and sialylated complex type N-oligosaccharides in human melanoma consistently increases in cells from metastatic sites, and support the view that carbohydrates are associated with the acquisition of the metastatic potential of tumor cells (Litynska et al. 2001; Ciolczyk-Wierzbicka et al. 2002). Glycosylation can be altered in various ways in malignancy. Classic reports of increased size of tumor cell–derived glycopeptides have been convincingly explained by an increase in β1–6 branching of N-glycans (Fig. 1.4), which results from enhanced expression of UDP-GlcNAc:N-glycan GlcNAc

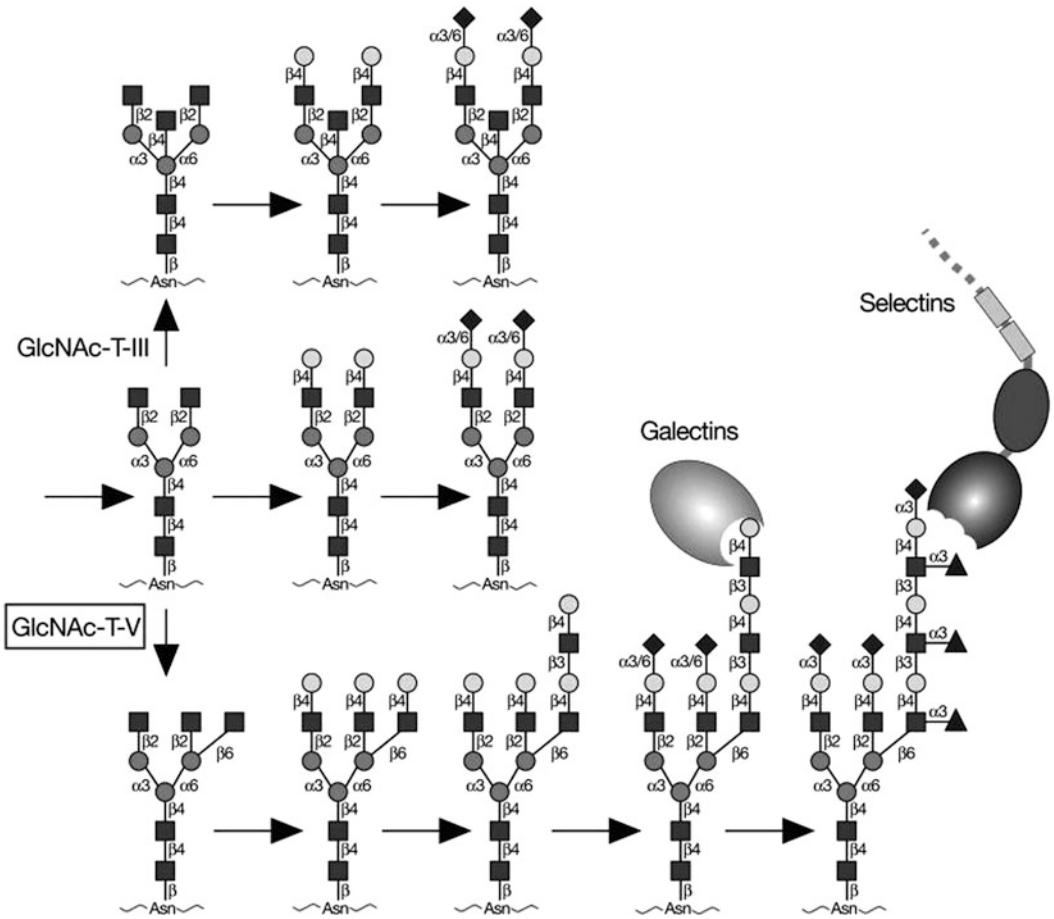

Fig.1.4 N-Glycan: Glycan covalently linked to an asparagine residue of a polypeptide chain in the consensus sequence: -Asn-X-Ser/Thr. Unless otherwise stated, the term N-glycan denotes the most common linkage region, Manβ1–4GlcNAcβ1–4GlcNAcβ1-N-Asn. The increased size of N-glycans that occurs upon transformation can be explained by an elevation in GlcNAc transferase-V (GNT-V) activity, which catalyzes the β1–6 branching of N-glycans. This, in turn, may lead to enhanced expression of poly-*N*-acetyllactosamines, which can also be sialylated and fucosylated. These structures are potentially recognized by galectins and selectins, respectively. The structural consequences of increased expression of GlcNAc transferase-III (GNT-III) are also shown (Adapted by permission from Varki et al. 2009 © The Consortium of Glycobiology Editors, La Jolla, California) ○ Galactose (Gal); ● Mannose (Man); ■ N-Acetylglucosamine (GlcNac); ◆ N-Acetylneuraminic acid (Neu 5Ac); ▲ Fucose (Fuc)

transferase V (GlcNAcT-V). Reports indicate that GlcNAcT-V plays an important part in the biology of cancer. Enhanced expression of another glycosyltransferase affecting N-glycan structure UDP-GlcNAc:N-glycan GlcNAc transferase III (GlcNAcT-III), which catalyzes the addition of the bisecting GlcNAc branch, has been reported in certain tumors, such as rat hepatomas. Although GlcNAcT-III expression appears to affect the biology of tumors, the results are not as clear-cut and consistent as those seen with GlcNAcT-V (Varki et al. 2009).

1.6.3 Protein-Carbohydrate Interactions in Immune System

The immune system consists of various types of cells and molecules that specifically interact with each other to initiate the host defense mechanism. Recent studies have shown that carbohydrates and lectins play an essential role in mediating such interactions. Carbohydrates, due to their chemical nature, can potentially form structures that are more variable than proteins and nucleic acids. The interactions between lectins and carbohydrates have been shown to be involved in such activities as opsonization of microorganisms, cell adhesion and migration, cell activation and differentiation, and apoptosis. The number of lectins identified in the immune system is increasing at a rapid pace. The development in this area has opened a new aspect in studying the immune system, and at the same time, provided new therapeutic routes for the treatment and prevention of disease.

Protein-carbohydrate interactions are used for intercellular communication. Mammalian cells are known to bear a variety of glycoconjugates. The discovery of plant lectins as biochemical reagents gave a great impulse to modern

glycobiology and in the biomedical research. Plant lectins have been fundamental in human immunological studies because some of them are mitogenic/activating to lymphocytes. The understanding the molecular basis of lectin-carbohydrate interactions and of the intracellular signaling evoked holds promise for the design of novel drugs for the treatment of infectious, inflammatory and malignant diseases. It may also be of help for the structural and functional investigation of glycoconjugates and their changes during physiological and pathological processes.

1.6.4 Glycosylation and the Immune System

1.6.4.1 Carbohydrate-Mediated Recognition in Immune System

Almost all key molecules involved in the innate and adaptive immune response are glycoproteins. In the cellular immune system, specific glycoforms participate in the folding, quality control, and assembly of peptide-loaded major MHC antigens and the T cell receptor complex. Recent evidence indicates that protein-glycan interactions play a critical role in different events associated with the physiology of T-cell responses including thymocyte maturation, T-cell activation, lymphocyte migration and T-cell apoptosis. Glycans decorating T-cell surface glycoproteins can modulate T-cell physiology by specifically interacting with endogenous lectins including selectins and galectins. Sometimes, the generation of peptide antigens from glycoproteins may require enzymatic removal of sugars before the protein can be cleaved. Oligosaccharides attached to glycoproteins at the junction between T cells and antigen-presenting cells help to orient binding faces, provide protease protection, and restrict nonspecific lateral protein-protein interactions. In the humoral immune system, all of the immunoglobulins and most of the complement components are glycosylated. Although a major function for sugars is to contribute to the stability of the proteins to which they are attached, specific glycoforms are involved in recognition events. Among carbohydrate-recognizing receptors of the innate immune system are the members of the C-type lectin family, which include the collectins and the selectins and which operate by ligating exogenous (microbial) or endogenous carbohydrates. In rheumatoid arthritis, an autoimmune disease, a galactosylated glycoforms of aggregated IgG may induce association with the mannose-binding lectin and contribute to the pathology.

1.6.4.2 The Impact of Differential Glycosylation on T Cell Responses

Protein-carbohydrate interactions may be controlled at different levels, including regulated expression of lectins during T-cell maturation and differentiation and the spatio-temporal regulation of glycosyltransferases and glycosidases, which create and modify sugar structures present in T-cell surface glycoproteins. Galactose (Gal)-containing structures are involved in both the innate and adaptive immune systems. Gal is a ligand for Gal/N-acetylgalactosmine (GalNAc) receptors and galectins. Galactose is part of the scaffold structure that synthesizes oligosaccharide ligands for selectins, siglecs and other lectins of the immune system. Gal residues are added to glycoproteins and glycolipids by members of a large family of galactosyltransferases. Specific galactosyltransferases have been shown to control cell adhesion and leukocyte functions. Antibodies need to be galactosylated for normal function, and under-galactosylated immunoglobulin (Ig) is associated with rheumatoid arthritis, while Gal is lacking in the IgA of patients with IgA nephropathy. Interactions involving Gal play important roles in host defenses; they can also result in serious pathophysiology. Galactosyltransferases represent potential targets for the control of cell growth and apoptosis, inflammation and infections (Brockhausen 2006). Galectins participate in a wide spectrum of immunological processes. These proteins regulate the development of pathogenic T-cell responses by influencing T-cell survival, activation and cytokine secretion. Administration of recombinant galectins or their genes into the cells modulate the development and severity of chronic inflammatory responses in experimental models of autoimmunity by triggering different immunoregulatory mechanisms. Thus galectins have potential use as novel anti-inflammatory agents or targets for immunosuppressive drugs, in autoimmune and chronic inflammatory disorders (Bianco et al. 2006; Daniels et al. 2002; Toscano et al. 2007).

1.6.5 Principles of Protein-Glycan Interactions

Emerging roles of carbohydrates and glycomimetics in drug design are being recognized. The information from these studies is useful for designing tailored compounds that inhibit a glycan-binding site. Understanding the principles of glycan–protein interactions led to the development of high-affinity inhibitors of carbohydrate-protein interactions such as for viral neuraminidase, which is involved in the removal of sialic acids from host glycoprotein receptors; these inhibitors have proven utility in reducing the duration and spread of influenza infection. However, knowledge of protein and carbohydrate structure alone does not ensure accurate prediction of function and biological activity. A well-known characteristic of protein–carbohydrate interactions is the low affinity of binding, usually in mM range, but the estimates of binding affinities are not accurate due

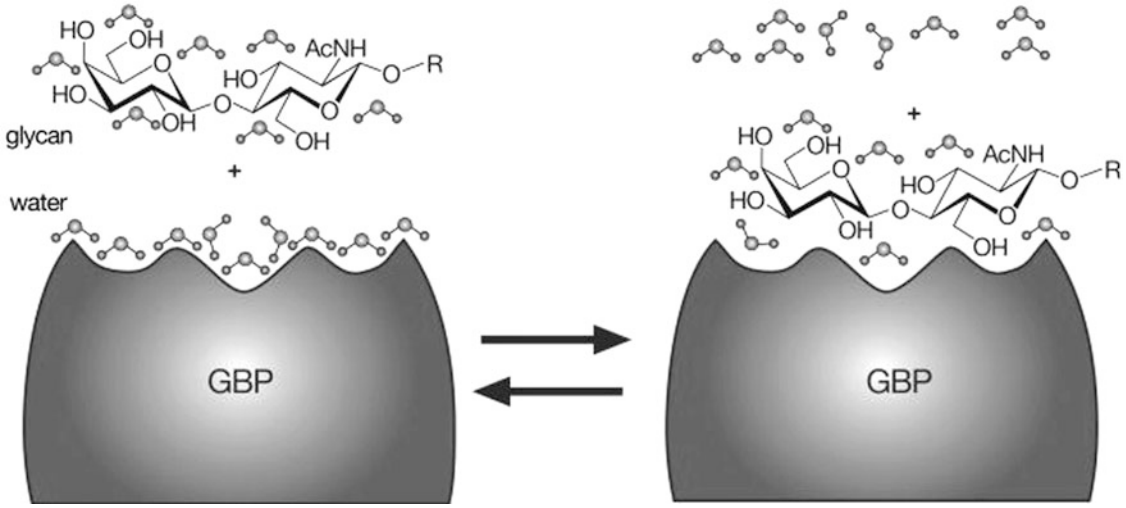

Fig. 1.5 Schematic diagram showing interaction between hydrated polyhydroxylated glycan and a hydrated protein-combining site of a GBP in water, resulting in the displacement of water (Reprinted with permission from Cummings and Esko, 2009 © The Consortium of Glycobiology Editors, La Jolla, California)

to several complications. As shown in Fig. 1.5, the overall process of binding typically involves the union of a hydrated polyhydroxylated glycan and a hydrated protein-combining site. If a surface on the glycan is complementary to the protein-combining site, water can be displaced and binding occurs. When the complex finally forms, it presents a new surface to the surrounding medium, which will also be hydrated. As solvation/desolvation energies are very large because of entropy from the disordering of water molecules, their contribution to binding cannot be reliably determined with existing models (Cummings and Esko 2009). Furthermore, glycans may undergo a conformational change upon binding, changing their internal energy and solvation. Schematic diagram of the binding of a glycan-binding protein (GBP) in water, which results in the displacement of water.bonding interactions in the combining site, the estimation of solvation energy changes is difficult. Another complicating issue concerns valency, which greatly increases the association of GBPs with their glycan ligands under biological conditions (Cummings and Esko 2009). The density of binding sites on the ligand can also affect the affinity of binding. Ligands for some GBPs may be glycoproteins that carry one or more multiantennary N-linked chains. Mucins present potentially hundreds of glycans, and their proximity can affect conformation and presentation of the ligands. Some polysaccharides, such as glycosaminoglycans, have multiple binding sites located along a single chain. In addition, nonglycan components, such as tyrosine sulfate, lipid, or peptide determinants, may also cooperate with glycans to provide relatively high affinity and specific interactions.

Glycan–protein complexes solved by X-ray crystallography and NMR spectroscopy revealed that the glycan-binding sites typically accommodate one to four sugar residues, although in some complexes, recognition extends over larger numbers of residues and may include aglycone components, such as the peptide or lipids to which the glycans are attached. Information on the three-dimensional structure of a glycan-protein complex can reveal the specificity of binding, changes in conformation that take place on binding, and the contribution of specific amino acids to the interaction. However, one would also like to determine the affinity of the interaction. Since the forces involved in the binding of a glycan to a protein are the same as for the binding of any ligand to its receptor (hydrogen bonding, electrostatic or charge interactions, van der Waals interactions, and dipole attraction), it is tempting to try to calculate their contribution to overall binding energy, which can be related to an affinity constant (K_a). Unfortunately, calculating the free energy of association is difficult for several reasons, including problems in defining the conformation of the unbound versus the bound glycan, changes in bound water within the glycan and the binding site, and conformational changes in the GBP upon binding.

The complete characterization of any binding interaction requires a quantification of the affinity, number of binding sites, and the thermodynamic parameters. Thermodynamic data, specifically enthalpy (ΔH) and entropy (ΔS), reveal the forces that drive complex formation and mechanism of action. Thermodynamics provide information on conformational changes, hydrogen bonding, hydrophobic interactions, and charge-charge interactions. These interactions are driven by a favourable enthalpy (heat is released), offset by the multiple contact points (hydrogen bonds, van der Waals' interactions and hydrophobic stacking) between the carbohydrate and the protein. Recent

studies have shown that in the binding site, polar–polar interactions are actually stronger than in the corresponding protein–protein interactions. One reason for this is shorter hydrogen bond distances, partly because hydrogen bonds involve charged groups as opposed to the polar–polar interactions in proteins, which mainly involve non-charged groups. In addition, highly-organized hydrogen-binding atoms and a higher frequency of hydrogen bonds per unit area result in a closely spaced protein–carbohydrate interface (Holgersson et al. 2005).

The relatively high association of enthalpies of protein–carbohydrate (as compared to protein–protein) interactions has been ascribed to a higher degree of hydrogen bonds per unit area and shorter distances between the molecules involved in the hydrogen binding. The amphiphilic nature of carbohydrates results in different interactions with water molecules in the vicinity. The hydrophobic ring core represents a field of low dielectricity, resulting in densely packed water molecules. The hydroxyl groups, on the other hand, form hydrogen bonds with the closest water molecules, thus forcing these molecules to arrange themselves in a configuration that is energetically less favourable than the ones formed by bulk water molecules (Fig. 1.5). During protein–carbohydrate complexation, water molecules tend to escape to the bulk with a concomitant decrease or increase in energy depending on their pre-existing molecular interactions. After protein–carbohydrate binding has occurred, the complex formed will be resolvated and the water molecules rearrange themselves according to the new surface exposed (Holgersson et al. 2005). The affinity of most single glycan–protein interactions is generally low (mM–μM K_D values). In nature, many GBPs are oligomeric or may be membrane-associated proteins, which allows aggregation of the GBP in the plane of the membrane. Many of the glycan ligands for GBPs are also multivalent. In the case of cholera toxin, five B subunits present in the holotoxin interact with five molecules of GM1 normally present in the cell membrane. The interaction of multiple subunits with a multivalent display of GM1 raises the affinity of interaction by several orders of magnitude (K_D of ~40 pM).

1.7 Applications of Lectin Research and Future Perspectives

Lectins are used in wide variety of areas as in separation of glycoproteins and glycoconjugates, histochemistry of cells and tissues, cell differentiation, and in tracing cell surface pathways. These molecules are of great interest to immunologists mainly because of their ability to interact with lymphocytes and to induce blast cell transformation. Purified lectins are important in a clinical setting because they are used for blood typing . Some of the glycolipids and glycoproteins on an individual's red blood cells can be identified by lectins (such as lecin from Dolichos biflorus for A1 blood group, Ulex europaeus lectin for detection of H blood group antigen, and lecin from Vicia graminea in identification of N blood group antigen). The anterograde labeling method is used to trace the path of efferent axons with PHA-L, a lectin from the kidney bean (Carlson 2007). A lectin (BanLec) from bananas inhibits HIV-1 in vitro (Swanson et al. 2010). Lectins can be used in biochemical warfare. One example of the powerful biological attributes of lectins is the biochemical warfare agent ricin. Ricin from seeds of castor oil plant cleaves nucleobases from ribosomal RNA resulting in inhibition of protein synthesis and cell death (Lord et al. 1994). Growing insights into the functionality of lectin-carbohydrate interactions are identifying attractive new targets for drug design and drug targeting (Gupta et al. 2009). As glycan recognition is regulated by the structure of the sugar epitope and also by topological aspects of its presentation, a suitable arrangement of ligands in synthetic glycoclusters has the potential to enhance their avidity and selectivity. If adequately realized, such compounds might find medical applications (Lyra and Diniz 2007). Lectins of algal origin whose antiviral properties make them candidate agents for prevention of viral transmission through topical applications include cyanovirin-N, *Microcystis viridis* lectin, scytovirin, and griffithsin. Although all these proteins exhibit significant antiviral activity, their structures are unrelated and their mode of binding of carbohydrates differs significantly.

1.7.1 Mannose Receptor-Targeted Vaccines

Targeting antigens to endocytic receptors on professional APCs such as DCs represents an attractive strategy to enhance the efficacy of vaccines. Such APC-targeted vaccines have an exceptional ability to guide exogenous protein antigens into vesicles that efficiently process the antigen for MHC class I and class II presentation. The specific targeting of nanomedicines to mannose receptors (MR) and related C-type lectin receptors, highly expressed in cells of the immune system, perform a useful strategy for improving the efficacy of vaccines and chemotherapy (Gupta et al. 2009). The MR and related C-type lectin receptors are particularly designed to sample antigens (self and non-self), much like pattern recognition receptors, to integrate the innate with adaptive immune responses. Certainly, a better understanding of the mechanism associated with the induction of immune responses as a result of targeting antigens to the MR, will be important in exploiting MR-targeted vaccines not only for mounting immune defenses against cancer and infectious disease, but also for specific induction

of tolerance in the treatment of autoimmune diseases (Ahlers and Belyakov 2009). The DC-SIGN whose expression is restricted to DCs has gained an increased attention because of its involvement in multiple aspects of immune function. Upon DC-SIGN engagement by mannose- or fucose-containing oligosaccharides, the latter leads to a tailored Toll-like receptor signaling, resulting in an altered DC-cytokine profile and skewing of Th1/Th2 responses (Svajger et al. 2010) (Refer Chap. 46).

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Part II

Intracellular Lectins

Lectins in Quality Control: Calnexin and Calreticulin 2

G.S. Gupta

2.1 Chaperons

A long-standing enigma has been the role of *N*-linked glycans attached to many proteins in the endoplasmic reticulum (ER) and their co- and posttranslational remodelling along the secretory pathway. Evidence is accumulating that intracellular animal lectins play important roles in quality control and glycoprotein sorting along the secretory pathway. Calnexin and calreticulin in conjunction with associated chaperones promote correct folding and oligomerization of many glycoproteins in the ER. The discovery that one of these glycan modifications, mannose 6-phosphate, serves as a lysosomal targeting signal that is recognized by mannose 6-phosphate receptors has led to the notion that lectins may play more general roles in exocytotic protein trafficking (Chaps. 3–6). In present and subsequent chapters we discuss the role of intracellular lectins in quality control (QC) and their role in understanding the mechanisms underlying protein traffic in the secretory pathway (Chaps. 3–7) (Table 2.1).

In eukaryotic cells, the ER plays an essential role in the synthesis and maturation of a variety of important secretory and membrane proteins. The ER is also considered one of the most important and metabolically relevant sources of cellular Ca^{2+}. The ability of ER to control Ca^{2+} homoeostasis has profound effects on many cell functions. To achieve its function the ER and its lumen contain a characteristic set of resident proteins that are involved in every aspect of ER function. For glycoproteins, the ER possesses a dedicated maturation system, which assists folding and ensures the quality of final products before ER release. A chaperone is a protein that binds transiently to newly synthesized proteins and assists in newly synthesized proteins in folding (Ellgaard and Helenius 2003; Trombetta and Parodi 2003). Essential components of this system include the lectin chaperones calnexin (Cnx) and calreticulin (Crt) and their associated co-chaperone ERp57, a glycoprotein specific thiol-disulfide oxidoreductase. The significance of this system is underscored by the fact that Cnx and Crt interact with practically all glycoproteins investigated to date, and by the debilitating phenotypes revealed in knockout mice deficient in either gene. Compared to other important chaperone systems, such as the Hsp70s, Hsp90s and GroEL/GroES, the principles whereby this system works at the molecular level are relatively poorly understood. However, structural and biochemical data have provided important new insights into this chaperone system and present a solid basis for further mechanistic studies. Both Crt and Cnx act as lectins which recognize CRD and act as molecular chaperones. Both of them bind monoglucosylated proteins and associate with the thiol oxidoreductase ERp57, which is a protein disulfide isomerase (PDI)-like protein resident in ER and promotes disulfide formation/isomerization in glycoproteins. Calreticulin, together with Cnx and ERp57 comprise the so-called "calreticulin/calnexin cycle", which is responsible for QC and folding in newly synthesized (glyco) proteins.

Crt/Cnx pathway is thought to monitor protein conformation through modification of N-linked glycans covalently attached to Asn residues through the activity of glycosyl transferases, glucosidases and chaperones. Folding enzymes such as PDI and ERp57 interact with polypeptides displaying non-native disulfide bonds, effectively reorganizing these covalent cross-links to their native pattern. ERp57, a thiol oxidoreductase that catalyzes disulfide formation in heavy chains of MHC class I molecules, also forms a mixed disulfide with tapasin within the class I peptide loading complex, stabilizing the complex and promoting efficient binding of peptides to class I molecules. ERp57 appears to play a structural rather than catalytic role within the peptide loading complex (Zhang et al. 2009). The PDI can help the formation of disulfide bonds that are the critical structure of protein secondary structure (Jun-Chao et al. 2006).

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Table 2.1 Lectins associated in quality control of proteins and the secretory pathway

Lectin	Localization	Sugar specificity	Major function
Calnexin	ER	Gluc + Man	Folding and degradation
Calreticulin	ER	Gluc + Man	Folding and degradation
F-Box Proteins	ER	Man	Degradation
EDEM	ER	Man	Degradation
CD-MPR[a]	LE	Man-6-P	Golgi-to-endosome transport
IGF-II/CI-MPR[b]	LE	Man-6-P	Golgi-to-endosome and plasma membrane-to -endosome transport
ERGIC-53	ERGIC	Man	ER-to-ERGIC transport
VIP36	cis-Golgi/ ERGIC	Man6–9	Retrieval?

[a]CD-MPR = Cation-dependent mannose 6-phosphate receptor
[b]IGF-II/CI-MPR = Insulin-like growth factor-II or cation-independent mannose 6-phosphate/receptor; ER = Endoplasmic reticulum; LE = late endosome

2.1.1 Calnexin

The Calnexin Protein

Calnexin is a 90-kDa integral membrane protein of the ER. It binds Ca^{2+} and functions as a chaperone in the transition of proteins from ER to outer cellular membrane. Calnexin has been cloned from placenta. A subdomain containing four internal repeats binds Ca^{2+} with highest affinity. This sequence is highly conserved when compared to calreticulin, and yeast and plant calnexin homologues. An adjacent subdomain, also highly conserved and containing four internal repeats, failed to bind Ca^{2+}. The carboxyl-terminal, cytosolic domain is highly charged and binds Ca^{2+} with moderate affinity. The calnexin amino-terminal domain (residues 1–253) also binds Ca^{2+}, in contrast to the amino-terminal domain of calreticulin, which is relatively less acidic. A subdomain containing four internal repeats binds Ca^{2+} with the highest affinity. This sequence is highly conserved when compared to Crt, and yeast and plant Cnx homologues. An adjacent subdomain, also highly conserved and containing four internal repeats, failed to bind Ca^{2+}. Cnx cDNA is highly conserved when compared to calreticulin, an Onchocerca surface antigen, and yeast and plant Cnx homologues. Comparison of mouse and rat calnexin sequences reveals very high conservation of sequence identity (93–98%), suggesting that calnexin performs important cellular functions. The gene for human calnexin is located on the distal end of the long arm of human chromosome 5 at 5q35 (Ellgaard et al. 1994; Tjoelker et al. 1994).

Calmegin: A Male Germ Cell Specific Homologue of Calnexin

During mammalian spermatogenesis, many specific molecules are expressed. A 93-kDa male meiotic germ cell-specific antigen (Meg 1) is exclusively expressed in germ cells from pachytene spermatocyte to spermatid stage (Watanabe et al. 1994). A cDNA from mouse testis showed this transcript of 2.3 kb in length and expressed only in testis and not in other somatic tissues or in ovary. The expression of the mRNA was first detected at pachytene spermatocyte stage of male germ cell development. The predicted protein consists of 611 amino acids, including a hydrophobic NH2 terminus characteristic of a signal peptide, two sets of internal repetitive sequences (four repeats of IPDPSAVKPEDWDD and GEWXPPMIPNPXYQ), and a hydrophilic COOH terminus. The deduced amino acid sequence showed 58% homology with dog Cnx and significant partial homology with Crt at repetitive sequence. The name calmegin was proposed for this antigen. Calmegin is a Ca^{2+}-binding protein that is specifically expressed in spermatogenesis (Watanabe et al. 1994). *Calmegin* gene contained GC-rich sequences and potential binding sites for AP2 and Sp1, but lacked TATA sequence. The CAT gene activity was detected exclusively in testes, indicating that the 330 bp calmegin 5′ sequence was sufficient for the testis-specific expression. The existence of testicular nuclear factors specifically bound to the putative promoter sequence was also demonstrated (Watanabe et al. 1995). The human homolog of mouse Calmegin showed 80% identity with the mouse calmegin and strong conservation of two sets of internal repetitive sequences (Ca^{2+} binding motif), and the hydrophilic COOH terminus, which corresponds to the putative ER retention motif. The transcript was 3 kb in length and was expressed exclusively in the testis. Human Calmegin gene was mapped to chromosome 4q28.3–q31.1 (c/r Gupta 2005). Calmegin functions as a chaperone for one or more sperm surface proteins that mediate the interactions between sperm and egg. The defective zona pellucida-adhesion phenotype of sperm from calmegin-deficient mice is reminiscent of certain cases of unexplained infertility in human males (Ikawa et al. 1997). These results suggest that spermatogenic cell endoplasmic reticulum has a unique calcium binding protein, calnexin-t, which appears to be a calnexin variant.

2.1.2 Calnexin Structure

The ER luminal segment of Cnx and its soluble paralog Crt share sequence similarity that is most pronounced in a central segment containing two proline-rich sequence motifs repeated in tandem (David et al. 1993). Motifs 1 and 2, which are repeated three times each in Crt and four times each in Cnx, have consensus sequences of I-DP(D/E) A-KPEDWD(D/E) and G-W-P-IN-P-Y, respectively. In addition, there are three segments of high sequence similarity, A, B, and C, with the last two flanking the repeat motifs (Fig. 2.1 and 2.2). The globular domain contains the oligosaccharide-binding site with amino acids that contact

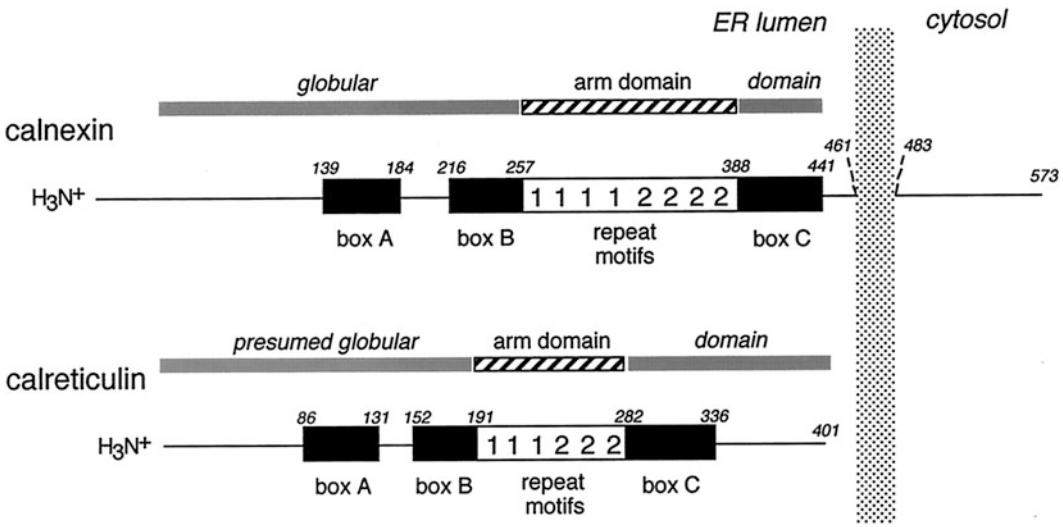

Fig. 2.1 Comparison of the linear sequences of CNX and CRT. Regions of sequence similarity are represented by *large rectangles*. The *white rectangles* correspond to segments with highest identity which are comprised of two sequence motifs repeated in tandem (indicated by the *numbers 1* and *2*). *Black rectangles* represent segments that share substantial sequence identity, and they are termed *boxes A*, *B*, and *C* to facilitate discussion. Two domains identified in the x-ray crystal structure of CNX are shown: an arm domain that corresponds to the repeat motifs (*hatched bar*) and a globular domain (*gray bar*). An arm domain has also been identified in CRT (*hatched bar*) which corresponds to the repeat motifs. The structure of the remainder of the molecule has not been solved but is presumed to form a globular domain analogous to that of CNX (*gray bar*) (Reprinted by permission from Leach et al. 2002 © The American Society for Biochemistry and Molecular Biology)

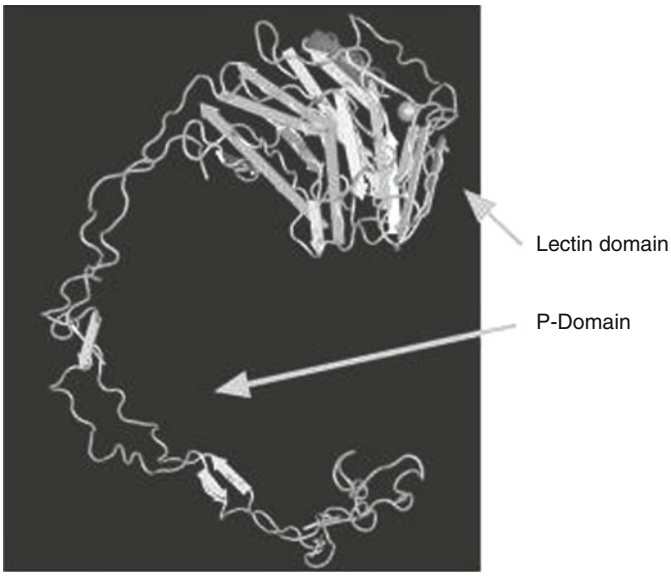

Fig. 2.2 Domain organization of calnaxin (Adapted with permission from Schrag et al. 2001 © Elsevier)

the terminal glucose residue. Each motif-1-repeat is paired with a motif-2-repeat on the opposite strand.

The lectin domain confers specific binding to glycoproteins bearing Asn-linked oligosaccharides of the form Glc1Man5–9GlcNAc2 (Leach et al. 2002; Ware et al. 1995). A 140-Å hairpin loop forms the arm domain, the tip of which includes the binding site for a thiol oxidoreductase, ERp57 (Leach et al. 2002; Pollock et al. 2004). In vitro studies have shown that the recruitment of a reduced glycoprotein to Cnx-ERp57 complex greatly enhances oxidative folding relative to ERp57 alone (Zapun et al. 1998). Both the globular and arm domains have been shown to bind Ca^{2+} but the crystal structure revealed only a single bound Ca^{2+} within the globular domain (Schrag et al. 2001). Although the complete structure of Crt is yet to be solved, ~39% overall sequence identity to Cnx, combined with conserved oligosaccharide binding specificity (Vassilakos et al. 1998), a shorter but similar arm domain structure as determined by NMR (Ellgaard et al. 2001a), and conserved ERp57 association properties (Frickel et al. 2002) all suggest a similar overall structure for the two chaperones. Ca^{2+} also binds to the globular and arm domains of Crt, which suggests that this ion is important for stabilizing both chaperones (Brockmeier and Williams 2006; Li et al. 2001).

2.1.3 Calnexin Binds High-Mannose-Type Oligosaccharides

In ER and in early secretory pathway, where repertoire of oligosaccharide structures is still small, the glycans play a pivotal role in protein folding, oligomerization, quality control, sorting, and transport. They are used as universal "tags" that allow specific lectins and modifying enzymes to establish order among the diversity of maturing glycoproteins (Yamashita et al. 1999). High-mannose-type oligosaccharides have been shown to play important roles in protein

quality control (QC). Several intracellular proteins, such as lectins, chaperones and glycan-processing enzymes, are involved in this process. These include Cnx/Crt, UDP-glucose:glycoprotein glucosyltransferase (UGGT), cargo receptors (such as VIP36 and ERGIC-53), mannosidase-like proteins (e.g. EDEM and Htm1p) and ubiquitin ligase (Fbs). They are thought to recognize high-mannose-type glycans with subtly different structures, although the precise specificities are yet to be clarified. Calnexin binds mostly with N-glycosylated proteins. Soluble Cnx binds specifically with Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoprotein. Findings suggested that once complexes between calnexin and glycoproteins are formed, oligosaccharide binding does not contribute significantly to the overall interaction (Leach et al. 2002; Ware et al. 1995).

2.1.4 Functions of Calnexin

Cnx/Crt as Classical Chaperones of Protein Folding

The polypeptide-binding site on Cnx and Crt permits them to function as classical chaperones capable of recognizing non-native features of protein folding intermediates and suppressing their aggregation. This function was initially uncovered through in vitro experiments that demonstrated that both Cnx and Crt can suppress the aggregation not only of glycoproteins bearing monoglycosylated oligosaccharides but that of nonglycosylated proteins as well (Culina et al. 2004; Rizvi et al. 2004; Thammavongsa et al. 2005). Aggregation suppression ability was enhanced in presence of physiological ER Ca^{2+} concentrations as well as millimolar ATP, the latter causing an increased hydrophobic surface on chaperones (Brockmeier and Williams 2006). Several studies have validated the existence of functional polypeptide-based interactions between either Cnx or Crt and folding glycoproteins in living cells. ER molecular chaperones of the calreticulin/calnexin cycle have overlapping and complementary but not redundant functions. The absence of one chaperone can have devastating effects on the function of the others, compromising overall QC of the secretory pathway and activating unfolded protein response (UPR)-dependent pathways.

A number of proteins have been demonstrated to bind calnexin. The 90-kDa phosphoprotein (p90) of ER formed stable and transient complexes with other cellular proteins, and associated with heavy chain of MHC class I protein. A truncated version of integral membrane glycoprotein Glut 1 (GT_{155}) interacts with calnexin in vitro. Reports highlight the importance of the calnexin cycle in the functional maturation of the g-secretase complex (Hayashi et al. 2009b). γ-secretase is a membrane protein complex that catalyzes intramembrane proteolysis of a variety of substrates including the amyloid precursor protein of Alzheimer disease. Nicastrin (NCT), a single-pass membrane glycoprotein that harbors a large extracellular domain, is an essential component of the γ-secretase complex. Lectin deficient mutants of Cnx were shown to interact with heavy chains of MHC class I molecules in insect cells and to prevent their rapid degradation (Leach and Williams 2004). Similarly, Crt-deficient mutants were found not only to interact with a broad spectrum of newly synthesized proteins and dissociate with normal kinetics, but it was also able to complement all MHC class I biosynthetic defects associated with Crt deficiency (Ireland et al. 2008). Studies are consistent with a model wherein Cnx and Crt associate with folding glycoproteins through both lectin- and polypeptide-based interactions thereby increasing the avidity of association relative to either interaction alone (Stronge et al. 2001). Binding of Cnx or Crt serves to prevent premature release of folding intermediates from the ER and promotes proper folding by suppressing off-pathway aggregation and by providing a privileged environment in which associated ERp57 promotes thiol oxidation and isomerization reactions (Zapun et al. 1998). The Crt is essential for normal Cnx chaperone function. In the absence of Crt, Crt substrates are not "picked up" by Cnx but accumulate in ER lumen, resulting in the activation of unfolded protein response (UPR), which is activated to induce transcription of ER-localized molecular chaperones (Shen et al. 2004). Pancreatic ER kinase (PERK) and Ire1a, UPR-specific protein kinases, and eIF2a are also activated in the absence of Crt (Knee et al. 2003).

Cnx/Crt Cycle

Calnexin binds only those N-glycoproteins which possess GlcNAc2Man9Glc1 oligosaccharides. Oligosaccharides with three sequential glucose residues are added to asparagine residues of the nascent proteins in the ER. The monoglucosylated oligosaccharides that are recognized by Cnx result from trimming of two glucose residues by sequential action of two glucosidases (GLS), I and II. GLS II can also remove third and last glucose residue (Hebert et al. 1995). The ER also contains a uridine diphosphate (UDP)-glucose, glycoprotein transferase (UGGT), which can re-glucosylate chains that have been glucose-trimmed. If the glycoprotein is not properly folded, the UGGT will add the glucose residue back onto the oligosaccharide thus regenerating the glycoprotein's ability to bind to calnexin. The improperly-folded glycoprotein chain thus loiters in the ER, risking the encounter with α-mannosidase (MNS1), which eventually sentences the underperforming glycoprotein to degradation by removing its mannose residue. If the protein is correctly translated, the chance of it being correctly folded before it encounters MNS1 is high. ATP and calcium ions are two of the cofactors involved in substrate binding for calnexin. Together, UGGT and GLS II establish a cycle of de-glucosylation and re-glucosylation.

Importantly, UGGT discriminates between folded and unfolded proteins, adding back a glucose residue to unfolded proteins only. This results in "rebuilding" of the monoglucosylated oligosaccharide on unfolded substrates, enabling them to interact with Crt and/or Cnx again. This de-glucosylation/glucosylation cycle may be repeated several times before a newly synthesized glycoprotein is properly folded (Roth et al. 2003) (Fig. 2.3). The lectin-oligosaccharide interaction is regulated by the availability of the terminal glucose on Glc1Man5–9Glc-NAc2 oligosaccharides. If folding of glycoprotein does not occur promptly, the folding sensor UGGT recognizes non-native conformers and reglucosylates *N*-glycans, thereby allowing re-entry into the chaperone cycle (Caramelo et al. 2004; Ritter et al. 2005; Taylor et al. 2004). Terminally folding-defective glycoproteins are further processed by demannosylation that diverts them from Cnx/Crt cycle into ERAD disposal pathway (Helenius and Aebi 2004). The lectin-binding site of Cnx and Crt is localized to the Ca^{2+} binding P-domain of the protein and the bound Ca^{2+} is essential for the lectin-like function of these proteins. Moreover, while glycoproteins are bound to Cnx and Crt, the disulphide bonds of the substrates are rearranged by the PDI activity associated with ERp57 suggesting that Crt binding to carbohydrates may be a 'signal' to recruit other chaperones to assist in protein folding. It should be emphasized, however, that monoglucosylated high-mannose carbohydrates may not be a prerequisite for substrate binding to Crt. For example, castanospermine and 1-deoxynojirimycin, inhibitors of the glucosidase II, do not affect association between Crt and Factor VIII or between Crt and mucin. Calreticulin also binds directly to PDI, ERp57, perforin, the synthetic peptide KLGFFKR and the DNA-binding domain of steroid receptors, indicating that chaperone function of Crt may involve both protein–protein and protein–carbohydrate interactions (Michalak et al. 1999).

Site of Interaction/Substrate Specificity

Although lectin sites of Cnx and Crt have been well defined through structural and mutagenesis studies (Schrag et al. 2001; Kapoor et al. 2004; Thomson and Williams 2005), less is known about the location and substrate specificity of the polypeptide-binding sites. Deletion mutagenesis of rabbit Crt as well as Cnx suggested that their abilities to suppress the aggregation of nonglycosylated proteins reside primarily within their globular domains (Leach et al. 2002; Xu et al. 2004). Furthermore, in vitro binding experiments with nonglycosylated proteins such as citrate synthase and malate dehydrogenase have indicated that both chaperones interact preferentially with non-native conformers, suggesting that they act as folding sensors in addition to the role provided by UDP-glucose:glycoprotein glucosytransferase (Saito et al. 1999). To characterize the specificity of the polypeptide-binding site of Crt, Sandhu et al. (2007) and Duus et al. (2008) examined a panel of peptides for their binding to Crt using a competitive ELISA. Peptide binding required a minimum peptide length of five residues that were hydrophobic in nature. In another study, a hydrophobic Crt-binding peptide was shown to compete with the ability of Crt to suppress the thermally induced aggregation of a soluble MHC class I molecule (Rizvi et al. 2004). Collectively, these findings indicate the presence of a site on Cnx and Crt that recognizes non-native protein conformers and that, in the case of Crt, exhibits specificity for hydrophobic peptide segments.

Brockmeier et al. (2009) investigated the location and characteristics of the polypeptide-binding function of Cnx under physiological conditions of ER lumen. Using an assay in which soluble ER luminal domain of Cnx (S-Cnx) suppresses the aggregation of nonglycosylated firefly luciferase at 37 °C and 0.4 mM Ca^{2+} (Brockmeier and Williams 2006), Brockmeier et al. showed that this aggregation suppression function resides within the globular lectin domain but is enhanced by the presence of the full-length arm domain. Direct binding experiments revealed a single site of peptide binding in globular domain at a location distinct from the lectin site. The site in globular domain is responsible for aggregation suppression and is capable of binding hydrophobic peptides with μM affinity ($K_D = 0.9$ μM). Furthermore, binding studies with peptides and non-native proteins of increasing size revealed that the arm domain contributes to the aggregation suppression function of S-Cnx not through direct substrate binding but rather by sterically constraining large polypeptide chains.

Cnx as a PACS-2 Cargo Protein

Proteins of Cytosolc Sorting (Pacs): CK2-phosphorylatable acidic clusters are hallmark interacting sequences for proteins of cytosolc sorting (PACS) protein family, which includes PACS-1 and PACS-2. The interaction of these acidic motifs with PACS proteins mediates a variety of intracellular steps that include trafficking between *trans*-Golgi (TGN) network and endosomes, localization to mitochondria and retention in ER. Cargo proteins can usually interact with both PACS-1 and PACS-2. Changing the amount of Cnx on plasma membrane could affect cell surface properties and might have implications on phagocytosis or cell–cell interactions. Hence, the amount of Cnx on plasma membrane could depend on cell type or cellular homeostasis, and it might be the result of regulated intracellular retention (c/r Myhill et al. 2008). Myhill et al. (2008) identified Cnx as a PACS-2 cargo protein on ER. Cnx interacts with PACS-2 using its acidic CK2 motif. Results suggest that the phosphorylation state of the calnexin cytosolic domain and its interaction with PACS-2 sort this chaperone between domains of the ER and the plasma membrane.

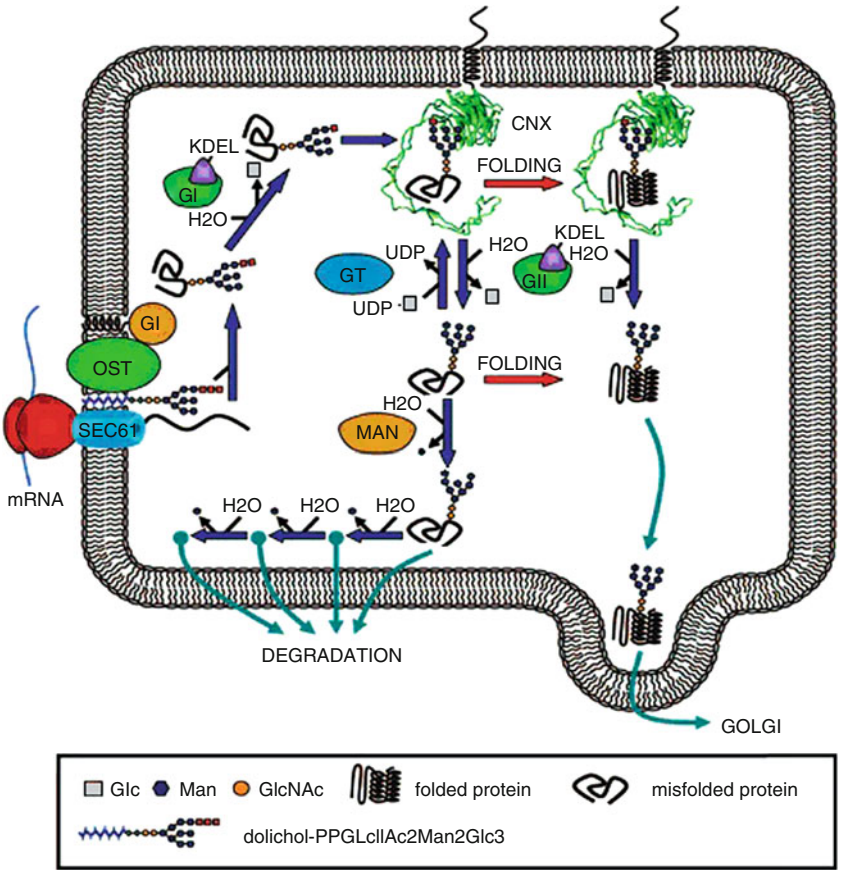

Fig. 2.3 Model proposed for the quality control of glycoprotein folding. Proteins entering the ER are *N*-glycosylated by the oligosaccharyltransferase (*OST*) as they emerge from the translocon. Two glucose units are removed by the sequential action of GI and GII to generate monoglucosylated species that are recognized by Cnx and/or Crt (only Cnx is shown), which is associated with ERp52. The complex between the lectins and folding intermediates/misfolded glycoproteins dissociates upon removal of the last glucose by GII and is reformed by GT activity. Once glycoproteins have acquired their native conformations, either free or complexed with the lectins, GII hydrolyzes the remaining glucose residue and releases the glycoproteins from the lectin anchors. These species are not recognized by GT and are transported to the Golgi. Glycoproteins remaining in misfolded conformations are retrotranslocated to the cytosol, where they are deglycosylated and degraded by the proteasome. One or more mannose residues may be removed during the whole folding process (Reprinted with permission from Caramelo and Parodi 2008 © American Society for Biochemistry and Molecular Biology)

In addition to folding intermediates, ribosomes and SERCA2b, Cnx also interacts with BAP31, an ER cargo receptor that mediates export of transmembrane proteins from the ER and shuttles them to the ER quality control compartment. Thus, Cnx can reach the plasma membrane and can also interact with numerous ER membrane proteins that are found on multiple domains of the ER such as the Mitochondria Associated Membrane (MAM). Although Cnx and other ER chaperones clearly localize to multiple cellular membranes, it is currently not understood whether the cell has mechanisms in place that control the distribution of chaperones between these various locations. Support for the hypothesis of a controlled distribution of ER proteins to specific membrane domains comes from pioneering studies on Cnx (Chevet et al. 1999; Roderick et al. 2000), which showed that PKC, ERK-1 and protein kinase CK2 (CK2) can phosphorylate the Cnx cytosolic domain. Phosphorylation by ERK-1 on serine 583 increases interaction of Cnx with ribosomes, but also interaction with SERCA2b. In addition, CK2 phosphorylation of serines 554 and 564 by CK2 synergizes with ERK-1 phosphorylation of serine 583 to promote interaction with ribosomes (Chevet et al. 1999). Hence, the Cnx phosphorylation state could lead to enrichment on the MAM and the rER, where these Cnx interactors are found. However, it is still unclear what happens to dephosphorylated v that has been demonstrated to exist in vivo (Myhill et al. 2008).

However, Calnexin deficiency is not embryonic lethal. This is amazing considering great structural and functional similarities between the Crt and Cnx chaperones. The $Cnx^{-/-}$ animals exhibit impaired motor function and die within the first 5 weeks of life. Studies with Crt and Cnx gene knockout mice indicate that these proteins are unable to compensate for the loss of each other, suggesting they have unique functions. The molecular chaperone function of calreticulin

and calnexin may only partially explain phenotypes of $Cnx^{-/-}$ and $Crt^{-/-}$ mice (Denzel et al. 2002).

2.1.5 Patho-Physiology of Calnexin Deficiency

ER and Oxidative Stresses Are Common Mediators of Apoptosis: The field of the ER stress in mammalian cells has expanded rapidly during the past decade, contributing to understanding of the molecular pathways that allow cells to adapt to perturbations in ER homeostasis. One major mechanism is mediated by molecular ER chaperones which are critical not only for quality control of proteins processed in the ER, but also for regulation of ER signaling in response to ER stress. Proteome analysis of human diploid fibroblasts (HDF) showed that Cnx significantly decreased with aging. Oxidative stress-induced expression of Cnx also attenuated in old HDF compared to young cells (Choi and Kim 2004). Ni and Lee (2007) reviewed the properties and functions of Cnx, Crt, and their role in development and diseases. Many of the new insights are derived from constructed mouse models where the genes encoding the chaperones are genetically altered, providing invaluable tools for examining the physiological involvement of the ER chaperones in vivo. Wei et al. (2008) uncovered that chemical disruption of lysosomal homeostasis in normal cells causes ER stress, suggesting a cross-talk between the lysosomes and the ER. Most importantly, chemical chaperones that alleviate ER and oxidative stresses are also cytoprotective in all forms of LSDs studied. It was proposed that ER and oxidative stresses are common mediators of apoptosis in both neurodegenerative and non-neurodegenerative LSDs. Hepatic ER stress induced by burn injury was associated with compensatory upregulation Cnx and Crt, suggesting that ER calcium store depletion was the primary trigger for induction of ER stress response in mice. Thus, thermal injury causes long-term adaptive and deleterious hepatic function characterized by significant upregulation of ER stress response (Song et al. 2009). Pre-administration of α-tocopherol is protective against oxidative renal tubular damage and subsequent carcinogenesis by ferric nitrilotriacetate (Fe-NTA) in rats. In addition to scavenging effects, α-tocopherol showed significantly beneficial effects in renal protection. Results suggest that α-tocopherol modifies glycoprotein metabolism partially by conferring mild ER stress (Lee et al. 2006).

Calnexin in Apoptosis Induced by ER Stress in *S. pombe*: Stress conditions affecting the functions of the endoplasmic reticulum (ER) cause the accumulation of unfolded proteins. ER stress is counteracted by the unfolded-protein response (UPR). However, under prolonged stress the UPR initiates a proapoptotic response. Mounting evidence indicate that the Cnx is involved in apoptosis caused by ER stress. Overexpression of Cnx in *Schizosaccharomyces pombe* induces cell death with apoptosis markers. Guerin et al. (2008) argue for the conservation of the role of calnexin in apoptosis triggered by ER stress, and validate *S. pombe* as a model to elucidate the mechanisms of Cnx-mediated cell death. The ER is highly sensitive to stresses perturbing the cellular energy levels and ER lipid or glycolipid imbalances or changes in the redox state or Ca^{2+} concentration. Such stresses reduce the protein folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins, a condition referred to as ER stress. When the capacity of the ER to fold proteins properly is compromised or overwhelmed, a highly conserved UPR signal-transduction pathway is activated. Guerin et al. (2008) further showed that the apoptotic effect of calnexin is counteracted by overexpression of Hmg1/2p, the *S. pombe* homologue of the mammalian antiapoptotic protein HMGB1 (high-mobility group box-1 protein). Interestingly, the overexpression of mammalian Cnx also induced apoptosis in *S. pombe*, suggesting the functional conservation of the role of Cnx in apoptosis. Inositol starvation in *S. pombe* causes cell death with apoptotic features. Observations indicated that Cnx takes part in at least two apoptotic pathways in *S. pombe*, and suggested that the cleavage of Cnx has regulatory roles in apoptotic processes involving Cnx (Guerin et al. 2009).

Calnexin in Biogenesis of Cystic Fibrosis: Deletion of phenylalanine at position 508 (δ F508) in first nucleotide-binding fold of cystic fibrosis transmembrane conductance regulator (CFTR) is the most common mutation in patients with cystic fibrosis. Although retaining functional Cl^- channel activity, this mutant is recognized as abnormal by cellular "quality control" machinery and is retained within ER. This intracellular retention was restricted to the immature (or ER-associated) forms of the CFTR proteins. Study indicated that Cnx retains misfolded or incompletely assembled proteins in ER and thus is likely to contribute to the mislocalization of mutant CFTR (Pind et al. 1994).

Cnx in Rod Opsin Biogenesis: Misfolding mutations in rod opsin are a major cause of the inherited blindness retinitis pigmentosa. A report from *Drosophila* rhodopsin Rh1 suggests the requirement of Cnx for its maturation and correct localization to R1–6 rhabdomeres (Rosenbaum et al. 2006). However, unlike *Drosophila* Rh1, mammalian rod opsin biogenesis does not appear to have an absolute requirement for Cnx. Other chaperones are likely to be more important for mammalian rod opsin biogenesis and quality control (Kosmaoglou and Cheetham 2008). Furthermore, the over-expression of Cnx leads to an increased accumulation of misfolded P23H opsin but not the correctly folded protein.

Finally, the increased levels of Cnx in the presence of the pharmacological chaperone 11-cis-retinal increase the folding efficiency and result in an increase in correct folding of mutant rhodopsin. These results demonstrate that misfolded rather than correctly folded rhodopsin is a substrate for Cnx and that the interaction between Cnx and mutant- misfolded rhodopsin can be targeted to increase the yield of folded mutant protein (Noorwez et al. 2009). Thus, Cnx preferentially associates with misfolded mutant opsins during retinitis pigmentosa.

Congenital Disorders: Autosomal dominant polycystic liver disease (PCLD), a rare progressive disorder, is characterized by an increased liver volume due to many fluid-filled cysts of biliary origin. Disease causing mutations in PRKCSH or SEC63 are found in approximately 25% of the PCLD patients. Hepatocystin is directly involved in the protein folding process by regulating protein binding to Cnx/Crt in the ER. A separate group of genetic diseases affecting protein N-glycosylation in the ER is formed by the congenital disorders of glycosylation (CDG). In distinct subtypes of this autosomal recessive multisystem disease specific liver symptoms have been reported that overlap with PCLD. Recent research revealed novel insights in PCLD disease pathology such as the absence of hepatocystin from cyst epithelia indicating a two-hit model for PCLD (Janssen et al. 2010)

Peripheral Neuropathies: Schwann cell-derived peripheral myelin protein-22 (PMP-22) when mutated or over-expressed causes heritable neuropathies with a unexplained "gain-of-function" ER retention phenotype. Missense point mutations in Gas3/PMP22 are responsible for the peripheral neuropathies Charcot-Marie-Tooth 1A and Dejerine Sottas syndrome. These mutations induce protein misfolding with the consequent accumulation of proteins in ER and the formation of aggresomes. In Trembler-J (Tr-J) sciatic nerves, prolonged association of mutant PMP-22 with Cnx is found. In 293A cells overexpressing PMP-22(Tr-J), Cnx and PMP-22 colocalize in large intracellular structures. Similar intracellular myelin-like figures were also present in Schwann cells of sciatic nerves from homozygous Trembler-J mice with no detectable activation of stress response pathway as deduced from BiP and CHOP expression. Sequestration of Cnx in intracellular myelin-like figures may be relevant to the autosomal dominant Charcot-Marie-Tooth-related neuropathies (Dickson et al. 2002). During folding PMP22 associates with calnexin. Calnexin interacts with the misfolded transmembrane domains of PMP22. The emerging models indicate for a glycan-independent chaperone role for calnexin and for the mechanism of retention of misfolded membrane proteins in the endoplasmic reticulum (Fontanini et al. 2005)

2.2 Calreticulin

2.2.1 General Features

Calreticulin (Crt) was first identified as a Ca^{2+}-binding protein of the muscle sarcoplasmic reticulum in 1974. Calreticulin is a ubiquitous protein, found in a wide range of species and in all nucleated cell types, and has a variety of important biological functions. Since its discovery (Ostwald and MacLennan 1974) from rabbit skeletal muscle it has been cloned in several vertebrates and invertebrates, and also higher plants (Michalak et al. 1999). There is no Crt gene in yeast and prokaryotes whose genomes have been fully sequenced. The human gene for calreticulin contains nine exons and eight introns. The deduced amino acid sequence indicates that calreticulin has a 17 amino acid hydrophobic signal sequence at its N terminus and that mature calreticulin contains 400 amino acids. The structure of calreticulin has been well characterized. It has at least three structural and functional domains.

The genomic configuration of the mouse and human calreticulin gene is shown in Fig. 2.4. The protein is encoded by a single gene (McCauliffe et al. 1992; Waser et al. 1997), and only one species of 1.9 kb mRNA encoding calreticulin has been identified. There is no evidence for alternative splicing of the calreticulin mRNA. The Crt gene consists of nine exons and spans approx. 3.6 kb or 4.6 kb of human or mouse genomic DNA respectively (Fig. 2.4). Human and mouse genes have been localized to chromosomes 19 and 8 respectively (McCauliffe et al. 1992; Rooke et al. 1997). The exon–intron organization of human and mouse genes is almost identical. The nucleotide sequences of the mouse and human gene show greater than 70% identity, with the exception of introns 3 and 6, indicating a strong evolutionary conservation of the gene. In the mouse gene these introns are approximately twice the size of the corresponding introns in the human gene. The promoter of the mouse and human calreticulin genes contain several putative regulatory sites, including AP-1 and AP-2 sites, GC-rich areas, including an Sp1 site, an H4TF-1 site, and four CCAAT sequences (McCauliffe et al. 1992; Waser et al. 1997). AP-2 and H4TF-1 recognition sequences are typically found in genes that are active during cellular proliferation. There is no obvious nuclear factor of activated T-cell (NF-AT) and nuclear factor κB (NF-κB) sites in the calreticulin promoter. Several poly (G) sequences, including GGGNNGGG motifs, are also found in the promoter regions of calreticulin and other ER/sarcoplasmic reticulum (SR) luminal proteins, including glucose-regulated protein 78 (Grp78) and Grp94. These motifs may therefore play a role in regulation of the expression of luminal ER proteins and in ER stress-

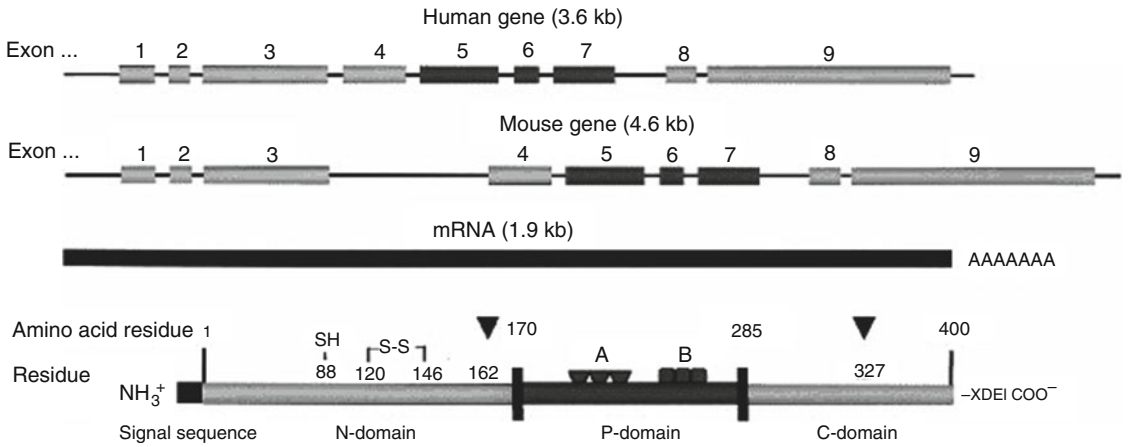

Fig. 2.4 The calreticulin gene: The Figure shows a schematic representation of the genomic configuration of domain structure of calreticulin protein. Structural predictions for calreticulin suggest that the protein has at least three structural and functional domains. Exons encoding the N domain (including the N-terminal signal sequence), and the C domain of calreticulin are in *grey* color while P is *black*. The N, P, and C domains are also presented in same color. The protein contains an N-terminal amino acid signal sequence (*black box*) and a C-terminal KDEL ER retrieval signal. The locations of 3 cysteine residues and the disulphide bridge in the N domain of calreticulin are indicated. The arrows indicate the location of potential glycosylation sites (residues 162 and 327). Repeats A (amino acid sequence PXXIXDPDAXKPEDWDE) and B (amino acid sequence GXWXPPXIXNPXYX) are also indicated (Reprinted with permission from Michalak et al. 1999; Biochem J. 344: 281–97 © The Biochemical Society)

dependent activation of the calreticulin gene (Michalak et al. 1999).

Depletion of Ca^{2+} stores induces severalfold activation of Crt promoter followed by increase in Crt mRNA and protein levels (Waser et al. 1997). Expression of Crt is also activated by bradykinin-dependent Ca^{2+} depletion of intracellular Ca^{2+} stores both in vitro and in vivo (Waser et al. 1997). The calreticulin promoter is activated by Zn^{2+} and heat shock. Expression of calreticulin is also induced by viral infection (Zhu 1996), by amino acid deprivation and in stimulated cytotoxic T-cells, further indicating that the calreticulin gene is activated by a variety of chemical and biological stresses. Since calreticulin has been implicated in a wide variety of cellular processes, the stress-dependent activation of the calreticulin gene may affect numerous biological and pathophysiological conditions (Michalak et al. 1999).

2.2.2 The Protein

Calreticulin is a 46-kDa protein with an N-terminal cleavable amino acid signal sequence and a C-terminal KDEL ER retrieval signal (Fig. 2.4). These specific amino acid sequences are responsible for targeting and retention of Crt in the ER lumen. Depending on species, Crt may have one or more potential N-linked glycosylation sites. The glycosylation pattern of the protein seems to be heterogeneous and does not appear to be a conserved property of the protein. The glycosylation of Crt is more common in plants than in animal cells (Navazio, et al. 1996). Heat shock may trigger glycosylation of calreticulin (Jethmalani, and Henle 1998); however, the functional consequence of this stress-induced glycosylation of the protein is presently not clear. Calreticulin has three cysteine residues, and all of them are located in the N-domain of the protein. Importantly, the location of these amino acid residues is conserved in calreticulin from higher plants to that in humans (Michalak et al. 1999). Two out of three cysteine residues found in the protein form a disulphide bridge (Cys^{120}–Cys^{146}) (Michalak et al. 1999; 2009), which may be important for proper folding of N-terminal region of Crt. ER localization of Crt is specified by two types of targeting signals, an N-terminal hydrophobic sequence that directs insertion into ER and a C-terminal KDEL sequence that is responsible for retention in the ER. Afshar et al. (2005) showed that Crt is fully inserted into ER, undergoes processing by signal peptidase, and subsequently undergoes retrotranslocation to the cytoplasm. C-terminal Ca^{2+} binding domain plays an important role in Crt retrotranslocation. Calreticulin is an ATP-binding protein but it does not contain detectable ATPase activity. Digestion of the protein with trypsin in presence of Mg^{2+} ATP protects the full-length protein. Results indicate that calreticulin may undergo frequent, ion-induced conformation changes, which may affect its function and its ability to interact with other proteins in lumen of ER (Corbett et al. 2000).

Calreticulin consists of various structural and functional domains. The N-domain of calreticulin, together with the central P-domain, is responsible for protein's chaperone function. Studies by Guo et al. (2003) and Michalak et al.

(2002a) with site-specific mutagenesis showed that mutation of a single His^{153} in calreticulin's N-domain destroys the protein's chaperone function. The P-domain of Crt (residues 181–290) contains a proline-rich region, forms an extended-arm structure, and interacts with other chaperones in the lumen of ER. The extended-arm structure is predicted to curve like that in Cnx that is similar to Crt, forming an opening that is likely to accommodate substrate binding, including the carbohydrate-binding site. As a molecular chaperone, Crt binds the monoglucosylated high mannose oligosaccharide (Glc1Man9GlcNAc2) and recognizes the terminal glucose and four internal mannoses in newly synthesized glycoproteins (Michalak et al. 2002b). Changes within ER, such as alterations in concentration of Ca^{2+}, Zn^{2+} or ATP, may affect the formation of these chaperone complexes and thus the ability of Crt to assist in protein folding (Trombetta and Parodi 2003).

Three Structural Domains

Similar to Cnx, Crt promotes the folding of proteins carrying N-linked glycans. Both proteins cooperate with an associated co-chaperone, the thiol-disulfide oxidoreductase ERp52. Three distinct structural domains have been identified in calreticulin: the amino-terminal, globular N-domain; the central P-domain; and the carboxyl-terminal C-domain (Michalak et al. 1999). NMR (Ellgaard et al. 2001), modeling (Michalak et al. 2002), and biochemical studies (Nakamura et al. 2001a) indicate that the globular N-domain and the "extended arm" P-domain of Crt may form a functional protein-folding unit (Michalak et al. 2002). This region of Crt contains a Zn^{2+} binding site and one disulfide bond, and it may also bind ATP (Baksh et al. 1995; Andrin et al. 2000; Corbett et al. 2000).

The exon–intron organization of the Crt gene suggests that the central P-domain of the protein may be encoded by exons 5, 6 and 7, whereas the first four exons and the last two exons may encode the N- and C-domain of the protein respectively (Fig. 2.4). Table 2.1 summarizes functional properties of these domains. The N-terminal part of the protein, encompassing the N- and P-domain of Crt, has the most conserved amino acid sequence (Michalak et al. 1999).

i). N-Domain

The N-terminal half of the molecule is predicted to be a highly folded globular structure containing eight anti-parallel β-strands connected by protein loops. The amino acid sequence of N-domain of Ctr is extremely conserved in all calreticulins. The N-domain binds Zn^{2+} (Baksh et al. 1995b, c; Andrin et al. 2000; Corbett et al. 2000) and it undergoes dramatic conformational changes (Michalak et al. 1999). Chemical modification of Crt has revealed that four histidines located in the N-domain of the protein (His^{25}, His^{82}, His^{128}, and His^{153}) are involved in Zn^{2+} binding (Baksh et al. 1995c). The Zn^{2+}-dependent conformational change in Crt affects its ability to bind to unfolded protein/glycoprotein substrates in vitro (Saito et al. 1999), suggesting that conformational changes in Crt may modify its chaperone function. ER of calreticulin-deficient cells with N-terminal histidine (His^{25}, His^{82}, His^{128}, and His^{153}) indicated that His^{153} chaperone function was impaired. Thus, mutation of a single amino acid residue in Crt has devastating consequences for its chaperone function, and may play a significant role in protein folding disorders (Guo et al. 2003). The N-domain interacts with the DNA-binding domain of the glucocorticoid receptor in vitro (Burns et al. 1994), with rubella virus RNA (Singh and Atreya 1994; Nakhasi et al. 1994), β-integrin and with protein disulphide-isomerase (PDI) and ER protein 57 (ERp57) (Baksh et al. 1995a, Corbett et al. 1999). Interaction of this region of Crt with PDI inhibits PDI chaperone function (Baksh et al. 1995a), but enhances ERp57 activity (Zapun et al. 1998). These protein–protein interactions are regulated by Ca^{2+} binding to the C-domain of Crt (Corbett et al. 1999). The N-domain of Crt also inhibits proliferation of endothelial cells and suppresses angiogenesis (Pike et al. 1998).

ii). P-Domain

The P-domain of Crt comprises a proline-rich sequence with three repeats of the amino acid sequence PXXIXDPDAXKPEDWDE (repeat A) followed by three repeats of the sequence GXWXPPXIXNPXYX (repeat B) (Fig. 2.5a). While the C-domain is responsible for the low-affinity and high-capacity Ca^{2+} binding, the P-domain of Crt binds Ca^{2+} with high affinity. The repeats may be essential for high-affinity Ca^{2+} binding of Crt (Baksh and Michalak 1991; Tjoelker et al. 1994) that interact with PDI (Corbett et al. 1999), NK2 homeobox 1 (Perrone et al. 1999), and perforin (Andrin et al. 1998; Fraser et al. 1998), a component of the cytotoxic T-cell granules. More importantly, repeats A and B are critical for the lectin-like chaperone activity of Crt (Vassilakos et al. 1998). The P-domain is one of the most interesting and unique regions of Crt because of its lectin-like activity and amino acid sequence similarities to other Ca^{2+}-binding chaperones, including Cnx (Bergeron et al. 1994), calmegin (Watanabe et al. 1994) and CALNUC, a Golgi Ca^{2+}-binding protein (Lin et al. 1998). However, the C-domain is less conserved than other domains of Crt (Michalak et al. 1999). Four amino acid residues (Glu^{239}, Asp^{241}, GLu^{243}, and Trp^{244}) at the tip of the 'extended arm' of P-domain are critical in chaperone function of Crt (Martin et al. 2006). Martin et al. (2006) focused studies on two cysteine residues (Cys^{88} and Cys^{120}), which form a disulfide bridge in N-terminal domain of Crt, a tryptophan residue located in CRD (Trp^{302}), and on certain residues located at the tip of "hairpin-like" P-domain of Crt (Glu^{238}, Glu^{239},

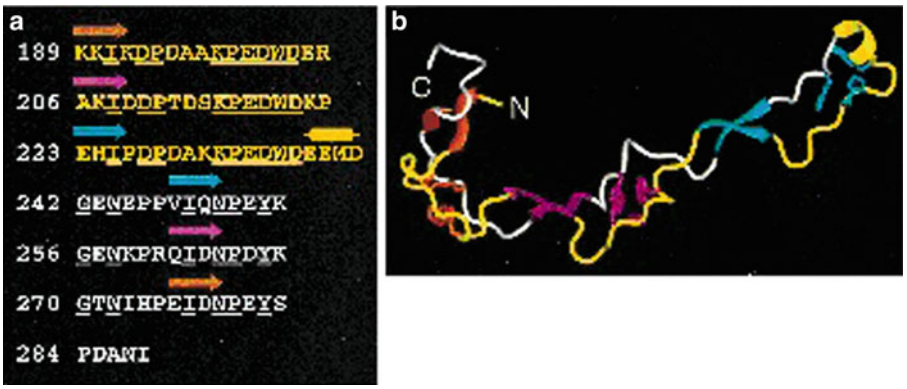

Fig. 2.5 (**a**). Alignment of the sequence repeats in rat Crt (189–288). The three 17-residue type 1 repeats are shown in yellow, and the three 14-residue type 2 repeats are white. The positions of the strands in three short antiparallel β-sheets are indicated by *orange*, *red*, and *blue arrows* above the sequence. The position of a helical turn, which includes a two-residue insert between the third and fourth repeat, is shown by a *yellow* cylinder above the sequence. Residues conserved in all three repeats of one type are underlined. (**b**). Bundles of the 20 energy-minimized conformers used to represent the NMR structure of Crt(189–288). (a) Superposition for best fit of the backbone atoms N, Ca, and C9 of the residues 219–258. (b) Superposition for best fit of the backbone atoms N, Ca, and C9 of the residues 189–209 and 262–284. In each drawing the polypeptide segments used for the superposition are colored *yellow*, and the remaining residues are *white*. (c) Cartoon of the conformer from *a* for which the *white* region is on the extreme *left*. The β-sheets and the helical turn on the extreme *right* are represented by ribbons and colored as in Fig. 4a. The same color code is used for the three associated hydrophobic clusters. The polypeptide segments that connect the β-strands are drawn as thin cylindrical rods, which are *yellow* for the type 1 repeats and white for the type 2 repeat (Reprinted with permission from Ellgaard et al. 2001a © National Academy of Sciences, USA)

Asp^{241}, Glu^{243}, and Trp^{244}). It was revealed that bradykinin-dependent Ca^{2+} release from ER was rescued by wild-type Crt and by Glu^{238}, Glu^{239}, Asp^{241}, and Glu^{243} mutants. Other amino acids mutants under study rescued the Crt-deficient phenotype only partially (~40%), or did not rescue it at all. Thus, amino acid residues Glu^{239}, Asp^{241}, Glu^{243}, and Trp^{244} at the hairpin tip of P-domain are critical in the formation of a complex between ERp57 and Crt. Although the Glu^{239}, Asp^{241}, and Glu^{243} mutants did not bind ERp57 efficiently, they fully restored bradykinin dependent Ca^{2+} release in $crt^{-/-}$ fibroblast cells.

The central, proline-rich P-domain of Crt, comprising residues 189–288, contains three copies of each of two repeat sequences (types 1 and 2), which are arranged in a characteristic '111222' pattern. The central proline-rich P-domain of Crt (189–288) shows an extended hairpin topology, with three short anti-parallel β-sheets, three small hydrophobic clusters, and one helical turn at the tip of the hairpin. The loop at the bottom of the hairpin consists of residues 227–247, and is closed by an anti-parallel β-sheet of residues 224–226 and 248–250. Two additional β-sheets contain residues 207–209 and 262–264, and 190–192 and 276–278. The 17-residue spacing of the β-strands in N-terminal part of hairpin and 14-residue spacing in the C-terminal part reflect the length of type 1 and type 2 sequence repeats. As a consequence of this topology the peptide segments separating the β-strands in the N-terminal part of the hairpin are likely to form bulges to accommodate the extra residues. Further, the residues 225–251 at the tip of Crt P-domain are involved in direct contacts with ERp52. The Crt P-domain fragment Crt(221–256) constitutes an autonomous folding unit, and has a structure highly similar to that of corresponding region in Crt(189–288). Of the 36 residues present in Crt(221–256), 32 form a well-structured core, making this fragment one of the smallest known natural sequences to form a stable non-helical fold in absence of disulfide bonds or tightly bound metal ions. Crt(221–256) comprises all the residues of intact P-domain that were shown to interact with ERp52.

The NMR structure of the rat calreticulin P-domain, comprising residues 189–288, Crt(189–288), shows a hairpin fold that involves the entire polypeptide chain, has the two chain ends in close spatial proximity, and does not fold back on itself. This globally extended structure is stabilized by three antiparallel β-sheets, with the β-strands comprising the residues 189–192 and 276–279, 206–209 and 262–265, and 223–226 and 248–251, respectively. The hairpin loop of residues 227–247 and the two connecting regions between the β-sheets contain a hydrophobic cluster, where each of the three clusters includes two highly conserved tryptophyl residues, one from each strand of the hairpin. The three β-sheets and the three hydrophobic clusters form a repeating pattern of interactions across the hairpin that reflects the periodicity of the amino acid sequence, which consists of three 17-residue repeats followed by three 14-residue repeats. Within the global hairpin fold there are two well-ordered subdomains comprising the residues 219–258, and 189–209 and 262–284, respectively. These are separated by

a poorly ordered linker region, so that the relative orientation of the two subdomains cannot be precisely described. The structure for Crt(189–288) provides an additional basis for functional studies of the abundant endoplasmic reticulum chaperone calreticulin (Ellgaard et al. 2001).

iii). C-Domain

The C-terminal region of Crt (C-domain) is highly acidic and terminates with the KDEL ER retrieval sequence (Fig. 2.1). The C-domain of Crt is susceptible to proteolytic cleavage and that the N- and P-domains form a proteolytically stable tight association. The C-domain of Crt binds over 25 mol of Ca^{2+}/mol of protein (Baksh and Michalak 1991), binds to blood-clotting factors (Kuwabara et al. 1995) and inhibits injury-induced restenosis (Dai et al. 1997). Ca^{2+} sensitivity, confined to the C-terminal part of the protein (C-domain), suggests that the C-domain of Crt may play a role of Ca^{2+} 'sensor' in ER lumen. Ca^{2+} binding to C-domain of Crt plays a regulatory role in the control of Crt interaction with PDI, ERp57 and perhaps other chaperones (Corbett et al. 1999). A modified form of calreticulin lacking the C-terminal hexapeptide including KDEL ER retention sequon has been isolated. Such a truncation may point to a mechanism that allows escape of Crt from ER (Hojrup and Roepstorff 2001). A transcription-based reporter assay revealed an important role for C-domain in Crt retrotranslocation (Afshar et al. 2005). At present, no structural information is available for C-domain which is involved in Ca^{2+} storage in the lumen of ER (Nakamura et al. 2001b).

A study by Jin et al. (2009) shows that only one of the three forms of the ER folding helper Crt of the plant Arabidopsis thaliana interacts with a mutated form of BRI1, the plasma membrane leucine-rich-repeat kinase receptor for brassinosteroids, plant-specific hormones playing important roles in plant growth. Arabidopsis CRT1 and CRT2 are very similar and have homologs in nonplant organisms, but the BRI1-interacting CRT3 seems to be a plant-specific form, with orthologs in higher and lower plant species (Persson et al. 2003). Gene coexpression analysis indicates that CRT3 can be grouped with stress resistance genes, whereas CRT1 and CRT2 are coexpressed mainly with other folding helpers. The important observations by Jin et al. (2009) point to plant-specific functional divergence in CRT family.

2.2.3 Cellular Localization of Calreticulin

Numerous studies confirmed ER localization of the protein in many diverse species, including plants. Besides its main location in ER (Opas et al. 1996), Crt has been found to reside in the nuclear envelope, the spindle apparatus of the dividing cells (Denecke et al. 1995), the cell surface (Gardai et al. 2005), and the plasmodesmata (Laporte et al. 2003; Chen et al. 2005), indicating that Crt is essential for normal cell function**.** The protein has also been localized to the cytoplasmic granules of the cytotoxic T-cell (Andrin et al. 1998; Fraser et al. 1998; Dupuis et al. 1993), sperm acrosomes (Nakamura et al. 1993), tick saliva (Jaworski et al. 1996), the cell surface (Arosa et al. 1999; Basu and Srivastava 1999), and it may even be secreted in the bloodstream. However, Crt has also been found outside the ER, such as within the secretory granules of cytotoxic lymphocytes, the cell surface of melanoma cells and virus-infected fibroblasts, and the cytosol and nucleus of several cell types (Arosa et al. 1999). Given its lectin-like properties, Crt is considered to be an ER chaperone involved in the assembly and folding of nascent glycoproteins. Surprisingly, there is a considerable controversy concerning cellular localization of Crt (Michalak et al. 1999). It was proposed that this may be due to the direct interaction between Crt and the DNA-binding domain of steroid receptors (Burns et al. 1994; Dedhar et al. 1994) and the cytoplasmic tail of α-integrin (Dedhar 1994). For calreticulin to bind to these molecules, the protein would have to be present in the nucleus and/or cytosol. However, to date there have been no reports on the identification of calreticulin or calreticulin-like protein in the cytosol. Calreticulin-like immunoreactivity was detected in the nucleus of some cells (Opas et al. 1996; Dedhar et al. 1994), in squamous carcinoma cell nuclei in response to ionizing radiation (Ramsamooj et al. 1995) or in nucleus of dexamethasone-treated LM(TK^-) cells (Roderick et al. 1997). However studies on the biosynthesis of MHC class I molecules have never reported associations of ER chaperones with MHC class I molecules outside the ER and Golgi compartments (c/r Arosa et al. 1999). In human peripheral blood T lymphocytes calreticulin is expressed at the cell surface, where it is physically associated with a pool of unfolded MHC class I molecules. Michalak et al. (1996) indicated that Crt is not a nuclear resident protein, and identification of the protein in the nucleus (Opas et al. 1996) was likely an artifact of immunostaining. There is also accumulating evidence for diverse roles for Crt localized outside ER, including reports suggesting important roles for Crt localized to the outer cell surface of a variety of cell types, in the cytosol, and in the extracellular matrix (ECM). Moreover, the addition of exogenous Crt rescues numerous Crt -driven functions, such as adhesion, migration, phagocytosis, and immunoregulatory functions of Crt-null cells.

2.2.4 Functions of Calreticulin

Calreticulin Is a Multi-Process Molecule

Calreticulin (also referred as calregulin, CRP55, CaBP3, mobilferrin and calsequestrin-like protein) is a multifunctional protein involved in many biological processes that include the regulation of Ca^{2+} homeostasis (Michalak et al. 1999), intercellular or intracellular signaling, gene expression (Johnson et al. 2001), glycoprotein folding (Helenius and Aebi 2004), and nuclear transport (Holaska et al. 2001). Overexpression of Crt enhanced apoptosis in myocardiac H9c2 cells under conditions inductive to differentiation with retinoic acid (Kageyama et al. 2002) or under oxidative stress (Ihara et al. 2006). Moreover, Crt regulates p53 function to induce apoptosis by affecting the rate of degradation and nuclear localization of p53 (Mesaeli and Phillipson 2004). Michalak et al. (1999, 2009) focussed on calreticulin, as a ER luminal Ca^{2+}-binding chaperone implicated in playing a role in many cellular functions, including lectin-like chaperoning, Ca^{2+} storage and signaling, regulation of gene expression, cell adhesion and autoimmunity. Several excellent reviews have been published concerning the structure and function of Crt in animals (Bedard et al. 2005; Johnson et al. 2001; Michalak et al. 1999, 2009) and in plants (Jia et al. 2009)

Calreticulin binds Ca^{2+} in the lumen of ER with high capacity and also participates in the folding of newly synthesized proteins and glycoproteins. Hence, it is an ER luminal Ca^{2+}-buffering chaperone. The protein is involved in regulation of intracellular Ca^{2+} homoeostasis and ER Ca^{2+} capacity. The protein impacts on store-operated Ca^{2+} influx and influences Ca^{2+}-dependent transcriptional pathways during embryonic development. It is a component of the calreticulin/calnexin pathway (Ellgaard et al. 1999). Both Crt and Cnx proteins cooperate with an associated co-chaperone, the thiol-disulfide oxidoreductase ERp57, which catalyzes the formation of disulfide bonds in Cnx and Crt-bound glycoprotein substrates. Calreticulin has been implicated to participate in many (perhaps too many) cellular functions. This strongly exemplifies the central role that the ER plays in a variety of cellular functions. It is not surprising, therefore, that any changes in calreticulin expression and function have profound effects on many cellular functions. There is also accumulating evidence for diverse roles for Crt localized outside the ER, including roles for Crt localized to the outer cell surface of a variety of cell types, in the cytosol, and in the extracellular matrix. Furthermore, the addition of exogenous Crt rescues numerous Crt-driven functions, such as adhesion, migration, phagocytosis, and immunoregulatory functions of Crt-null cells. Nonetheless, it has become clear that Crt is a multicompartmental protein that regulates a wide array of cellular responses important in physiological and pathological processes, such as wound healing, the immune response, fibrosis, and cancer (Gold et al. 2010). Notwithstanding, there is a widespread agreement that calreticulin performs two major functions in the ER lumen: (1) chaperoning and (2) regulation of Ca^{2+} homoeostasis.

In a soluble form, and in association with the homologous membrane bound protein Cnx, Crt binds to glycoproteins, preventing aggregation and allowing the proteins to attain their correct folding conformation (Bedard et al. 2005). Calreticulin also plays an important role in maintenance of cellular calcium homeostasis (Michalak et al. 1992). By regulating the amount of free and bound calcium within the lumen of the endoplasmic reticulum, calreticulin affects many different cellular functions, including cell shape, adhesion and motility (Bedard et al. 2005). Other activities that influence the various roles that this multi-functional protein plays include its capacity to bind to zinc (Baksh et al. 1995b, c) and a number of hormone receptors (Burns et al. 1994; Dedhar et al. 1994).

Calreticulin as a Chaperone

Calreticulin forms part of the quality control systems of ER for newly synthesized proteins.similar to calnexin. Both chaperones participate in the ‘quality-control’ process during the synthesis of a variety of molecules, including ion channels, surface receptors, integrins, MHC class I molecules, and transporters. Signicant progress has been made in understanding how Crt/Cnx act jointly with other ER chaperones and assisting proteins, in correct folding of proteins in ER. Molecular chaperones prevent the aggregation of partially folded proteins, increase the yield of correctly folded proteins and assembly, and also increase the rate of correctly folded intermediates by recruiting other folding enzymes. Researches document that Crt functions as a lectin-like molecular chaperone for many proteins (c/r Michalak et al. 1999, 2009).

Correct folding of a protein is determined in large part by the sequence of the protein, but it is also assisted by interaction with enzymes and chaperones of the ER. Calreticulin, calnexin, and ERp57 are among the endoplasmic chaperones that interact with partially folded glycoproteins and determine if the proteins are to be released from ER to be expressed, or alternatively, if they are to be sent to proteosome for degradation (Bedard et al. 2005; Michalak et al. 2009). Calnexin and calreticulin share many substrates and may form a link of lectin-like chaperones handing over the glycoproteins from one to the other to ensure proper folding (Bass et al. 1998; Helenius and Aebi 2004; Van Leeuwen et al. 1996a, b; Wada et al. 1995). Proposed functions for calreticulin range from chaperoning in ER to antithrombotic effects at the cell surface, and from the regulation of Ca^{2+} signaling to the modulation of gene expression

Table 2.2 Putative functions of calreticulin domains[a]

(a) Structural features and function	P-domain	C-domain
Calreticulin CRT ensuring proper protein folding and preventing aggregation. Proceeded by the N-terminal signal sequence targeting the protein to the ER lumen; highly conserved amino acid sequence; potential phosphorylation site; potential glycosylation site (bovine proteins); putative autokinase activity; inhibits PDI activity; suppresses tumors; inhibits angiogenesis	The P-domain contains the lectin site and a high-affinity Ca-binding region and is proline-rich; the lectin site recognizes N-linked oligosaccharide processing intermediates of glycoproteins and prolonged interaction with misfolded proteins initiates rejection and subsequent direction to the proteasome for degradation. CRT also engages in direct protein–protein interactions. CRT amino acid sequence shows similarity to calnexin, calmegin and CANLUC.	The acidic carboxy-terminal C-domain contains the high-capacity, low-affinity Ca^{2+}, binding sequence and terminates in a KDEL (lysine, aspartic acid, glutamic acid, leucine) sequence for ER retrieval; Putative glycosylation site; antithrombotic activity; prevents restenosis Ca^{2+} 'sensor' of calreticulin–protein interactions. CRT was shown to exist outside the ER by retrotranslocation to the cytoplasm through C-domain (Afshar et al. 2005).
(b) Ion binding		
binds Zn^{2+}	High-affinity Ca^{2+}-binding site	High-capacity Ca^{2+}-binding site
(c) Molecules binding		
Binds to DNA-binding domain of steroid receptor; binds to α-subunit of integrin; binds rubella RNA; interacts with PDI and ERp57; weak interactions with perforin. CRT directly binds to the identical amino-acid sequence, GFFKR, in the alpha integrin cytoplasmic tails. The binding site in the N-terminus of CRT that binds heparin-binding domain I, to mediate TSP-1 signaling, has been localized to amino-acid residues 19–36	Binds to a set of ER proteins; strong interactions with PDI; strong interactions with perforin; lectin-like chaperone site. Cell surface CRT binds to the carbohydrate constituent (mannose) of the cell adhesion and basement membrane protein, laminin, important in cell migration through integrin binding	Binds a set of ER proteins; binds factor IX and factor X; binds to cell surface

[a]Michalak et al. (1999; Gold et al. (2006)

and cellular adhesion (Table.2.2). Two major functions of calreticulin in the ER lumen, ie, chaperoning and regulation of Ca^{2+} homeostasis, were intensively investigated and well characterized.

The assembly of MHC class I molecules is one of the most widely studied examples of protein folding in ER. It is also one of the most unusual cases of glycoprotein quality control involving ERp57 and chaperones Cnx/Crt. Pulse-chase experiments showed that Crt was associated with several proteins ER in T lymphocytes and suggested that it was expressed at the cell surface. The cell surface 46-kDa protein co-precipitated with Crt is unfolded MHC-I. Results show that after T cell activation, significant amounts of Crt are expressed on T cell surface, where they are found in physical association with a pool of β2-free MHC class I molecules (Arosa et al. 1999). Calreticulin promotes folding of HLA class I molecules to a state at which they spontaneously acquire peptide binding capacity. However, it does not induce or maintain a peptide-receptive state of class I-binding site, which is likely to be promoted by one or several other components of class I loading complexes (Culina et al. 2004).

MHC class I molecules consist of two non-covalently linked subunits, an integral membrane glycoprotein (β chain), a small soluble protein [β_2-microglobulin (β_2-m)], and a peptide of eight to eleven residues. The multi-step assembly of MHC class-I heavy chain with β_2-m and peptide is facilitated by these ER-resident proteins and further tailored by the involvement of a peptide transporter, aminopeptidases, and the chaperone-like molecule tapasin (Wearsch and Cresswell 2008). In mouse and human, β chains associate with calnexin soon after its synthesis via interactions with both immature glycans and with residues in the transmembrane domain of β chains. In human chain, β_2-m binding to β chain displaces Cnx, and the resulting β chain–β_2-m heterodimer binds Crt. MHC class I expression and transport to the cell surface are not changed in Cnx-deficient cells, suggesting that Cnx is not essential for MHC class I synthesis and transport. These findings indicate, albeit indirectly, that Crt can function in the absence of Cnx. Wearsch and Cresswell (2008) presented the roles of these general and class I-specific ER proteins in facilitating the optimal assembly of MHC class I molecules with high affinity peptides for antigen presentation. Bass et al. (1998) demonstrated a chaperone function for Cnx/Crt in human insulin receptor (HIR) folding in vivo and also provided evidence that folding efficiency and homo-dimerization are counter balanced.

ER Luminal Ca^{2+} and Calreticulin Function

Ca^{2+} is released from the ER and taken up to the ER lumen. Ca^{2+} storage capacity of ER lumen is enhanced by Ca^{2+}-binding chaperones. These include calreticulin, Grp94, immunoglobulin-heavy-chain-binding protein (BiP; Grp78), PDI, ERp72 and ER/calcistorin. Reduction of $[Ca^{2+}]_{ER}$ (ER Ca^{2+}-depletion conditions) leads to accumulation of misfolded

proteins, activation of expression of ER chaperones and ER–nucleus and ER–plasma membrane 'signaling'. Ca^{2+} depletion inhibits ER–Golgi trafficking, blocks transport of molecules across the nuclear pore and affects chaperone function. Clearly, changes of the ER luminal $[Ca^{2+}]_{ER}$ have profound effects at multiple cellular sites, including the structure and function of the ER luminal Ca^{2+}-binding chaperones (Michalak et al. 1999).

Several studies of mammalian Crts have elucidated a number of key physiological functions, including the regulation of Ca^{2+} homeostasis and Ca^{2+}-dependent signal pathways (Michalak et al. 2002b; Gelebart et al. 2005), and integrin-dependent Ca^{2+} signaling at the extra-ER sites in mammalian cells (Coppolino et al. 1997; Krause and Michalak, 1997), and molecular chaperone activity in the folding of many proteins (Denecke et al. 1995; Williams, 2006). Ca^{2+} is a universal signaling molecule in the cell cytosol and can affects several processes in ER lumen, including modulation of chaperone–substrate and protein–protein interactions. For example, binding of carbohydrate to calreticulin and calnexin occurs at high $[Ca^{2+}]_{ER}$ and it is inhibited at low $[Ca^{2+}]_{ER}$ similar to Ca^{2+} depletion in stores. Several steps during chaprone action of both Cnx and Crt are regulated by Ca^{2+}. Studies suggest that C-domain of Crt plays a role of Ca^{2+} 'sensor' in the ER lumen. Association of Crt with Grp94, Grp78 and Cnx, maturation of thyroglobulin and apolipoprotein B may be regulated by Ca^{2+} binding.. The structure of Crt provides a unique feature enabling it to perform several functions in the ER lumen, while responding to continuous fluctuations of the free $[Ca^{2+}]_{ER}$. Calreticulin also affects Ca^{2+} homoeostasis (see below), and, in one case, the protein may even be taking advantage of its chaperone, lectin-like activity to modulate Ca^{2+} fluxes across the ER membrane (Leach et al. 2002; Michalak et al. 1999).

Calreticulin and Regulation of Ca^{2+} Homoeostasis: Calreticulin has two Ca^{2+}-binding sites: a high-affinity, low-capacity site ($K_d = 1$ μM; $B_{max} = 1$ mol of Ca^{2+}/mol of protein) in the P-domain and a low-affinity high-capacity site ($K_d = 2$ mM; $B_{max} = 25$ mol of Ca^{2+}/mol of protein) in the C-domain. Overexpression of Crt in a variety of cells does not affect the cytoplasmic $[Ca^{2+}]$; however, it does result in an increased amount of intracellularly stored Ca^{2+}. Interestingly, Ca^{2+}-storage capacity of the ER is not changed in the calreticulin-deficient embryonic stem cells (ES) or mouse embryonic fibroblasts (MEF) (Michalak et al. 1999). It is reported that changes in intracellular Ca^{2+} homeostasis modulate the rate of apoptosis as in molecular chaperones and radiation-induced apoptosis of gliomas (Brondani et al. 2004). Using human glioma cell lines, overexpression of Crt modulated radiosensitivity of human glioblastoma cells by suppressing Akt/protein kinase B signaling for cell survival via alterations of cell Ca^{2+} homeostasis. The level of CRT was higher in neuroglioma H4 cells than in glioblastoma cells (U251MG and T98G), and was well correlated with the sensitivity to γ-irradiation (Okunaga et al. 2006).

Calreticulin Functions Outside ER

There is considerable evidence to indicate that Crt is found outside ER, although how the protein relocates from ER to outside of the ER remains unclear. Functions of Crt outside the ER include modulation of cell adhesion, integrin-dependent Ca^{2+} signaling, and steroid-sensitive gene expression as well as mRNA destabilization both in vitro and in vivo. One major controversy in Crt research field concerns the mechanisms involved in Crt-dependent modulation of functions outside ER (Yokoyama and Hirata 2005). Outside ER Crt is known to modulate nuclear-hormone receptor-mediated gene expression (Burns et al. 1994; Michalak et al. 1996), control of cell adhesion (Opas et al. 1996; Fadel et al. 1999; Fadel et al. 2001; Goicoechea et al. 2002), and integrin-dependent Ca^{2+} signaling in vitro and in vivo (Coppolino et al. 1997; Michalak et al. 1999). It is also involved in blood function and development (Kuwabara et al. 1995; Andrin et al. 1998; Mesaeli et al. 1999). In addition, Crt appears to play a role in the immune system (Guo et al. 2002) and apoptosis. For example, Crt-dependent shaping of Ca^{2+} signaling was found to be a critical contributor to the modulation of the T cell adaptive immune response (Porcellini et al. 2006). Surface Crt mediates muramyl dipeptide-induced RK13 cell apoptosis through activating the apoptotic pathway (Chen et al. 2005). One major controversy in the calreticulin field concerns the mechanisms involved in Crt-dependent modulation of functions outside ER.

During apoptosis, both CRT expression and the concentration of nitric oxide (NO) are increased. By using S-nitroso-l-cysteine-ethyl-ester, an intracellular NO donor and inhibitor of APLT, phosphotidylserine (PS) and CRT externalization occurred together in an S-nitrosothiol-dependent and caspase-independent manner. Furthermore, the CRT and PS are relocated as punctate clusters on the cell surface. Thus, CRT induced nitrosylation and its externalization with PS could explain how CRT acts as a bridging molecule during apoptotic cell clearance (Tarr et al. 2010b).

Calreticulin and Cell Adhesiveness: Calreticulin may be involved in integrin function and cell adhesion. Crt binds to KXFF($^{K}/_{R}$)R synthetic peptide, a region corresponding to conserved amino acid sequence found in C-terminal tail of β-subunit of integrin. It was suggested that Crt may bind to the C-terminal cytoplasmic tail of β-integrin and modulate its function (Coppolino et al. 1997; Leung-Hagesteijn et al. 1994). Differential adhesiveness correlates inversely with

the expression level of mRNA and protein for the focal contact-associated cytoskeletal protein, vinculin, and the calreticulin. Furthermore, an inverse relationship exists between the level of calreticulin and the level of total cellular phosphotyrosine (Tyr(P)) such that the cells underexpressing calreticulin display a dramatic increase in the abundance of total cellular Tyr(P). This suggests that the effects of calreticulin on cell adhesiveness may involve modulation of the activities of protein-tyrosine kinases or phosphatases (Fadel et al. 1999). In addition, Crt can regulate cell adhesion indirectly from the ER lumen via modulation of gene expression of adhesion-related molecules such as vinculin and β-catenin (Opas et al. 1996; Fadel et al. 1999, 2001). It has also been shown that calreticulin associates transiently with the cytoplasmic domains of integrin α subunits during spreading and that this interaction can influence integrin-mediated cell adhesion to extracellular matrix (Coppolino et al. 1997; Leung-Hagesteijn et al. 1994; Goicoechea et al. 2002). Calreticulin serves as a cytosolic activator of integrin and a signal transducer between integrins and Ca^{2+} channels on the cell surface (Kwon et al. 2000).

Calreticulin can also modulate cell adhesion from the cell surface and mediate cell spreading on glycosylated laminin (Goicoechea et al. 2002) and thrombospondin-induced focal adhesion disassembly (Goicoechea et al. 2000; Pallero et al. 2008). Thrombospondin (TSP) is a member of a group of extracellular matrix proteins that exist in both soluble and extracellular matrix forms and regulates cellular adhesion (Goicoechea et al. 2002). When exposed to cells in its soluble form, thrombospondin has primarily anti-adhesive effects characterized by a reorganization of stress fibers and loss of focal adhesion plaques. A 19-amino acid sequence (aa 17–35) in the N-terminal heparin-binding domain of thrombospondin, referred to as the hep I peptide, has been shown to be sufficient for focal adhesion disassembly. Since, calreticulin can modulate cell adhesion from the cell surface and mediate thrombospondin-induced focal adhesion disassembly (Goicoechea et al. 2000), it was suggested that interactions between calreticulin and thrombospondin are Zn^{2+}- and Ca^{2+}-dependent and involve the RWIESKHKSDFGKFVLSS sequence in the N-terminal region of the N-domain of calreticulin (Goicoechea et al. 2002). TSP binding to Crt-LRP1 signals resistance to anoikis (Pallero et al. 2008).

Calreticulin Affects β-Catenin-Associated Pathways: It was shown that differential adhesiveness correlates inversely with the expression level of mRNA and protein for cytoskeletal protein, vinculin, and the Crt. Calreticulin has been shown to be important in cell adhesion. Furthermore, an inverse relationship existed between the level of Crt and the level of total cellular phosphotyrosine (Tyr(P)) such that cells underexpressing Crt display a dramatic increase in the abundance of total cellular Tyr(P). In either cell type, spatial distributions of Crt and Tyr(P) are complementary, with the former being confined to ER and the latter being found outside of it. Among proteins that are dephosphorylated in cells that over-express Crt is β-catenin, a structural component of cadherin-dependent adhesion complex and a part of the Wnt signaling pathway. To investigate the mechanisms behind Crt dependent modulation of cell adhesiveness, Fadel et al. (2001), using mouse L fibroblasts differentially expressing Crt, showed that stable over-expression of ER-targeted Crt correlates with an increased adhesiveness in transformed fibroblasts, such that their cohesion resembles that of epithelial cells in culture. Fadel et al. (2001) suggest that the changes in cell adhesiveness may be due to Crt-mediated effects on a signaling pathway from the ER, which impinges on Wnt signaling pathway via cadherin/catenin protein system and involves changes in the activity of protein-tyrosine kinases and/or phosphatases. Results suggest that calreticulin may play a role in a signaling pathway from ER, involving protein-tyrosine kinases and/or phosphatases This suggests that the effects of Crt on cell adhesiveness may involve modulation of the activities of protein-tyrosine kinases or phosphatases (Fadel et al. 1999, 2001). Protein phosphorylation/dephosphorylation of tyrosine is a major mechanism for regulation of cell adhesion. Although the mechanism(s) are still elusive, it is conceivable that the effects of Crt overexpression on cell adhesion may be due to Crt effects on a signaling pathway, which includes the vinculin/catenin–cadherin protein system and may involve changes in activity of tyrosine kinases and/or phosphatases. A direct implication of this for cell–substratum interactions is that calreticulin effects may target primarily focal-contact-mediated adhesion (Michalak et al. 1999).

Wound Repair: Crt was originally shown to be the biologically active component of a hyaluronic acid isolate from fetal sheep skin that accelerated wound healing in animal experimental models of cutaneous repair. Nanney et al. (2008) showed roles for exogenous Crt in both cutaneous wound healing and diverse processes associated with repair. Topical application of Crt to porcine excisional wounds enhanced the rate of wound re-epithelialization. The in vitro bioactivities provide mechanistic support for the positive biological effects of Crt observed on both the epidermis and dermis of wounds in vivo, underscoring a significant role for Crt in the repair of cutaneous wounds (Nanney et al. 2008).

Interaction of Calreticulin with C1q: C1q immobilized on a hydrophobic surface, exposed to heat-treatment or bound to Igs showed a strong, rapid and specific binding of Crt.

When both proteins were present in equal amounts in solution, no interaction could be demonstrated. Binding between C1q and Crt could be inhibited by serum amyloid P component. Results suggest Crt as a potential receptor for an altered conformation of C1q as occurs during binding to Igs. Thus, the chaperone and protein-scavenging function of Crt may extend from the ER to the topologically equivalent cell surface, where it may contribute to the elimination of immune complexes and apoptotic cells (Steinø et al. 2004).

Calreticulin in Signal Transduction: As indicated earlier, Crt is also found on cell surface of many cell types where it serves as a mediator of adhesion and as a regulator of the immune response. Calreticulin, present on the extracellular surface of mouse egg plasma membrane, is increased in perivitelline space after egg activation. The extracellular Crt appears to be secreted by vesicles in the egg cortex that are distinct from cortical granules. An anticalreticulin antibody binds to extracellular Crt on live eggs and inhibits sperm-egg binding but not fusion. In addition, engagement of cell surface Crt by incubation of mouse eggs in the presence of anticalreticulin antibodies results in alterations in the localization of cortical actin and the resumption of meiosis as indicated by alterations in chromatin configuration, decreases in cdc2/cyclin B1 and MAP kinase activities, and pronuclear formation. These events occur in absence of any observable alterations in intercellular calcium. These studies suggest that Crt functionally interacts with the egg cytoskeleton and can mediate transmembrane signaling linked to cell cycle resumption. Evidences suggest a role for Crt as a lectin that may be involved in signal transduction events during or after sperm-egg interactions at fertilization (Tutuncu et al. 2004).

Lessons from Calreticulin-Deficient Mice

The calreticulin-deficient mouse, created by the homologous-recombination, is embryonically lethal at 14.5–16.5 days *post coitus*. Calreticulin-deficient embryos most likely die from a lesion in cardiac development. In the adult, calreticulin is expressed mainly in non-muscle and smooth-muscle cells, and is only a minor component of the skeletal and cardiac muscle. However, the calreticulin gene is activated during cardiac development, concomitant with an elevated expression of the protein, which decreases sharply in the newborn heart. Therefore it was not surprising that calreticulin-deficient mice die from heart failure. Calreticulin may in general must play an important role in the formation of heart (Michalak et al. 2002a).

Calreticulin knockout ($Crt^{-/-}$) mice die at the embryonic stage due to impaired heart development, making it impossible to study mature $Crt^{-/-}$ CTLs and NK cells. To specifically investigate the role of Crt in CTL lytic function, Sipione et al. (2005) generated CTL lines from splenocytes derived from these mice and showed that in absence of Crt, CTL cytotoxicity is impaired. Sipione et al. (2005) suggest that Crt is dispensable for the cytolytic activity of granzymes and perforin, but it is required for efficient CTL-target cell interaction and for the formation of the death synapse. It was proposed that Crt may be involved in the mechanisms underlying target recognition by CTLs and/or formation of the death synapse. Calreticulin-deficient ES cells had impaired integrin-mediated adhesion, supporting the observation that changes in expression of calreticulin affect cell adhesion.

Impaired p53 Expression in Crt-Deficient Cells: The tumor suppressor protein, p53 is a transcription factor that not only activates expression of genes containing the p53 binding site but also can repress the expression of some genes lacking this binding site. Overexpression of wild-type p53 leads to apoptosis and cell cycle arrest. The level of Crt has been correlated with the rate of apoptosis. Crt-deficient cells ($crt^{-/-}$) demonstrated that Crt function is required for the stability and localization of the p53 protein. The observed changes in p53 in the $crt^{-/-}$ cells are due to the nuclear accumulation of Mdm2 (murine double minute gene). These results, lead us to conclude that Crt regulates p53 function by affecting its rate of degradation and nuclear localization (Mesaeli and Phillipson 2004).

Calreticulin and Steroid-Sensitive Gene Expression

Calreticulin is also found in nucleus, which suggests that it may play a role in transcriptional regulation. Calreticulin binds to the DNA-binding domain of steroid receptors and transcription factors containing the amino acid sequence KXFF($^{K}/_{R}$)R and prevents their interaction with DNA in vitro (Michalak et al. 1999). With the exception of the peroxisome-proliferator-activated receptor ('PPAR')–retinoid X heterodimers (Winrow et al. 1995), transcriptional activation by glucocorticoid, androgen, retinoic acid and vitamin D_3 receptors in vivo is modulated in cells overexpressing calreticulin (Burns et al. 1994; 1997; Dedhar et al. 1994; Desai et al. 1996; Michalak et al. 1996; St-Arnaud et al. 1995; Sela-Brown et al. 1998 Wheeler et al. 1995).

Outside ER, Crt appears to be affected by varios factors. The mRNA levels of Crt increased as a function of time after UV irradiation in transformed human keratinocytes (HaCaT cells) (Szegedi et al. 2001). A developmentally expressed cytosolic, trophoblast-specific, high Mr 57-kDa Ca-binding protein (CaBP) plays an important role in regulating and/or shuttling cytosolic Ca and represents the primary mechanism

in fetal Ca homeostasis. The full-length cDNA of the mouse CaBP shows significant homology to Crt. In addition, the action of parathyroid hormone related protein (PTHrP) on placental trophoblast Ca transport is likely to involve the regulation of CaBP expression to handle the increasing Ca requirements of developing fetus (Hershberger and Tuan 1998).

Calreticulin binds to the synthetic peptide KLGFFKR, which is almost identical to an amino acid sequence in the DNA-binding domain of the superfamily of nuclear receptors. The amino terminus of calreticulin interacts with the DNA-binding domain of the glucocorticoid receptor and prevents the receptor from binding to its specific glucocorticoid response element. Calreticulin can inhibit the binding of androgen receptor to its hormone-responsive DNA element and can inhibit androgen receptor and retinoic acid receptor transcriptional activities in vivo, as well as retinoic acid-induced neuronal differentiation.

Crt and Glucocorticoid Receptor Pathways: Calreticulin play an important role in the regulation of glucocorticoid-sensitive pathway of expression of the hepatocytes specific genes during development (Burns et al. 1997). ER Crt but not cytosolic Crt is responsible for inhibition of glucocorticoid receptor-mediated gene expression. These effects are specific to Crt, since over-expression of ER lumenal proteins (BiP, ERp72, or calsequestrin) had no effect on glucocorticoid-sensitive gene expression. The N domain of Crt binds to the DNA binding domain of glucocorticoid receptor in vitro. However, the N + P domain of Crt, when synthesized without ER signal sequence, does not inhibit glucocorticoid receptor function in vivo Michalak et al. (1996) suggest that Crt and glucocorticoid receptor may not interact in vivo and that the Crt-dependent modulation of the glucocorticoid receptor function may therefore be due to a Crt-dependent signaling from the ER. Wnt signaling pathway is a multicomponent cascade involving interaction of several proteins and found to be important for development and function of various cells and tissues. There is increasing evidence that Wnt/beta-catenin pathway constitutes also one of the essential molecular mechanisms controlling the metabolic aspects of osteoblastic cells. However, in bone, glucocorticoids (GCs) have been reported to weaken Wnt signaling. Calreticulin, known to bind the DNA binding domain of glucocorticoid receptor (GR), was found to be involved in the GR-mediated down-regulation of Wnt signaling. Furthermore, GR and β-catenin were shown to exist in same immunocomplex, while interaction between Crt and beta-catenin was observed only in the presence of GR as a mediator molecule. In addition, the GR mutant lacking Crt binding ability impaired the complex formation between beta-catenin and Crt. Together with GR, β-catenin could thus be co-transported from the nucleus in a Crt-dependent way (Olkku and Mahonen A, 2009).

Overexpression of calreticulin in mouse L fibroblasts inhibits glucocorticoid-response-mediated transcriptional activation of a glucocorticoid-sensitive reporter gene and of the endogenous, glucocorticoid-sensitive gene encoding cytochrome P450. This indicates that calreticulin may be important in gene transcription, regulating the glucocorticoid receptor and perhaps other members of the super-family of nuclear receptors (Burns et al. 1994). Thus, calreticulin can act as an important modulator of the regulation of gene transcription by nuclear hormone receptors. These are surprising findings, since calreticulin is an ER-resident protein and steroid receptors are found in the cytoplasm or in the nucleus. What could be a physiological or pathophysiological relevance of calreticulin (ER)-dependent modulation of gene expression? Up-regulation of the calreticulin gene may correlate with increased resistance to steroids. For example, calreticulin is one of the androgen-sensitive genes in prostate cancer (Zhu et al. 1998; Zhu and Wang 1999; Wang et al. 1997). Steroid-dependent regulation of expression of calreticulin may affect differential sensitivity of patients to steroid therapies.

Over-expression of calreticulin and calsequestrin impairs cardiac function, leading to premature death. Calreticulin is vital for embryonic development, but also impairs glucocorticoid action. Glucocorticoid overexposure during late fetal life causes intra-uterine growth retardation and programmed hypertension in adulthood. In view of the known associations between cardiac calreticulin overexpression and impaired cardiac function, targeted up-regulation of calreticulin may contribute to the increased risk of adult heart disease introduced as a result of prenatal overexposure to glucocorticoids (Langdown et al. 2003; Michalak et al. 2002a). The neural cell adhesion molecule, N-CAM inhibits the proliferation of rat astrocytes both in vitro and in vivo. Exposure of astrocytes to N-CAM in vitro, the levels of mRNAs for glutamine synthetase and calreticulin increased while mRNA levels for N-CAM decreased. Glutamine synthetase and calreticulin are known to be involved in glucocorticoid receptor pathways. Inhibition of rat cortical astrocyte proliferation in culture by dexamethasone, corticosterone, and aldosterone suggests that astrocyte proliferation is in part regulated by alterations in glucocorticoid receptor pathways, which may involve Crt (Crossin et al. 1997).

Regulation by Androgens: Calreticulin is an intracellular protein in prostatic epithelial cells. Its expression in prostate is much higher than that in seminal vesicles, heart, brain, muscle, kidney, and liver. The expression of Crt in prostate is conserved evolutionarily. After castration, Crt mRNA and

protein are down-regulated in the prostate and seminal vesicles and restored by androgen replacement. Because Crt is a major intracellular Ca^{2+}-binding protein with 1 high-affinity and 25 low-affinity Ca binding sites, observations suggest that Crt is a promising candidate that mediates androgen regulation of intracellular Ca^{2+} levels and/or signals in prostatic epithelial cells (Zhu et al. 1998). As expected, androgen protects androgen-sensitive LNCaP but not androgen-insensitive PC-3 cells from cytotoxic intracellular Ca^{2+} overload induced by Ca^{2+} ionophore A23182. Observations suggest that Crt mediates androgen regulation of the sensitivity to Ca^{2+} ionophore-induced apoptosis in LNCaP cells (Zhu and Wang, 1999; Meehan et al. 2004).

Estrogens: While estrogens are mitogenic in breast cancer cells, the presence of estrogen receptor α (ERα) clinically indicates a favorable prognosis in breast carcinoma. Calreticulin that could interact with amino acids 206–211 of ERα reversed hormone-independent ERα inhibition of invasion. However, since Crt alone also inhibited invasion, it was proposed that this protein probably prevents ERα interaction with another unidentified invasion-regulating factor. The inhibitor role of the unliganded ER was also suggested in three ERα-positive cell lines, where ERα content was inversely correlated with cell migration. It was concluded that ERα protects against cancer invasion in its unliganded form, probably by protein-protein interactions with the N-terminal zinc finger region, and after hormone binding by activation of specific gene transcription (Platet et al. 2000).

Neonatal treatment with diethylstilbestrol (DES) leads to disruption of spermatogenesis in adult animals after apparently normal testicular development during puberty indicating aberrant androgen action in DES-exposed adult hamsters. Analyses revealed that mRNA levels for AR-responsive genes calreticulin, SEC-23B, and ornithine decarboxylase were significantly decreased in DES-exposed animals and that neonatal DES exposure impairs the action of androgens on target organs in male hamsters (Karri et al. 2004).

Crt Enhances Transcriptional Activity of TTF-1: Calreticulin binds to thyroid transcription factor-1 (TTF-1), a homeodomain-containing protein implicated in the differentiation of lung and thyroid. The interaction between calreticulin and TTF-1 appears to have functional significance because it results in increased transcriptional stimulation of TTF-1-dependent promoters. Calreticulin binds to the TTF-1 homeodomain and promotes its folding, suggesting that the mechanism involved in stimulation of transcriptional activity is an increase of the steady-state concentration of active TTF-1 protein in the cell. It was also demonstrated that calreticulin mRNA levels in thyroid cells are under strict control by the thyroid-stimulating hormone, thus implicating calreticulin in the modulation of thyroid gene expression by thyroid-stimulating hormone (Perrone et al. 1999). The thyroid hormone receptor α1 (TRα) directly interacts with calreticulin, and point to the intriguing possibility that TRα follows a cooperative export pathway in which both calreticulin and CRM1 (Exportin) play a role in facilitating efficient translocation of TRα from the nucleus to cytoplasm (Grespin et al. 2008).

The increased level of calreticulin in activated T-cells suggests that the protein may be part of the Ca^{2+}-dependent transduction pathway(s) in stimulated T-cells, including activation of NF-AT. Therefore stress-dependent stimulation of the T-cells results in the activation of both the NF-AT and calreticulin pathways. Development of the heart as an organ must inflict a tremendous stress on cardiomyocytes. Therefore it is tempting to speculate that similar stress-induced signaling pathways are essential during cardiac development and the activation of the immune system.

Destabilization of 3′-Untranslated Region of mRNA by Crt

Angiotensin II is hypertrophic for cultured adult rat aortic vascular smooth muscle cells (VSMC), whereas platelet-derived growth factor and serum are hyperplastic. Hyperplastic and hypertrophic growth are accompanied by similar changes in protein expression, suggesting that both types of growth require up-regulation of the protein synthesis and folding machinery such as calreticulin and HSPs (Patton et al. 1995). Angiotensin II plays a central role in cardiovascular homeostasis and downregulates type 1 angiotensin II (AT_1) receptor, which appears to involve several mechanisms. The AT1 receptor plays a pivotal role in the pathogenesis of hypertension and atherosclerosis. AT1 receptor expression is regulated posttranscriptionally via destabilization of AT1 receptor mRNA by mRNA binding proteins. Nickening et al. discovered that mRNA binding protein, Crt, binds to the cognate sequence bases 2175–2195 within the 3′ untranslated region of the AT_1 receptor mRNA (Nickenig et al. 2002). Angiotensin II stimulation, which causes destabilization of AT_1 receptor mRNA, causes phosphorylation of Crt. This region comprises aAUUUUA hexamer and is considerably AU-rich. Phosphorylation of Crt is essential for binding of the AT1 receptor mRNA. Findings imply an important role of serine dephosphorylation and tyrosine phosphorylation on Crt mediated AT1 receptor mRNA stability in VSMC (Mueller et al. 2008).

Glucose transport in mammalian cells is mediated by a family of structurally related glycoproteins, the glucose transporters (GLUTs) (McGowan et al. 1995). Totary-Jain et al. report that calreticulin destabilized GLUT-1 mRNA expression in primary bovine aortic endothelial cells and smooth muscle cells under high glucose conditions

(Totary-Jain et al. 2005). They identified Crt as a specific destabilizing trans-acting factor that binds to a 10-nucleotide cis-acting element (CAE 2181–2190) in the 3′-untranslated region of GLUT-1 mRNA. CAE 2181–2190–Crt complex, which is formed in VSMCs and endothelial cells exposed to hyperglycemic conditions, renders GLUT-1 mRNA susceptible to degradation. RNA–protein interactions have been shown to influence many processes, including translation, RNA stability, mRNA transport and localization, splicing, and polyadenylation (Qi and Pekala, 1999, Yokoyama and Hirata 2005).

Calreticulin binds to antibodies in certain sera of systemic lupus and Sjogren patients that contain anti-Ro/SSA antibodies. Systemic lupus erythematosus is associated with increased autoantibody titers against Crt, but Crt is not a Ro/SS-A antigen. Earlier papers referred to Crt as an Ro/SS-A antigen, but this was later disproven. Increased autoantibody titer against human Crt is found in infants with complete congenital heart block of both the IgG and IgM classes.

2.2.5 Structure-Function Relationships in Calnexin and Calreticulin

The three-dimensional structure of the lumenal domain of the lectin-like chaperone calnexin determined to 2.9 A resolution reveals an extended 140 A arm inserted into a beta sandwich structure characteristic of legume lectins. The extended arm is curved, forming an opening, which likely accommodates specific substrates. The glucose-binding site of calnexin is located on the surface of the globular domain, facing the extended arm. The arm is composed of tandem repeats of two proline-rich sequence motifs which interact with one another in a head-to-tail fashion. Identification of the ligand binding site establishes calnexin as a monovalent lectin, providing insight into the mechanism by which the calnexin family of chaperones interacts with monoglucosylated glycoproteins. The globular domain of calnexin also contains a Ca2+ binding site and one disulfide bond. Another disulfide bond is located near the tip of the extended arm (Schrag et al. 2001). A model of calreticulin 3D structure predicts that the N-domain of calreticulin is globular and contains a glucose binding site and a disulfide bridge. The P-domain is also predicted to contain the unusual extended arm structure identified in calnexin. The globular N-domain together with the extended arm P-domain of calreticulin and calnexin may form a functional "protein-folding module". The C-terminal region of calreticulin, which is highly acidic, binds Ca2+ with high capacity and is involved in Ca^{2+} storage in the lumen of the ER in vivo (Nakamura et al. 2001b). Ca^{2+} binding to calreticulin and, consequently, changes in the ER Ca^{2+} storage capacity, affect the protein's chaperone function and thereby influence the "quality control" of the secretory pathway. Calreticulin interacts in a Ca^{2+}-dependent manner with other ER chaperones, modulating their function (Corbett et al. 2000).

Lectin-Deficient Crt Retains Full Functionality as a Chaperone: Calreticulin uses both a lectin site specific for $Glc_1Man_{5\text{-}9}GlcNAc_2$ oligosaccharides and a polypeptide binding site to interact with nascent glycoproteins. The latter mode of substrate recognition is controversial. To examine the relevance of polypeptide binding to protein folding in living cells, in Crt-deficient mutants, class I molecules exhibit inefficient loading of peptide ligands, reduced cell surface expression and aberrantly rapid export from ER. It suggested that Crt can use nonlectin-based modes of substrate interaction to effect its chaperone and quality control functions on class I molecules in living cells. Furthermore, lectin-deficient Crt bound to a similar spectrum of client proteins as wild-type Crt and dissociated with similar kinetics, suggesting that lectin-independent interactions are common place in cells that may be regulated during client protein maturation (Ireland et al. 2008).

2.2.6 Pathophysiological Implications of Calreticulin

Cardiovascular System

It is interesting that high glucose augments calreticulin expression in vascular smooth muscle cells and endothelial cells (Qi and Pekala 1999), although the mechanism and patho-physiological significance of these findings in the vascular cells has not yet been investigated. Dai et al. (1997) documented a profound inhibitory effect of intravenous administration of calreticulin on intimal hyperplasia in rat iliofemoral arteries after balloon injury in vivo. Because calreticulin can be found in extracellular locations including the blood, and it has been associated with regulation of immune responses, calreticulin has also been implicated in a number of pathological processes. The calreticulin gene knock-out study also indicates that the protein plays a role in the development of the heart (Gelebart et al. 2005; Michalak et al. 2008). It is shown that carticulin is upregulated in the heart during the middle stages of embryogenesis, whereas it is expressed at a low level after birth. Further studies are required to unravel the pathophysiological roles of calreticulin in the pathogenesis of various diseases.

Calreticulin and Cardiac Pathology: Calreticulin deficiency is embryonic lethal because it causes lesions during cardiac development (Mesaeli et al. 1999). However, overexpression of the protein in developing and postnatal heart leads to bradycardia, complete heart block and sudden death.

Ultrastructural evidence indicates that the deficiency associated with the absence of calreticulin in the heart may be due to a defect in the development of the contractile apparatus and/or a defect in development of the conductive system as well as a metabolic abnormality. Michalak et al. (2004) postulate that calreticulin and endoplasmic reticulum plays an important role in cardiac development and postnatal pathologies (Michalak et al. 2004).

Calreticulin-Deficient Mouse: Cells isolated from $Crt^{-/-}$ embryos have impaired agonist-induced Ca^{2+} release (Nakamura et al. 2001a), inhibited nuclear import of the transcription factors NF-ATc1, Mef2c and p53, modified sensitivity to apoptosis, compromised function of calnexin, and activated unfolded proteins response (UPR) indicating a major impact of calreticulin deficiency on ER and cellular functions. Remarkably, $Crt^{-/-}$ mice are rescued by expression of constitutively active calcineurin in the heart and exhibit severe postnatal pathology with death 7–35 days after birth (Guo et al. 2002). Calreticulin-deficient animals that have been rescued with cardiac expression of calcineurin go on to develop severe metabolic problems in cholesterol, lipid, and carbohydrate metabolism. The underlying cause of the metabolic aberrations in these mice is not understood but it indicates that many metabolic processes rely on ER function. Calreticulin expression is high in embryonic heart and declines sharply after birth, probably due to transcriptional control of the calreticulin gene. High expression of calreticulin in the heart of transgenic mice results in early postnatal death. Animals over-expressing calreticulin in the heart develop bradycardia associated with sinus node dysfunction, complete cardiac block, and death due to intractable heart failure. This indicates that calreticulin plays a role in the pathology of the heart's conductive system (Nakamura et al. 2001b).

Deletion of Crt gene leads to defects in the heart and the formation of omphaloceal. These defects could both be due to changes in the extracellular matrix composition. Matrix metalloproteinases (MMP)-2 and MMP-9 are two of the MMPs which are essential for cardiovascular remodeling and development. Wu et al. (2007) demonstrated that there is a significant decrease in the MMP-9 and increase in the MMP-2 activity and expression in $Crt^{-/-}$ deficient cells, and a significant increase in the expression of membrane type-1 matrix metalloproteinase (MT1-MMP).

Human Pregnancy and Pre-Eclampsia

Evidence indicates that pre-eclampsia involves widespread activation of maternal endothelial cells. Calreticulin has been shown to have both pro- and anti-inflammatory effects in vitro and in whole animals. In normal human pregnancy and in pre-eclampsia, there was a significant increase (5-fold) in calreticulin in plasma in term pregnant women compared with women who were not pregnant. Results indicate that calreticulin is increased in peripheral maternal blood early in pregnancy and remains elevated throughout normal gestation and that there is a further increase in calreticulin in pre-eclampsia (Gu et al. 2008). Calreticulin can be released into extracellular environment in some circumstances. For example, there is a tenfold increase in Crt in the blood of patients with systemic lupus erythematosus (Eggleton et al. 1997). The sources and roles of extracellular Crt are not clear. Nevertheless, evidence indicates that extracellular as well as intracellular Crt can also affect many cellular functions including adhesion, migration and proliferation (Bedard et al. 2005). In particular, its effects on vascular endothelial cells may be relevant to the normal pregnancy and pre-eclampsia.

Crt Interacts with HIV Envelope Protein

Calreticulin binds in vitro to a number of proteins isolated from ER. In cells expressing recombinant HIV envelope glycoprotein, gp160 bound transiently to calreticulin. The binding kinetics of calnexin and calreticulin to gp160 were very similar. Data suggested that most of the gp160 associated with calreticulin was also bound to calnexin but that only a portion of gp160 associated with calnexin was also bound to calreticulin (Otteken and Moss 1996).

Despite ER targeting and retention signals, calreticulin is also located within the nucleus where its presence increases due to its interaction with glucocorticoid receptors (Roderick et al. 1997). Therefore, Crt can inhibit steroid-regulated gene transcription by preventing receptor binding to DNA. Over-expression of Crt gene in B16 mouse melanoma cells resulted in a decrease in retinoic acid (RA)-stimulated reporter gene expression. Purified Crt inhibited the binding of endogenous RAR to a β-RA response element oligonucleotide, only if added prior to the addition of oligonucleotide. Cyclic AMP increased the expression of Crt. Cyclic AMP may act to antagonize RA action by both decreasing RAR expression and stimulating Crt levels (Desai et al. 1996).

Crt Is a Human Rheumatic Disease-Associated Autoantigen

Reports indicate that Crt is a human rheumatic disease-associated autoantigen. This protein shares an intimate relationship with Ro/SS-A autoantigen complex. Calcium ionophore, heat shock, and heavy metals such as zinc and cadmium are consistently found to increase Crt transcriptional activities in A431 cells (a human epidermoid squamous carcinoma cell line) under transient transfection conditions. Studies suggest that Crt is regulated at transcriptional level, and like some other LE-related autoantigens, Crt appears to function as a heat shock/stress-response gene (Nguyen et al. 1996). CRT was present at higher

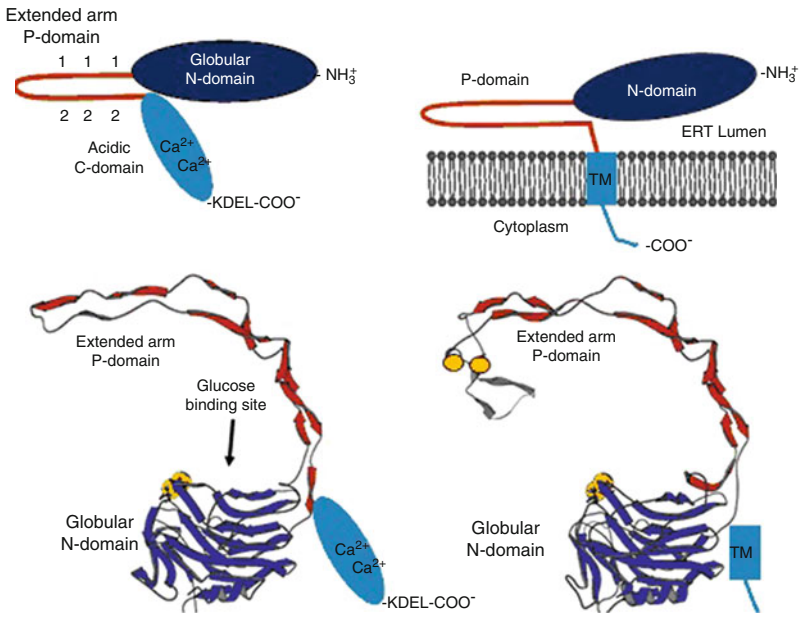

Fig 2.6 Structural models of calnexin and of calreticulin: *Left* panel shows schematic representation of calreticulin domains. *Right* panel shows a model of calnexin based on crystallographic studies of Cnx (1JHN). *Yellow* balls represent cysteines, which form an S-S bridge. Putative glucose-binding site is indicated (Adapted with permission from Schrag et al. 2001 © Elsevier and with permission from Michalak et al. 1999 Biochem J. 344: 281–97 © The Biochemical Society)

concentrations in the plasma and synovial fluid of RA patients. CRT had the capacity to bind directly to FasL, and inhibiting FasL- mediated apoptosis of Jurkat T cells, and might play a role in inhibiting apoptosis of inflammatory T cells in RA (Tarr et al. 2010a).

2.2.7 Similarities and Differences Between Cnx and Crt

Cnx performs the same service for soluble proteins as does calreticulin. Both proteins, Cnx and Crt, have the function of binding to oligosaccharides containing terminal glucose residues, thereby targeting them for degradation. Structural studies suggest that both proteins consist of a globular domain and an extended arm domain comprised of two sequence motifs repeated in tandem (Fig. 2.6). The primary lectin site of Cnx and Crt resides within the globular domain, but the results also point to a much weaker secondary site within the arm domain, which lacks specificity for monoglucosylated oligosaccharides. For both proteins, a site of interaction with ERp57 is centered on the arm domain, which retains ~50% of binding compared with full-length controls. This site is in addition to a Zn^{2+}-dependent site located within the globular domain of both proteins. Finally, calnexin and calreticulin suppress the aggregation of unfolded proteins via a polypeptide binding site located within their globular domains but require the arm domain for full chaperone function (Leach et al. 2002).

In normal cellular function, trimming of glucose residues off the core oligosaccharide added during N-linked glycosylation is a part of protein processing. If "overseer" enzymes note that residues are misfolded, proteins within the RER will re-add glucose residues so that other Calreticulin/Calnexin can bind to these proteins and prevent them from being exported from ER to Golgi. This leads these aberrantly folded proteins down a path whereby they are targeted for degradation.

Calnexin and Calreticulin at Mitochondrial Membranes: ER chaperones, particularly Ca^{2+}-binding chaperones (Cnx, Crt, and BiP), were also found to be compartmentalized at mitochondrial membranes (MM) (Hayashi and Su 2007; Myhill et al. 2008). Under physiological conditions, these chaperones serve as high-capacity Ca^{2+}-binding proteins at ER (Hendershot 2004). Calreticulin provides up to 45% of the Ca^{2+}-buffering capacity for a pool of the IP3-sensitive Ca^{2+} inside the ER (Bastianutto et al. 1995). The compart-mentalized chaperones at the MAM therefore serve as high-capacity Ca^{2+} pools in the ER. In addition, independent of its Ca^{2+}-buffering capacity in the ER, calreticulin inhibits IP3 receptor-mediated Ca^{2+} signaling by using its high-affinity-low-capacity Ca^{2+}-binding domain (Camacho and Lechleiter 1995). Further, calreticulin regulates the activity of Ca^{2+}-ATPase, providing dynamic control of ER Ca^{2+} homeostasis (Li and Camacho 2004). Calnexin

can also regulate the activity of Ca^{2+}-ATPase via a direct protein-protein interaction (Roderick et al. 2000). In addition, the activity and action of calnexin and calreticulin are regulated by other chaperones or proteins most likely occurring at the MAM of the ER (Hayashi et al. 2009a).

Cnx/Crt in MHC Class I Assembly Pathway: MHC class I molecules are ligands for T-cell receptors of $CD8^+$ T cells and inhibitory receptors of natural killer cells. Assembly of the heavy chain, light chain, and peptide components of MHC class I molecules occurs in ER. The folding and assembly of class I molecules is assisted by molecular chaperones and folding catalysts that comprise the general ER quality control system which also monitors the integrity of the process, disposing of misfolded class I molecules through ER associated degradation (ERAD). Fu et al. (2009) showed that reduced class I expression in Crt deficient cells can be restored by direct delivery of peptides into the ER or by incubation at low temperature.

Crt deficient cells exhibited a TAP-deficient phenotype in terms of class I assembly, without loss of TAP expression or functionality. In the absence of Crt, ERp57 is up-regulated, which indicates that they collaborate with each other in class I antigen processing. Specific assembly factors and generic ER chaperones, collectively called the MHC class I peptide loading complex (PLC), are required for MHC class I assembly. Calreticulin has an important role within the PLC and induces MHC class I cell surface expression. Interactions with ERp57 and substrate glycans are important for the recruitment of calreticulin into the PLC and for its functional activities in MHC class I assembly. The glycan and ERp57 binding sites of calreticulin contribute directly or indirectly to complexes between calreticulin and the MHC class I assembly factor tapasin and are important for maintaining steady-state levels of both tapasin and MHC class I heavy chains. The generic polypeptide binding sites per se are insufficient for stable recruitment of calreticulin to PLC substrates in cells. However, such binding sites could contribute to substrate stabilization in a step that follows the glycan and ERp57-dependent recruitment of calreticulin to the PLC (Del Cid et al. 2010).

Association of HLA Class I Antigen Abnormalities with Disease Progression in Malignancies: MHC class I molecules are crucial in presenting antigenic peptide epitopes to cytotoxic T lymphocytes. Proper assembly of MHC class I molecules is dependent on several cofactors, e.g. chaperones Cnx and Crt residing in ER. Lectin deficient mutants of Cnx were shown to interact with heavy chains of major MHC class I molecules in insect cells and to prevent their rapid degradation. Similarly, lectin-deficient Crt was found not only to interact with a broad spectrum of newly synthesized proteins and dissociate with normal kinetics, but it was also able to complement all MHC class I biosynthetic defects associated with Crt deficiency. MHC class Ia downregulation has been repeatedly described on melanoma cells and is thought to be involved in failure of immune system to control tumor progression. Alterations in the expression of chaperones Cnx/Crt may have important implications for MHC class I assembly, peptide loading, and presentation on the tumor cell surface and thus may contribute to immune escape phenotype of tumor cells. Metastatic melanoma lesions exhibited significant downregulation of Cnx as compared to primary melanoma lesions. In contrast, Crt was expressed in melanoma cells of primary as well as of metastatic lesions. Data suggest that chaperone-downregulation, particularly Cnx-downregulation, may contribute to the metastatic phenotype of melanoma cells in vivo. Consistently, conserved chaperone expression in metastatic melanoma lesions may be a useful criterion for selection of patients for treatment with T cell-based immunotherapies (Dissemond et al. 2004). However, mutant human cells lacking Cnx, infected with recombinant vaccinia viruses encoding mouse MHC class I molecules, K^d, K^b, K^k, D^d, D^b, and L^d, indicated that Cnx is not required for the efficient assembly of MHC class I molecules with TAP-dependent or independent peptides (Prasad et al. 1998; Mehta et al. 2008). The IFN-γ inducible proteasome subunits LMP2 and LMP7, TAP1, TAP2, Cnx, Crt, ERp57, and tapasin are strongly expressed in the cytoplasm of normal prostate cells, whereas HLA class I heavy chain (HC) and β_2-microglobulin are expressed on cell surface. Most of antigen processing machinery (APM) components was downregulated in a substantial number of prostate cancers. Thus HLA class I APM component abnormalities are mainly due to regulatory mechanisms, play a role in the clinical course of prostate cancer and on the outcome of T cell-based immunotherapies (Dissemond et al. 2004; Seliger et al. 2010).

2.2.8 Calreticulin in Invertebrates

Crt is highly conserved in eukaryotic cells, which is indicated by sequence analysis on the deduced amino acids of the known Crt cDNA clones from several mammalian species and other organisms including nematode, fruit fly (Smith, 1992), marine snail (Kennedy et al. 1992), clawed frog (Treves et al. 1992), rainbow trout (Stephen et al. 2004), and Cotesia rubecula (Zhang et al. 2006). Kennedy et al. (1992) identified Crt in Aplysia where it was enriched in presynaptic varicosities. The steady-state level of Crt mRNA in Aplysia sensory neurons increases during the maintenance phase of long-term sensitization. This mRNA increase in expression late, some time after training, is consistent with

the idea that long-term neuromodulatory changes underlying sensitization may depend on a cascade of gene expression in which the induction of early regulatory genes leads to the expression of late effector genes (Kennedy et al. 1992).

A human Ro/SS-A (Ro) autoantigen of 60-kDa, homologous to Crt and Aplysia "memory molecule" has a molecular mass, isoelectric point, and significant amino acid sequence similar to Aplysia californica snail neuronal protein 407 (McCauliffe et al. 1990). These homologies suggest that this Ro protein has a very basic cellular function(s) which may in part involve calcium binding (McCauliffe et al. 1990). The Ro autoantigens consist of at least four immunologically distinct proteins which are recognized by autoantibodies typically found in sera from patients with primary Sjogren's syndrome and in subsets of patients with lupus erythematosus. The mouse cDNA-encoded amino acid sequence was found to be 94% homologous to the human Ro sequence and is 100% homologous to murine calreticulin, a high affinity calcium-binding protein which resides in the endoplasmic and sarcoplasmic reticulum. The amino acid sequence of rabbit Crt is 92% homologous to both murine Crt and human Ro. Onchocerca volvulus and Drosophila melanogaster also have molecules that are highly homologous to human Ro.

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P-Type Lectins: Cation-Dependent Mannose-6-Phosphate Receptor

3

G.S. Gupta

In eukaryotic cells, post-translational modification of secreted proteins and intracellular protein transport between organelles are ubiquitous features. One of the most studied systems is the *N*-linked glycosylation pathway in the synthesis of secreted glycoproteins (Schrag et al. 2003). The *N*-linked glycoproteins are subjected to diverse modifications and are transported through ER and Golgi apparatus to their final destinations in- and outside the cell. Incorporation of cargo glycoproteins into transport vesicles is mediated by transmembrane cargo receptors, which have been identified as intracellular lectins. For example, mannose 6-phosphate receptors (Ghosh et al. 2003) function as a cargo receptor for lysosomal proteins in the *trans*-Golgi network, whereas ERGIC-53 (Zhang et al. 2003) and its yeast orthologs Emp46/47p (Sato and Nakano 2003) are transport lectins for glycoproteins that are transported out of ER.

3.1 The Biosynthetic/Secretory/Endosomal Pathways

In eucaryotic cells, proteins destined for secretion are first inserted into ER and then transported by a process of vesicle budding and fusion, through the Golgi complex and then to the cell surface. Various compartments that comprise this secretory pathway, despite being interconnected by vesicular traffic, differ in their lipid and protein composition. The maintenance of these differences requires that the incorporation of molecules into vesicles is a selective process, and that vesicles are directed to specific target membranes. Much effort has been directed in recent years in understanding these processes, and the ways in which they are integrated to produce organelles of characteristic size, morphology and composition.

3.1.1 Organization of Secretory Pathway

The early secretoty pathway (ESP) is defined as sequential compartments comprising the cisternal/tubular ER, pre-Golgi intermediates [also referred to as vesicular tubular clusters (VTCs) or ER-Golgi intermediates (ERGIC)] and the cis-Golgi network (CGN), the Golgi stacks and trans-Golgi network (TGN) as a final sorting station. The basic function of this pathway includes transport of proteins destined for secretion from ER to Golgi and further to the plasma membrane (PM) (Fig. 3.1). Synthesized material can also be recycled to ER from the Golgi. The compartments are connected with each other by vesicular traffic which mediates the transport of cargo between different organelles and also controls the composition and homeostasis of the structures (Rothman and Wieland 1996). The ESP transports biosynthetic material from ER to Golgi complex. Lipids, proteins and carbohydrates are modified and transported through Golgi to TGN in which they are sorted and packed into vesicles for further transport to various destinations. At TGN, specific sorting signals in the cargo molecules and the cellular sorting machineries are responsible for directing the cargo either to the PM, to regulated secretory granules, or to the endosomal/lysosomal system (Le Borgne and Hoflack 1998). The bulk flow secretory pathway operates in all cells and it leads to a continuous unregulated secretion or transport to the PM. Some specialized cells also possess a distinct regulated secretory pathway in which certain specific proteins are secreted to extracellular space in response to external signal(s). In general, the secretory pathway provides a framework by which proteins undergo a series of posttranslational modifications including proteolytic processing, folding and glycosylation (Storrie et al. 2000).

3.1.1.1 Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a eukaryotic organelle that forms an interconnected network of tubules, vesicles, and cisternae within cells. The general structure of the ER is an extensive membrane network of cisternae (sac-like structures) held together by the cytoskeleton. The phospholipid membrane encloses a space, the cisternal space (or lumen), which is continuous with the perinuclear space but separate from

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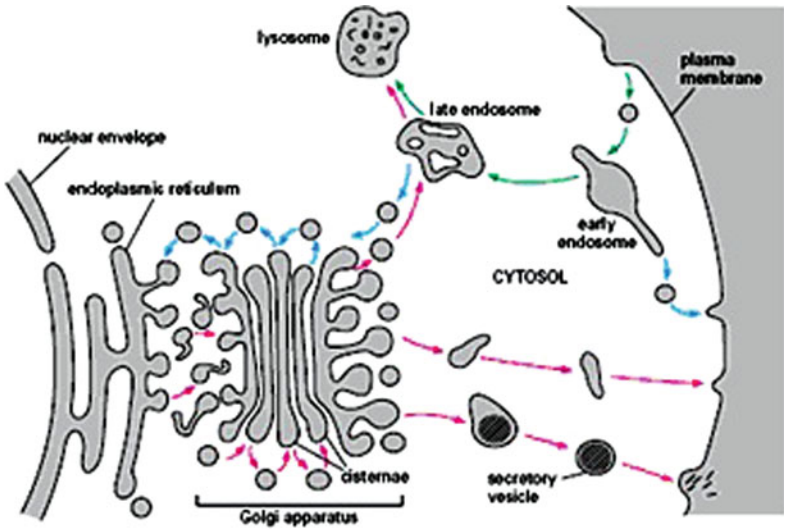

Fig. 3.1 A schematic representation of the different vesicle transport pathways originating from the *trans*-Golgi network (Reprinted by permission from from Le Borgne and Hoflack 1998 © Elsevier). *Arrows* indicate transport steps that are known to occur, but not discussed in detail in this article

the cytosol. The functions of the endoplasmic reticulum vary greatly depending on the exact type of endoplasmic reticulum and the type of cell in which it resides. The ER is the entry station for all proteins of the synthetic/secretory pathway and consists of nuclear envelope, rough ER (rER), smooth ER (sER), transitional ER (tER), and intermediate compartment (IC) (Marie et al. 2009; Lippincott-Schwartz et al. 2000). Rough endoplasmic reticula synthesize proteins, while smooth endoplasmic reticula synthesize lipids and steroids, metabolize carbohydrates and steroids, and regulate calcium concentration, drug metabolism, and attachment of receptors on cell membrane proteins. Different subcompartments of ER have characteristic biochemical and physiological properties and they serve specific subcellular functions. Structurally ER is seen as a three-dimensional, reticular network of continuous tubules and sheets creating the largest membranous organelle of the cell. Functionally ER is responsible for the synthesis and processing of secreted proteins, membrane proteins and organelle resident proteins. The ER is also part of a protein sorting pathway and seen as a compartment that participates in the assembly, sorting, and degradation of proteins as well as in the regulation of intracellular calcium concentration (Harter and Wieland 1996). It is, in essence, the transportation system of the eukaryotic cell. From ER, properly folded and assembled proteins are further transported via IC to the Golgi complex by specific carrier vesicles which bud on the ER and move to cis-Golgi membranes. Folded proteins may remain in the ER if it is their home compartment or else they are transported to the secretory pathway. COPII, a coat complex which forms a main structure of transport vesicles, is responsible for forward transport of cargo from the ER to Golgi complex. COPI vesicles in their turn carry cargo between Golgi and ER and in intra-Golgi transport (Barlowe et al. 1994; Letourneur et al. 1994; Schekman and Orci 1996). Sorting of synthesized proteins at the ER occurs by selective incorporation of secretory and membrane proteins into vesicles that bud from the ER (Pelham 1996; Wieland and Harter 1999). The majority of ER resident proteins are retained in the ER through a specific carboxy terminal retention motif. This motif is composed of four amino acids at the end of the protein sequence. The most common retention sequence is KDEL (*lys-asp-glu-leu*). However, variation on KDEL does occur and other sequences can also give rise to ER retention. There are three KDEL receptors in mammalian cells, and they have a very high degree of sequence identity. The functional differences between these receptors remain to be established. The lumenal soluble proteins of the ER carry a specific carboxyterminal KDEL signal which prevents the secretion of these proteins. Those ER-resident KDEL proteins which have escaped the ER, are recycled back from the Golgi to ER by COPI vesicles (Poussu 2001). Secretory proteins, mostly glycoproteins, are moved across the ER membrane. Proteins that are transported by the ER and from there throughout the cell are marked with an address tag called a signal sequence. The N-terminus (one end) of a polypeptide chain contains a few amino acids that work as an address tag, which are removed when the polypeptide reaches its destination. Proteins that are destined for places outside the ER are packed into transport vesicles and moved along the cytoskeleton toward their destination.

3.1.1.2 Golgi Complex

The Golgi is a stack of polarized tubular/saccular compartments with a defined cis- to trans content reflecting the presence of specialized processing enzymes that extensively modify newly synthesized proteins. The membrane-bound stacks are known as cisternae (singular: *cisterna*). Between four and eight stacks are usually present, although, in some protists as many as 60 have been observed. Each cisterna comprises a flattened membrane disk, and carries Golgi enzymes to help or to modify cargo proteins that travel through them. They are found in both plant and animal cells and the overall morphology of the ER-Golgi system can vary between cell types such as in budding to higher eukaryotes. The Golgi has earlier been viewed as a static station for the processing of secretory material, but now it seems that Golgi undergoes continuous remodeling. Traditionally, Golgi has been viewed as a series of stable compartments, named the *cis*-, medial- and *trans*-Golgi, as well as TGN (Glick 2000). The *trans*- face of the *trans*-Golgi network is the face from which vesicles leave the Golgi. These vesicles then proceed to later compartments such as the cell membrane, secretory vesicles or late endosomes. New *cisternae* form at the *cis*-Golgi network. The *cis*- and *trans*-Golgi networks are thought to be specialised *cisternae* leading in and out of the Golgi apparatus.

The Golgi apparatus is integral in modifying, sorting, and packaging macromolecules for cell secretion (exocytosis) or use within the cell. The Golgi apparatus plays an important role at the crossroads of the secretory pathway. It receives freshly synthesized proteins and lipids from the ER, modifies them, and then distributes cargo to various destinations. Proteins coming from ER to Golgi enter the organ on its tubulovesicular cis-face, travel across the stacks, and leave the Golgi on its trans-face. Cis-Golgi network not only receives the material from the ER but is also involved in sorting and recycling of lipids and proteins to the ER. On the way through the Golgi, newly synthesized glycoproteins are subjected to several posttranslational modifications such as ordered remodeling of their N-linked oligosaccharide side chains and biosynthesis of O-linked glycans. To effect such modifications, the Golgi complex is organized as polarized stacks of flattened cisternae enriched in transmembrane processing enzymes. To be able to send cargo even long distances through the cytoplasm, the Golgi complex is closely associated with the cytoskeleton. It is situated around the microtubule organizing center and is surrounded by actin cytoskeleton and actin-binding proteins (Holleran and Holzbaur 1998).

Enzymes within the cisternae are able to modify the proteins by addition of carbohydrates (glycosylation) and phosphates (phosphorylation). For example, the Golgi apparatus adds a mannose-6-phosphate label to proteins destined for lysosomes. The Golgi plays an important role in the synthesis of proteoglycans, which are molecules present in the extracellular matrix of animals. It is also a major site of carbohydrate synthesis. This includes the production of glycosaminoglycans (GAGs), long unbranched polysaccharides which the Golgi then attaches to a protein synthesised in the endoplasmic reticulum to form proteoglycans. Enzymes in the Golgi polymerize several of these GAGs via a xylose link onto the core protein. Another task of the Golgi involves the sulfation of certain molecules passing through its lumen via sulphotranferases that gain their sulphur molecule from a donor called PAPs. This process occurs on the GAGs of proteoglycans as well as on the core protein. The level of sulfation is very important to the proteoglycans' signalling abilities as well as giving the proteoglycan its overall negative charge.

3.1.1.3 Trans-Golgi Network

The trans-Golgi network is the site of the sorting and final exit of cargo from the Golgi. It refers to the trans-side of the Golgi and structurally it is seen as a sacculotubular network. The structure and the size of TGN varies remarkably from one cell type to another: in cells with a low number of secretory granules but with an extensive lysosomal system, TGN is massive, while secretory cells showing small or large secretory granules typically possess small TGN or even lack it (Clermont et al. 1995). Newly synthesized proteins traverse the Golgi stack until they reach the TGN. The trans-Golgi network sorts the proteins into several types of vesicles. Clathrin-coated vesicles carry certain proteins to lysosomes. At TGN, cargo molecules are sequestered into coated vesicles and directed to their correct destinations. For example, proteins with specific recognition signals are packed into clathrin-coated vescicles (CCVs) and transported to endosomal/lysosomal system in a selective pathway (Marks et al. 1997). Proteins carrying specific sorting signals are targeted and transported to plasma membrane through a so-called constitutive pathway (Pearse and Robinson 1990). In specialized cells producing large quantities of particular products in response to extracellular stimuli (e.g. hormonal or neural stimuli), there exists another secretion pathway leading to cell surface called the regulated secretory pathway (Traub and Kornfeld 1997). Other proteins are packaged into secretory vesicles for immediate delivery to the cell surface. Still other proteins are packaged into secretory granules, which undergo regulated secretion in response to specific signals. The sorting function of the Golgi apparatus allows the various organelles to grow while maintaining their distinct identities.

3.1.1.4 ER-Golgi Intermediate Compartment (ERGIC)

Protein traffic moving from ER to Golgi complex in mammalian cells passes through the tubulovesicular membrane clusters of the ERGIC, the marker of which is the lectin

ERGIC-53. Because the functional borders of the intermediate compartment (IC) are not well defined, the spatial map of the transport machineries operating between the ER and the Golgi apparatus remains incomplete (Fig. 3.1). However, studies showed that the IC consists of interconnected vacuolar and tubular parts with specific roles in pre-Golgi trafficking. The identification of ERGIC-53 has added to the complexity of the exocytic pathway of higher eukaryotic cells. Ffractional analysis of the ERGIC from Vero cells suggested that in the secretory pathway of Vero cells O-glycan initiation and sphingomyelin as well as glucosylceramide synthesis mainly occur beyond the ERGIC in the Golgi apparatus (Schweizer et al. 1994; Appenzeller-Herzog and Hauri 2006). Marie et al. (2009) provided novel insight into the compartmental organization of the secretory pathway and Golgi biogenesis, in addition to a direct functional connection between the IC and the endosomal system, which evidently contributes to unconventional transport of the cystic fibrosis transmembrane conductance regulator to the cell surface. The ERGIC defined by ERGIC-53 also participates in the maturation of (or is target for) several viruses such as corona virus, cytomegalovirus, flavivirus, poliovirus, Uukuniemi virus, and vaccinia virus. Understanding the targeting of viruses and viral proteins to the ERGIC could lead to development of general approaches for viral interference. Further analysis of the ERGIC-53 as a marker protein should provide novel results about the mechanisms controlling traffic in the secretory pathway (Chap. 7).

3.1.1.5 Protein Coats

Coatomer Protein Complex II (COPII) and COPI

Membrane traffic between the ER and the Golgi complex is regulated by two vesicular coat complexes, called coatomer protein complex II (COPII) and COPI. In addition, cells contain numerous clathrin/adaptor complexes—with each coat budding vesicles from a discrete subcellular location. COPII has been implicated in the selective packaging of anterograde cargo into coated transport vesicles budding from ER. In higher eukaryotes, transport from the ER is initiated by COPII mediated budding of vesicular carriers, but this is restricted to specialized, long-lived subdomains of the ER, the ER-exit sites (ERES). ERGIC clusters containing ERGIC-53 are close to but clearly distinct from ERES and delineate the subsequent stage in transport to the Golgi. The ERES are thought to generate transit vesicles and pleiomorphic tubular carriers through the activity of COPII coat machinery to yield pre-Golgi intermediates. In mammalian cells, these vesicles coalesce to form tubulo-vesicular transport complexes (TCs), which shuttle anterograde cargo from the ER to Golgi complex.

In contrast to COPII, COPI-coated vesicles are proposed to mediate recycling of proteins from the Golgi complex to the ER (David et al. 1999). The binding of COPI to COPII-coated TCs, however, has led to the proposal that COPI binds to TCs and specifically packages recycling proteins into retrograde vesicles for return to the ER. Observations, consistent with biochemical data, suggest a role for COPI within TCs *en route* to the Golgi complex. By sequestering retrograde cargo in the anterograde-directed TCs, COPI couples the sorting of ER recycling proteins to the transport of anterograde cargo (David et al. 1999; Rowe et al. 1996).

As observed, COPII-coated vesicles form on the ER to transport newly synthesized cargo to Golgi complex. Three proteins—Sec23/24, Sec13/31, and the ARF-family GTPase Sar1—are sufficient to bud ~60-nm COPII vesicles from native ER membranes and from synthetic liposomes. The COPII coat components coordinate to create a vesicle by locally generating membrane curvature and populating the incipient bud with the appropriate cargo (Lee and Miller 2007; Wiseman et al. 2007). COPII budding is initiated by the activation of Sar1 to its GTP-bound form, causing it to translocate to the membrane and embed an N-terminal α-1 helix in the bilayer (Fath et al. 2007).

Sequential Mode of Action for COPII and COPI

Exocytic transport from the ER to the Golgi complex has been visualized in living cells using a chimera of the temperature-sensitive glycoprotein of vesicular stomatitis virus and green fluorescent protein (ts-G-GFP[ct]). Upon shifting to permissive temperature, ts-G-GFP(ct) concentrates into COPII-positive structures close to ER, which then build up to form an intermediate compartment or transport complex, containing ERGIC-53 and the KDEL receptor, where COPII is replaced by COPI. These structures appear heterogenous and move in a microtubule-dependent manner toward the Golgi complex. These results suggest a sequential mode of COPII and COPI action and indicate that the transport complexes are ER-to-Golgi transport intermediates from which COPI may be involved in recycling material to the ER (Scales et al. 2000).

3.1.1.6 Clathrin Coated Vesicles (CCV)

Clathrin coats contain both clathrin and clathrin adaptor proteins. While clathrin acts as the scaffold, the clathrin adaptors bind to protein and lipid cargo. Specific cargos are recruited into clathrin-coated vesicles with the aid of CLASP proteins (clathrin-associated sorting proteins), such as ARH and Dab2. Clathrin-associated protein complexes are believed to interact with the cytoplasmic tails of membrane proteins, leading to their selection and concentration. At least 20 clathrin adaptors have been identified, which share a common design composed of a compact domain plus a long unstructured region that binds the clathrin beta-propeller. The two major types of clathrin adaptor complexes are: heterotetrameric adaptor protein

(AP) complexes, and the monomeric GGA (Golgi-localising, γ-adaptin ear domain homology, ARF-binding proteins) adaptors. Whereas clathrin heavy chain provides the structural backbone of the clathrin coat, it was suggested that clathrin light chains (CLCs) are not required for clathrin-mediated endocytosis but are critical for clathrin-mediated trafficking between the TGN and the endosomal system. In CLC-deficient mice CI-MPRs cluster near the TGN leading to a delay in processing of the lysosomal cathepsin D. In mammalian cells CLCs function in intracellular membrane trafficking by acting as recruitment proteins for huntingtin-interacting protein 1-related (HIP1R), enabling HIP1R to regulate actin assembly on clathrin-coated structures (Poupon et al. 2008).

3.1.1.7 Adaptor Protein Complexes

Adaptor protein (AP) complexes are found in coated vesicles and clathrin-coated pits. AP complexes connect cargo proteins and lipids to clathrin at vesicle budding sites, as well as binding accessory proteins that regulate coat assembly and disassembly. There are different AP complexes in mammals. AP1 is responsible for the transport of lysosomal hydrolases between the TGN and endosomes. AP2 associates with the plasma membrane and is responsible for endocytosis. The AP-1 and AP-2 complexes are the most abundant adaptors in CCVs, but clathrin-mediated trafficking can still occur in the absence of any detectable AP-1 or AP-2. AP3 is responsible for protein trafficking to lysosomes and other related organelles. AP4 is less well characterised. AP-4 is localized mainly in the Golgi complex, as well as on endosomes and transport vesicles. Mammary epithelial cells contain an unexpectedly high quantity of clathrin coated vesicles. Analysis of CCV adaptor composition showed that approximately 5–10% of total APs consist of AP-2 in mammary gland CCV whereas it represents approximately 70% of the total APs from bovine brain CCV. Relatively high quantities of furin and CI-MPR were detected in mammary CCV. AP-1 and the CI-MPR were localized in Golgi-associated vesicles and on the membrane of secretory vesicles. CCV in lactating mammary epithelial cells are involved in the transcytotic pathway, in sorting at the TGN and in the biogenesis of casein-containing secretory vesicles (Pauloin et al. 1999).

3.1.1.8 Lysosomes and Role of Mannose 6-Phosphate

The main function of lysosomes in the cell is the degradation of internalized material by lysosomal enzymes, the acid hydrolases. These enzymes are synthesized in the ER and during their maturation in the Golgi apparatus they acquire the mannose 6-phosphate (M6P) recognition marker. Most of these soluble hydrolases are transported to lysosomes through specific M6P receptors. The receptor-

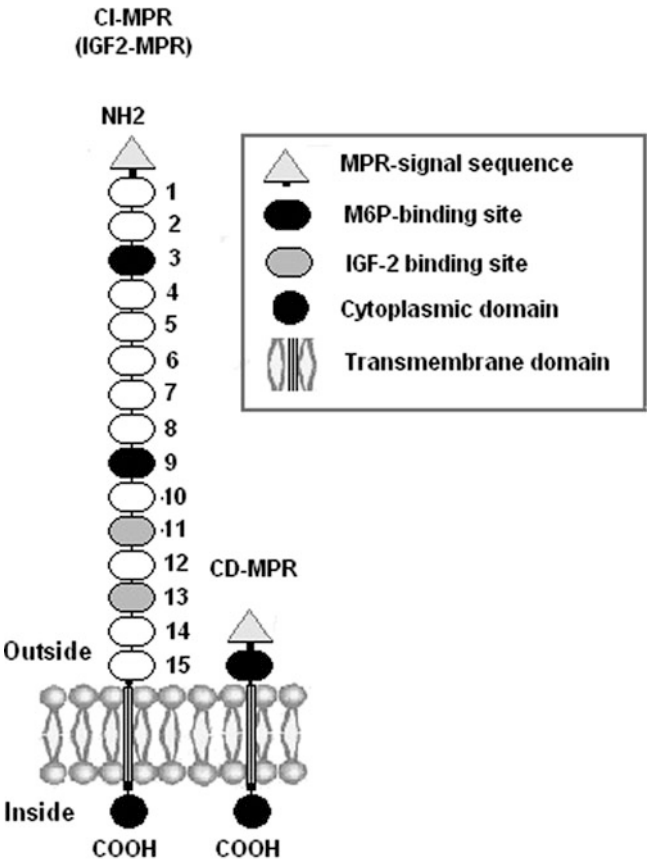

Fig. 3.2 Schematic diagram of the full-length MPRs. The MPRs are type I transmembrane glycoproteins that consist of an N-terminal (NH2) signal sequence, an extracytoplasmic region, a single transmembrane domain, and a C-terminal (COOH) cytoplasmic tail (Hancock et al. 2002)

enzyme complexes segregated in TGN are transferred to the lysosomal compartments *via* the secretory pathway through clathrin-coated vesicles.In mammalian cells, the targeting of newly synthesized hydrolases, as well as others ligands with M6P residues on their N-linked oligosaccharides, to the lysosomes depends on their recognition by two specific M6P receptors at the TGN: the cation-dependent M6P receptor (CD-MPR) and the cation-independent M6P receptor (CI-MPR), also called mannose 6-phosphate/insulin-like growth factor II receptor in eutherian mammals, in which it binds IGF-II (Kornfeld 1992; Varki and Kornfeld 2009) (Fig. 3.2). These two receptors (M6PRs) are considered sorting receptors because of their routing function. In the pre-lysosomal compartment, acidity induces release of enzymes from both MPR, which are then recycled into the Golgi apparatus. However, the CI-MPR, which participates to this cellular routing, is also anchored to the cell surface membrane and can internalize extracellular ligands (Kornfeld 1992; Munier-Lehmann et al. 1996; Dahms 1996). Thus, the pool of lysosomal acid hydrolases comes both from in situ synthesis, which involves the two

M6P receptors, and from endocytosis through the CI-MPR (Ni et al. 2006; Nykjaer et al. 1998). It is now well accepted that the TGN is the major site where proteins are sorted from the biosynthetic pathway for efficient delivery to endosomes. There, the MPRs and their bound ligands are segregated into nascent clathrin-coated vesicles, probably together with other transmembrane proteins destined to the endosomes/lysosomes. After budding and uncoating, these Golgi-derived vesicles fuse with endosomal compartments where the MPRs discharge their bound ligands. While the soluble lysosomal enzymes are directed toward the lysosomes, the MPRs recycle back to the TGN (Duncan and Kornfeld 1988) or to the cell surface where they are found in small amounts at steady state. At the plasma membrane, the MPRs undergo endocytosis via clathrin-coated pits like many other cell surface receptors (Fig. 3.1).

3.2 P-Type Lectin Family: The Mannose 6-Phosphate Receptors

The best-characterized function of two MPRs, CI-MPR and CD-MPR of 46 kDa and 300 kDa respectively, is their ability to direct the delivery of approximately 60 different newly synthesized soluble lysosomal enzymes bearing M6P on their N-linked oligosaccharides to lysosome. The CI-MPR is a multifunctional protein which binds at the cell surface to two distinct classes of ligands, the M6P bearing proteins and IGF-II. In addition to its intracellular role in lysosome biogenesis, the CI-MPR, but not the CD-MPR, participates in a number of other biological processes by interacting with various molecules at the cell surface. Though, the major function of CI-MPR is to bind and transport M6P-enzymes to lysosomes, but it can also modulate the activity of a variety of extracellular M6P-glycoproteins (i.e., latent TGFβ precursor, urokinase-type plasminogen activator receptor, Granzyme B, growth factors, Herpes virus). The synthesis and potential use of high affinity M6P analogues able to target this receptor have been described. Several M6P analogues with phosphonate, carboxylate or malonate groups display a higher affinity and a stronger stability in human serum than M6P itself. These derivatives can be used to favour the delivery of specific therapeutic compounds to lysosomes, notably in enzyme replacement therapies of lysosomal diseases or in neoplastic drug targeting (Gary-Bobo et al. 2007). In addition, their potential applications in preventing clinical disorders, which are associated with the activities of other M6P-proteins involved in wound healing, cell growth or viral infection, have been discussed.The list of extracellular ligands recognized by this multifunctional receptor has grown to include a diverse spectrum of M6P-containing proteins as well as several non-M6P-containing ligands. Structural studies have shown how these two receptors use related, but yet distinct, approaches in the recognition of phosphomannosyl residues (Dahms et al. 2008).

The two receptors, which share sequence similarities, constitute the P-type family of animal lectins. The CD-MPR (46 kDa) and the CI-MPR (300 kDa) are ubiquitously expressed throughout the animal kingdom and are distinguished from all other lectins by their ability to recognize phosphorylated mannose residues (Fig. 3.2). The P-type lectins play an essential role in the generation of functional lysosomes within the cells of higher eukaryotes by directing newly synthesized lysosomal enzymes bearing M6P signal to lysosomes. At the cell surface, the IGF2R/CI-MPR also binds to the nonglycosylated polypeptide hormone, IGF-II, targeting this potent mitogenic factor for degradation in lysosomes. The two MPRs have overlapping function in intracellular targeting of newly synthesized lysosomal proteins, but both are required for efficient targeting. Their main function is to transport lysosomal enzymes from TGN to the pre-lysosomal compartment. MPRs are conserved in the vertebrates from fish to mammals and show non-identical distribution among sub-cellular fractions in liver (Dahms and Hancock 2002; Messner et al. 1989; Messner 1993). Although much has been learned about the MPRs, it is unclear how these receptors interact with the highly diverse population of lysosomal enzymes. It is known that the terminal M6P is essential for receptor binding. Mannose receptor-enriched membranes of liver sinusoidal cells contain significant levels of CD-MPR, but not the CI-MPR. Both CD-MPRs and CI-MPRs bind their M6P-tagged cargo in the lumen of the Golgi apparatus in the cell. The CD-MPR shows greatly enhanced binding to M6P in the presence of divalent cations, such as manganese. M6P-containing proteins can be purified on immobilized MPRs. The sequences of zebrafish (*Danio rerio*) CD-MPR and CI-MPR (Nolan et al. 2006) indicate that targeting of lysosomal enzymes by MPRs is an ancient pathway in vertebrate cell biology. Yadavalli and Nadimpalli (2008) reported putative MPR receptors from starfish. Structural comparison of starfish receptor sequences with other vertebrate receptors gave structural homology with the vertebrate MPR-46 protein. The expressed protein in $mpr^{-/-}$ mouse embryonic fibroblast cells efficiently sorts lysosomal enzymes within the cells establishing a functional role for this protein (Yadavalli and Nadimpalli 2008). The insect cell line *Sf9* infected with a recombinant baculovirus containing the gene for human prorenin, cultured in presence of ^{3}H-mannose do not synthesize high-mannose-type oligosaccharides containing M6P, and consequently it appears unlikely that these cells utilize the MPR mediated pathway for targeting of lysosomal enzymes (Aeed and Elhammer 1994). Recently P-type CRD-like domains have been found in proteins with different architectures to the MPRs, and have been termed MPR homology (MRH) domains. Some of the MRH domains in

non-MPR proteins are known to have sugar-binding activity, and glycan recognition may be a general function of the MRH domain.

3.2.1 Fibroblasts MPRs

The turnover of the phosphomannosyl receptor in fibroblasts is very slow, in contrast with its rate of internalization in endocytosis, and that its rate of degradation is not greatly altered by a variety of agents that affect lysosomal protein turnover and/or receptor-mediated endocytosis (Creek and Sly 1983). The MPR, on the surface of fibroblasts, accounts for the intracellular transport of newly synthesized enzymes to the lysosome. Fibroblasts MPRs internalized oligosaccharides of known specific activity bearing a single phosphate in monoester linkage with K_{uptake} of 3.2×10^{-7} M, whereas oligosaccha- rides bearing two phosphates in monoester linkage were internalized with a K_{uptake} of 3.9×10^{-8} M (Natowicz et al. 1983).

3.2.2 MPRs in Liver

During perinatal development, the CI-MPR expression decreases progressively from 18-day fetuses to adults, whereas the CD-MPR showed a transient decrease in newborn and at the fifth day after birth in rats. Both receptors localize to hepatocytes at all ages and, additionally, the CD-MPR was reactive in megakaryocytes at early stages. In adult rat liver, CIMPR is detected intensely in hepatocytes and weakly in sinusoidal Kupffer cells and interstitial cells in Glisson's capsule. A high level of expression of CI-MPR mRNAs in hepatocytes and of CD-MPR mRNA in Kupffer cells was detected by in situ hybridization. Differential changes during perinatal development and adults suggest that two MPRs play distinct roles during organ maturation (Romano et al. 2006; Waguri et al. 2001). It was found that the activity of glycosidases changes during development, reaching a peak at the tenth day after birth and correlated with the expression and binding properties of CD-MPR. It was suggested that lysosome maturation in rat liver occurs around tenth day after birth, and that the CD-MPR may participate in that event. In hepatocytes of MPR-deficient neonatal mice, lysosomal storage occurs when both MPRs are lacking, whereas deficiency of CI-MPR only has no effect on the ultrastructure of the lysosomal system (Schellens et al. 2003). A biochemical comparison between autophagosomes and amphisomes from rat liver showed that the amphisomes were enriched in early endosome markers [the asialoglycoprotein receptor and the early endosome-associated protein as well as in a late endosome marker (CI-MPR)]. Amphisomes would thus seem to be capable of receiving inputs both from early and late endosomes (Berg et al. 1998). In human HepG2 and BHK cells, the two receptors were identified at the same sites: the trans-Golgi reticulum (TGR), endosomes, electron-dense cytoplasmic vesicles, and the plasma membrane. It was suggested that the two MPRs exit TGR via same coated vesicles. However, on arrival in the endosomes CD-MPR is more rapidly than CI-MPR segregated into associated tubules and vesicles (ATV) which probably are destined to recycle MPRs to TGR (Klumperman et al. 1993).

3.2.3 MPRs in CNS

The two related but distinct MPRs have been localized in neurons of mouse CNS, with more intense labeling in the medial septal nucleus, the nucleus of the Broca's diagonal band, layers IV-VI of the cerebral neocortex, layers II-III of the entorhinal cortex, the habenular nucleus, the median eminence, several nuclei and structures of the brainstem, the Purkinje cell layer of the cerebellum, and in the ventral horn of the spinal cord. Although intense reactivities of both MPRs were observed in the same groups of neurons in the same regions, the spatial differences in immunoreactive intensity for CI-MPR were greater, particularly in the telencephalon such as the basal forebrain and cerebral cortex, than those for CD-MPR. While CD-MPR is ubiquitously necessary for the general function of neurons, the CI-MPR is selectively necessary for certain region- and neurotransmitter-specific functions of neurons (Konishi et al. 2005). In rat brain during perinatal development, the expression of CI-MPR decreases progressively from fetuses to adults, where as CD-MPR increases around the tenth day of birth, and maintained there after. This shows that the two receptors play a different role in rat brain during perinatal development; CD-MPR being mostly involved in lysosome maturation (Romano et al. 2005). Given the critical role of endosomal-lysosomal (EL) system in the clearance of abnormal proteins, it is likely that the increase in the CI-MPR and components of the EL system in surviving neurons after 192-IgG-saporin represents an adaptive mechanism to restore the metabolic/structural abnormalities induced by the loss of cholin-ergic neurons (Hawkes et al. 2006).

A lysosomal enzyme binding receptor protein from monkey brain shows protein kinase activity and undergoes phosphorylation on serine and tyrosine residues. The lysosomal enzyme fucosidase and M6P, which are ligands for the receptor, stimulated the activity of protein phosphatase associated with the receptor protein. A phosphorylation/dephosphorylation mechanism may be operative in the ligand binding and functions of the receptor (Panneerselvam and Balasubramanian 1993).

The prion encephalopathies are characterized by accumulation of the abnormal form PrPsc of a normal host gene

product PrPc in the brain. In search of the mechanism and site of formation of PrPsc from PrPc in ME7 scrapie-infected mouse brain, it was found that proteinase K-resistant PrPsc is enriched in subcellular structures which contain CI-MPR, ubiquitin-protein conjugates, β-glucuronidase, and cathepsin B, termed late endosome-like organelles. The organelles may act as chambers for the conversion of PrPc into infectious PrPsc in murine model of scrapie (Arnold et al. 1995).

In neuroendocrine cells sorting of proteins from immature secretory granules (ISGs) occurs during maturation and is achieved by CCV containing AP-1. The MPRs are detected in ISGs of PC12 cells and more than 80% of the ISGs contained furin. Fifty percentage at most of the ISGs contained CI-MPR. Dittié et al. (1999) suggested the presence of two populations of ISGs: those that have both MPRs and furin, and those which contain only furin. It was shown that binding of adapter protein-1 (AP-1) requires casein kinase II phosphorylation of CI-MPRfusion protein, and in particular phosphorylation of Ser-2474.

The β-amyloid deposits in the brains of all patients of Alzheimer's disease (AD). Stephens and Austen (1996) defined major location of β-amyloid precursor protein fragments possessing the Asp-1 N-terminus of β-amyloid as the TGN or late endosome on the basis of colocalisation with a mAb to the CI-MPR. The co-localisation suggested that the p13 fragment and MPR are trafficked by alternative pathways from TGN. Hawkes and Kar (2004) delineated the role of the CI-MPR in the CNS, including its distribution, possible importance as well as its implications in neurodegenerative disorders such as AD.

3.2.4 CI-MPR in Bone Cells

The osteoclast is a polarized cell which secretes large amounts of newly synthesized lysosomal enzymes into an apical extracellular lacuna where bone resorption takes place. Osteoclast expresses large amounts of immunoreactive CI-MPR, despite the fact that most of the lysosomal enzymes it synthesizes are secreted. In osteoclast, M6P receptors are involved in the vectorial transport and targeting of newly synthesized lysosomal enzymes, presumably via a constitutive pathway, to the apical membrane where they are secreted into the bone-resorbing compartment. This mechanism could insure polarized secretion of lysosomal enzymes into the bone-resorbing lacuna (Baron 1989). The rapid inhibition of bone resorption by calcitonin involves the vesicular translocation of the apical membranes and the rapid arrest in the synthesis and secretion of lysosomal enzymes in osteoclasts (Baron et al. 1990). IGF2R/CI-MPR is present in rat calvarial osteoblasts. Osteoblasts bind IGF-II with high affinity ($K_D \sim 2.0$ nM). The osteoblastic Ca^{2+} response to IGF-II is caused by an intracellular release of Ca^{2+} which is mediated by the IGF-II/CI-MPR (Martinez et al. 1995). The phosphorylated monosaccharide, M6P stimulates alkaline phosphatase produced by osteoblasts. Glucose-6-phosphate and fructose-1-phosphate also stimulated osteoblast alkaline phosphatase production, but not to the same extent as M6P. Since, the stimulatory effect of M6P is similar to that of IGF-II, it supports similar mechanism for signal transduction for both IGF-II and M6P (Ishibe et al. 1991).

Secretory ameloblasts possesses strong immunoreactivity for MPR in the supranuclear Golgi region and in the cytoplasm between the Golgi region and the distal junctional complexes, where as cathepsin B immunoreactivity was mainly seen in the distal portion of Tomes' process, which was unreactive for MPR immunogenicity. Since MPR and lysosomal enzymes were also detected on the ruffled border of osteoclasts adjacent to alveolar bone, report provides strong evidence for a similarity between the maturation process in enamel, as mediated by ameloblasts, and bone resorption mediated by osteoclasts (Al Kawas et al. 1996).

3.2.5 Thyroid Follicle Cells

Thyroglobulin (Tg), the major secretory product of thyrocytes, is the macromolecular precursor of thyroid hormones. The Tg has been shown to be phosphorylated and to carry M6P signal in terminal position. In porcine thyroid follicle cells, the CI-MPR is primarily located in elements of the endocytic pathway such as coated pits and endosomes. This localization of the CI-MPR in thyrocytes differs from the receptor sites in other cell types by the rare occurrence of CI-MPR in cisternae of the Golgi complex. The CI-MPR in thyrocytes might be unable to bind and to convey Tg efficiently. The receptor is, however, a binding site for Tg at the apical plasma membrane and may, therefore, be involved in the binding of Tg and its transfer from the follicle lumen to lysosomes (Lemansky and Herzog 1992; Scheel and Herzog 1989). Using antibodies against Tg and CI-MPR, Kostrouch et al. (1991) suggested three types of endocytic structures: those slightly positive for MPR and ArS-A, those strongly positive for both markers, and those only positive for ArS-A. These compartments exhibited the properties of early endosomes (EE), late endosomes (LE), and lysosomes (L), respectively. The data indicate that internalized Tg molecules are transported to EE and then transferred from EE to LE.

3.2.6 Testis and Sperm

M6P receptors have been isolated from germ cells and Sertoli cells present in testes. Isolated mouse pachytene

spermatocytes and round spermatids synthesize predominantly the 46 kDa CD-MPR and only low levels of the 270 kDa CI-MPR. In contrast, Sertoli cells synthesized substantial amounts of the CI-MPR, but little of CD-MPR. Like germ cells, Sertoli cells in primary culture endocytosed ^{125}I-M6P-bearing ligands at levels that were about 10% of the endocytic activity measured for 3T3 fibroblasts. This indicates that both spermatogenic and Sertoli cells have surface MPRs capable of mediating endocytosis (O'Brien et al. 1989, 1993). Tsuruta and O'Brien (1995) and Tsuruta et al. (2000) provided evidence that IGF-II/Cl-MPR ligands secreted by Sertoli cells can modulate gene expression in spermatogenic cells and strongly suggest that they are important in the regulation of spermatogenesis. Moreno (2003) studied the dynamics of some components of the endosome/lysosome system, as a way to understand the complex membrane trafficking circuit established during spermatogenesis and suggested that the CI-MPR could be involved in membrane trafficking and/or acrosomal shaping during spermiogenesis.

A single CI-MPR transcript, approximately 10 kb in size, was present in mouse spermatogenic and Sertoli cells. Like the CI-MPR protein, its mRNA transcript was more abundant in Sertoli cells than in spermatogenic cells from adult testes. The CD-MPR was the predominant MPR synthesized by pachytene spermatocytes or round spermatids. Multiple CD-MPR transcripts were detected in these cells, including a 2.4-kb CD-MPR mRNA that was indistinguishable from CD-MPR transcripts in somatic tissues and Sertoli cells. Results suggested that alternate polyadenylation signals are used to produce multiple CD-MPR transcripts in spermatogenic cells (O'Brien et al. 1994). Low molecular weight M6P-receptors from bovine testis exhibited two isoforms with Mr of 45,000 (MPR-2A) and 41,000 (MPR-2B). These isoforms contain a common polypeptide core, but differ in their carbohydrate content (Li and Jourdian 1991). Two (pro)renin receptors have been characterized so far, the MPR and a specific receptor called (P)RR for (pro)renin receptor. Each receptor controls a different aspect of renin and prorenin metabolism. The MPR is a clearance receptor, whereas (P)RR mediates their cellular effects by activating intracellular signaling and up-regulating gene expression.

Belmonte et al. (1998) demonstratesd that α-mannosidase from rat epididymal fluid is a ligand for phosphomannosyl receptors on the sperm surface. Evidence is also presented that the CI-phosphomannosyl receptors are responsible for the interaction with alpha-mannosidase. These findings suggest a new role for extracellular transport mediated by the M6P receptor. Both MPRs undergo changes in distribution as spermatozoa passed from rete testis to cauda epididymis. CI-MPR was concentrated in the dorsal region of the head in rete testis sperm and that this labeling extended to the equatorial segment of epididymal spermatozoa. CD-MPR, however, changed from a dorsal distribution in rete testis, caput, and corpus to a double labeling on the dorsal and ventral regions in cauda spermatozoa; staining for either CI-MPR or CD-MPR increased from rete testis to epididymis. Changes in MPRs distribution may be related to a maturation process, which suggests new roles for the phosphomannosyl receptors (Belmonte et al. 2000). The targeted disruption of either MPR does not result in decreased acrosomal targeting efficiency (Chayko and Orgebin-Crist 2000).

3.2.7 MPRs During Embryogenesis

The MPR46 showed high expression at the sites of hemopoiesis and in the thymus while MPR300 was highly expressed in the cardiovascular system. Late in embryogenesis (day 17.5) a wide variety of tissues expressed the receptors, but still the expression pattern was almost non-overlapping. This unexpected spatially and temporally expression pattern points to specific functions of the two MPRs during mouse embryogenesis (Matzner et al. 1992).

3.3 Cation-Dependent Mannose 6-Phosphate Receptor

3.3.1 CD-MPR- An Overview

The cation-dependent mannose 6-phosphate receptor (or CD-MPR) is one of two proteins that bind M6P tags on acid hydrolase precursors in the Golgi apparatus that are destined for transport to the endosomal-lysosomal system. The CD-MPR recognizes the phosphomannosyl recognition marker of lysosomal enzymes. Homologues of CD-MPR are found in all eukaryotes. The CD-MPR is a type I transmembrane protein with a single transmembrane domain. The extracytoplasmic/lumenal M6P binding-domain consists of 157 amino acid residues. The bovine CD-MPR is composed of a 28-residue amino-terminal signal sequence, a 159-residue extracytoplasmic region, a 25-residue transmembrane region, and a 67-residue carboxyl-terminal cytoplasmic domain. The extracytoplasmic region of the CD-MPR contains 6 cysteine residues that are involved in the formation of three intramolecular disulfide bonds that play an essential role in the folding of the receptor (Wendland et al. 1991) (Fig. 3.2).

The CD-MPR from P388D1 macrophages lacks 215-kDa MPR. An identical protein was purified from bovine liver. The MPR binds efficiently to phosphomannosyl monoester-containing ligands in presence of MnCl2. The receptor contains both high mannose (or hybrid)- and complex-type oligosaccharide units on the basis of sensitivity to digestion with endo-beta-N-acetylglucosaminidase H and endo-β-N-acetylglucosaminidase F. The 46-kDa CD-MPR and the 215-kDa CI-MPR not only differ in their properties but are also immunologically distinct (Hoflack and Kornfeld 1985).

The receptor from human liver has a subunit molecular size of 43 kDa. It is rich in hydrophobic and charged amino acids and contains threonine at the N-terminus. The receptors from human and rat liver are antigenically related. Both are immunologically distinct from the CI-MPR of 215-kDa from human liver. The CD receptor exists in solution as a dimer or tetramer (Dahms and Hancock 2002). Modification of arginine and histidine residues reduced the binding of the receptor to immobilized ligands. Presence of M6P during modification of arginine residues protected the binding properties of the receptor, suggesting that arginine is a constituent of the M6P binding site of the receptor (Stein et al. 1987c). PC12 cells express CI-MPR, but not CD-MPR as much. The CD-MPR preferentially transports cathepsin B in PC12 cells, and cathepsins B and D participate in the regulation of PC12 cell apoptosis (Kanamori et al. 1998).

The cDNA clones encoding entire sequence of bovine 46-kDa CD-MPR, in *Xenopus laevis* oocytes results in a protein that binds specifically to phosphomannan-Sepharose and a deduced 279 amino acid sequence reveals a single polypeptide chain that contains a putative signal sequence and a transmembrane domain. The microsomal membranes containing the receptor and the location of the five potential N-linked glycosylation sites indicate that the receptor is a transmembrane protein with an extracytoplasmic amino terminus. This extracytoplasmic domain is homologous to the approximately 145 amino acid long repeating domains present in the 215-kDa CI-MPR (Dahms et al. 1987). The full-length cDNA for the goat CD-MPR46 protein was expressed in MPR deficient cells. It exhibits oligomeric nature as observed in the other species. The binding and sorting functions of the expressed protein to sort cathepsin D to lysosomes were similar to natural protein (Poupon et al. 2007). 46-kDa MPR mediates transport of endogenous but not endocytosis of exogenous lysosomal enzymes. Internalization of receptor antibodies indicated that the failure to mediate endocytosis of lysosomal enzymes is due to an inability of surface 46-kDa MPR to bind ligands rather than its exclusion from the plasma membrane or from internalization (Stein et al. 1987a, b).

3.3.2 Human CD-MPR

c-DNA clones for the human CD-MPR from a human placenta encoding the nucleotide sequence of the 2463-bp cDNA insert includes a 145-bp 5′ untranslated region, an ORF of 831 bp corresponding to 277 amino acids (Mr = 30,993), and a 1487-bp 3′ untranslated region. The deduced amino acid sequence is colinear with that determined by amino acid sequencing of the N-terminus peptide (41 residues) and nine tryptic peptides (93 additional residues). The receptor is synthesized as a precursor with a signal peptide of 20 amino acids. The hydrophobicity profile of the receptor indicates a single membrane-spanning domain, which separates an N-terminal region containing five potential N-glycosylation sites from a C-terminal region lacking N-glycosylation sites. Thus the N-terminal (Mr = 18,299) and C-terminal (Mr less than or equal to 7,648) segments of the mature receptor are assumed to be exposed to the extracytosolic and cytosolic sides of the membrane, respectively. The gene for the receptor is located on human Chromosome 12 *p13* (Ghosh et al. 2003).

The human MPR46 gene is distributed over 12 kb and divided into seven exons (110–1573 bp). All the intron/exon borders agree with the consensus sequences of splice junctions. Exon 1 codes for a 5′ untranslated sequence. The ATG initiation codon begins with the second nucleotide in exon 2. A signal sequence of 26 amino acid residues is followed by the extracytoplasmic (luminal) domain, which extends to exon 5. The transmembrane domain of the receptor spans exons 5 and 6 and the cytoplasmic domain is encoded by exons 6 and 7. The latter domain also codes for an extended 3′ untranslated sequence. The transcription-initiation site was defined by primer extension. The sequence upstream of the cap site has strong promoter activity and contains structural elements characteristic of promoters found in housekeeping genes. No correlation between the genomic organization and known protein domains of the MPR46 was apparent (Klier et al. 1991). Moreover, the sequence of about 150 amino acids within the luminal domain of MPR46, which is homologous to the 15 repeats that constitute the luminal domain of the MPR300, does not correlate with intron/exon borders. MPR46 and MPR300 have therefore diverged from a common ancestral gene before introduction of the present intron sequences.

3.3.3 Mouse CD-MPR

A cDNA clone for mouse MPR revealed a single open reading frame that codes for a protein of 278 residues. It shows an over-all amino-acid identity of 93% with the human receptor. Nine non-conservative amino-acid exchanges are found in the luminal domain, one non-conservative exchange of hydrophobic amino acids is in the transmembrane domain, while the cytoplasmic receptor tails are identical. All five potential N-glycosylation sites are conserved as well as amino acids that are important for ligand binding (Arg^{137} and His^{131}) and disulfide pairing (Cys^{32} and Cys^{78}, Cys^{132} and Cys^{167}, Cys^{145} and Cys^{179}). The absolute identity in the cytoplasmic MPR46 tail suggests the importance of this amino-acid sequence for the intracellular routing of the MPR46 (Köster et al. 1991). The 278-amino acid sequence deduced from the cDNA for the murine MPR46 shows 19 amino acid differences from that of the human MPR46, none of which are found in the 68-amino acid cytoplasmic tail. Binding of ligand to the murine MPR46 in permeabilized cells showed a pH optimum of 6.5, was completely inhibited

by M6P, and was stimulated by divalent cations. Mn^{2+} was more effective than Ca^{2+} or Mg^{2+}. Endocytosis was demonstrated at pH 6.5 and was stimulated four- to sevenfold by Mn^{2+}. In its responsiveness to divalent cations and its preference for Mn^{2+}, the murine 46MPR resembled the bovine 46MPR more than the human 46MPR. It was no more efficient than the human 46MPR in correcting the sorting defect of IGF-IIR/MPR-deficient mouse L cells (Ma et al. 1991).

3.3.3.1 Pseudogene of Mouse CD-MPR

Ludwig et al. (1992) cloned the mouse CD-MPR gene and also a very unusual processed-type CD-MPR pseudogene. Both are present at one copy per haploid genome and map to chromosomes 6 and 3, respectively. Comparison of the complete 10-kb sequence of the functional gene with the cDNA indicates that it contains seven exons. Exon 1 encodes the 5′-untranslated region of the mRNA, the others (exons 2–7) encode the luminal, transmembrane, and cytoplasmic domains of the CD-MPR. Exon 7 also contains a 1.2-kb-long 3′-untranslated region of the mRNA. A unique transcription-initiation site was determined by primer extension of mouse liver mRNA. The promoter elements in the 5′ upstream region of this site resemble those contained in genes constitutively transcribed. However, Northern blot analysis demonstrates that the CD-MPR is variably expressed in adult mouse tissues and during mouse development. The pseudogene, which is flanked by direct repeats, is almost colinear with the cDNA indicating that it presumably arose by reverse transcription of an mRNA. However, the pseudogene differs from the cDNA. It contains at its 5′ end, an additional 340-nucleotide (nt) sequence homologous to the promoter region of the functional gene. This sequence exhibits some promoter activity in vitro. Furthermore, a 24-nt insertion interrupts the region homologous to the 5′-noncoding region of the cDNA. In the functional gene, this 24-nt sequence occurs between exon 1 and 2, where it is flanked by typical consensus sequences of exon/intron boundaries. Therefore, it may represent an additional exon of the functional gene. These two features of the pseudogene suggest that expression of the CD-MPR gene may be regulated by use of different promoters and/or alternative splicing.

3.4 Structural Insights

3.4.1 N-Glycosylation Sites in CD-MPR

The bovine CD-MPR contains five potential N-linked glycosylation sites, four of which are utilized. CD-MPR mutants lacking various potential glycosylation sites showed that the presence of a single oligosaccharide chain, particularly at position 87 significantly enhanced its M6P-binding ability when compared with non-glycosylated receptors. It was suggested that N-glycosylation of the bovine CD-MPR facilitates the folding of the nascent polypeptide chain into a conformation that is conducive for intracellular transport and ligand binding (Zhang and Dahms 1993). A soluble truncated form CD-MPR encoding only the extracytoplasmic region, Stop155, and a truncated glycosylation-deficient form of the CD-MPR, Asn81/Stop155, which has been modified to contain only one N-linked glycosylation site at position 81 instead of five, were purified from baculovirus-infected High Five insect cells. The extracellular region of the CD-MPR is sufficient for high-affinity binding and that oligosaccharides at positions 31, 57, and 87 do not influence ligand binding (Marron-Terada et al. 1998). The recombinant insect-produced CD-MPR existed as a dimer in the membrane. The cytoplasmic domains of the MPRs are sufficient to determine the steady-state distribution of the full-length proteins (Dahms and Hancock 2002; Mauxion et al. 1995).

Mammalian cell lysosomal enzymes or phosphorylated oligosaccharides derived from them are endocytosed by a MPR found on the surface of fibroblasts. Studies suggest that two residues of M6P in phosphomonoester linkage but not diester linkage (PDE) are essential for a high rate of uptake. The lysosomal enzymes of the slime mold *Dictyostelium discoideum* are also recognized by the MPR on these cells; however, none of the oligosaccharides from these enzymes contain two phosphomonoesters. Instead, most contain multiple sulfate esters and two residues of M6P in an unusual PDE linkage. Further study shows that nearly all of the α-mannosidase molecules contain the oligosaccharides required for uptake, and that each tetrameric, holoenzyme molecule has sufficient carbohydrate for an average of 10 Man8GlcNAc2 oligosaccharides. Results suggest that the interactions of multiple, weakly binding oligosaccharides, especially those with 2 PDE, are important for high rate of uptake of the slime mold enzymes. The conformation of the protein may be important in orienting the oligosaccharides in a favorable position for binding to MPR (Freeze 1985).

3.4.2 3-D Structure of CD-MPR

Roberts et al. (1998) reported the three-dimensional structure of a glycosylation-deficient, yet fully functional form of the extracytoplasmic domain of the bovine CD-MPR (residues 3–154) complexed with M6P at 1.8 A resolution. The extracytoplasmic domain of the CD-MPR crystallizes as a dimer, and each monomer folds into a nine-stranded flattened beta barrel, which bears a striking resemblance to avidin (Fig. 3.3). The distance of 40 A between the two ligand-binding sites of the dimer provides a structural basis for the observed differences in binding affinity exhibited by the CD-MPR toward various lysosomal enzymes.

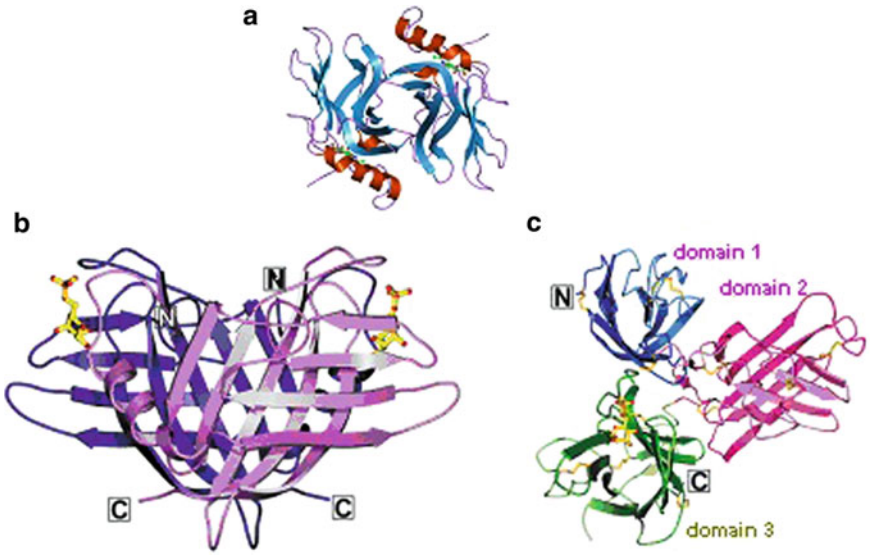

Fig. 3.3 Crystal structure of CD-MPR (46 kDa in humans) (**a**) (PDB ID: 1KEO). (**b**) Crystal structure of the extracytoplasmic region (residues 3–154) of the bovine CD-MPR in the presence of an oligosaccharide, pentamannosyl phosphate (PDB 1 C39). Note that only the terminal Man-6-P (*gold* ball-and-stick model) is shown for clarity. Both monomers (*light purple* and *dark purple*) of the CD-MPR dimer are shown in this ribbon diagram. The N-terminus (N) and C-terminus (C) are boxed. (**c**) Crystal structure of the N-terminal three domains (residues 7–432) of the bovine CI-MPR (PDB 1SZO). The N- and C-terminus of the protein encoding domain 1 (*blue*), domain 2 (*pink*), and domain 3 (*green*) are indicated. The location of Man-6-P (*gold* ball-and-stick model) is shown (Reprinted with permission from Dahms et al. 2008 © Oxford University Press)

Studies using synthetic oligosaccharides indicated that the binding site encompasses at least two sugars of the oligosaccharide. Olson et al. (1999b) reported the structure of the soluble extracytoplasmic domain of a glycosylation-deficient form of the bovine CD-MPR complexed to pentamannosyl phosphate. This construct consists of the amino-terminal 154 amino acids (excluding the signal sequence) with glutamine substituted for asparagine at positions 31, 57, 68, and 87. The binding site of the receptor encompasses the phosphate group plus three of the five mannose rings of pentamannosyl phosphate. Receptor specificity for mannose arises from protein contacts with the 2-hydroxyl on the terminal mannose ring adjacent to the phosphate group. Glycosidic linkage preference originates from the minimization of unfavorable interactions between the ligand and receptor.

Recent advances in the structural analyses of both CD-MPR and CI-MPR have revealed the structural basis for phosphomannosyl recognition by these receptors and provided insights into how the receptors load and unload their cargo. A surprising finding is that the CD-MPR is dynamic, with at least two stable quaternary states, the open (ligand-bound) and closed (ligand-free) conformations, similar to those of hemoglobin. Ligand binding stabilizes the open conformation; changes in the pH of the environment at the cell surface and in endosomal compartments weaken the ligand-receptor interaction and/or weaken the electrostatic interactions at the subunit interface, resulting in the closed conformation (Kim et al. 2009).

CD-MPR Adopts at Least Two Different Conformations

Crystallographic studies of CD-MPR have identified 11 amino acids within its carbohydrate binding pocket. Mutant receptors containing a single amino acid substitution toward a lysosomal enzyme showed that substitution of Gln^{66}, Arg^{111}, Glu^{133}, or Tyr^{143} results in a >800-fold decrease in affinity, suggesting that these four amino acids are essential for carbohydrate recognition by CD-MPR. Furthermore, Asp^{103} has been identified as the key residue which mediates the effects of divalent cations on the binding properties of the CD-MPR. The MPRs encounter a variety of conditions as they travel to various compartments where they bind and release their ligands. Key to their function is pH-dependence of ligand-protein interaction. Cells treated with reagents that raise the pH of endosomal/lysosomal compartments exhibit decreased sorting of lysosomal enzymes to lysosomes and a concomitant increase in the secretion of these enzymes into the medium (Imort et al. 1983). This observation implies that it is essential for MPRs to release their ligands in the acidic environment of endosomes in order to be able to recycle back to the TGN to retrieve additional lysosomal enzymes. To determine whether different pH conditions elicit conformational changes in the receptor that alters ligand binding affinities, CD-MPR structures were obtained under different conditions representing various environments encountered by the receptor: bound state at pH 6.5 and pH 7.4 and unbound state at pH 6.5 and pH 4.8 (Olson et al. 2002, 2008) (Fig. 3.4).

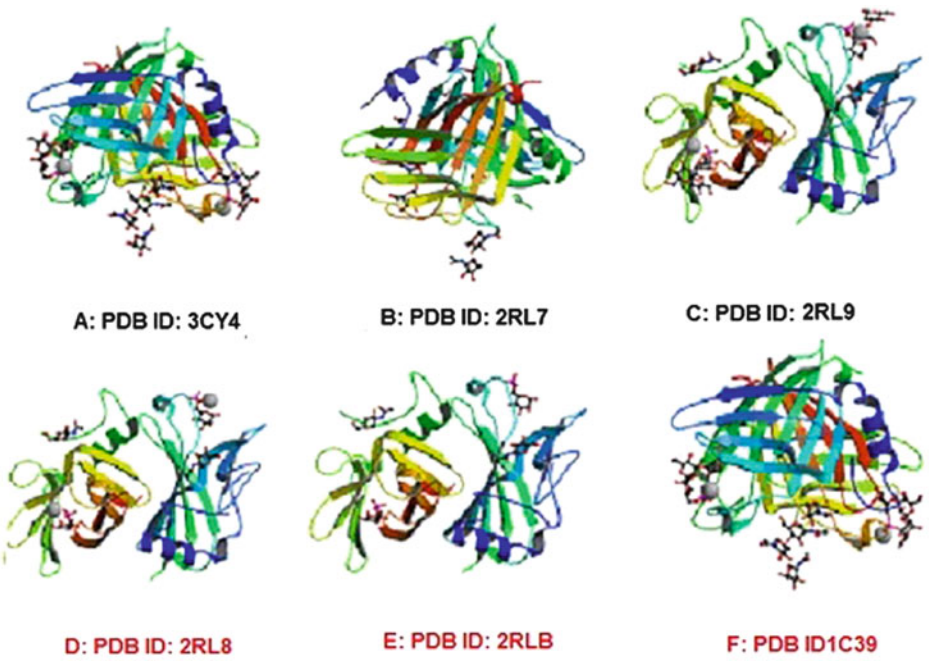

Fig. 3.4 **Crystal Structure of bovine CD-MPR.** (**a**) at pH 7.4 (Asymmetric Unit) (PDB ID: 3CY4 DOI:dx.doi.org); (**b**) at pH 4.8 (PDB ID: 2RL7); (**c**) at pH 6.5 bound to trimannoside (PDB ID: 2RL9 DOI:dx.doi.org); (**d**) at pH 6.5 bound to M6P (PDB ID: 2RL8 DOI:dx.doi.org); (**e**) at pH 6.5 bound to M6P in absence of Mn (PDB ID: 2RLB) DOI:dx.doi.org; (**f**) bound to pentamannosyl phosphate (PDB ID:1 C39)

These structures of CD-MPR were categorized into one of two conformations: an "open" conformation found in all structures containing ligand in the binding pocket and a "closed" conformation found in all structures missing bound carbohydrate (Dahms et al. 2008). Unlike what has been observed in other lectins, the structure of the ligand-free CD-MPR differs considerably from the ligand-bound form in that changes in both quaternary structure and positioning of loops involved in sugar binding, along with changes in the spacing of the two carbohydrate binding sites in the dimeric receptor (the Cα atoms of His^{105} located in Loop C are ~34 Å apart in the open conformation and ~26 Å apart in the closed conformation). Loop D (residues Glu^{134}–Cys^{141}) exhibits the most dramatic change in position, with Val^{138} displaying the largest displacement (Cα–Cα distance of 16 Å). The CD-MPR conformation differs dramatically from other lectins in an unbound state, where water molecules fill the shallow binding grooves of other most lectins in absence of bound sugar. Instead of essential side chain interactions being shifted from the carbohydrate hyroxyls to water, the pocket of CD-MPR undergoes restructuring: loop D swings into the binding pocket in the absence of ligand and provides contacts that hold essential residues in the proper orientation so that they are maintained in a "ready-state" to accept ligand. The two conformations also display a dramatic difference in their quaternary structure that can be described globally as a scissoring and twisting motion between the two subunits of the dimer. Results indicate that the CD-MPR is dynamic and must be able to transition between two conformations as it moves to different organelles, with changing environment of each (Dahms et al. 2008; Olson et al. 2008).

Based on these structures, distinct mechanisms for the dissociation of lysosomal enzymes at the cell surface and under the acidic conditions of the endosome were proposed for the CD-MPR (Olson et al. 2008). His^{105} is the only residue of the receptor in which a titratable side chain is involved in binding the phosphate group of M6P. Deprotonation of His^{105} and the phosphate moiety of M6P appear to be key elements in the release of ligand at the cell surface: loss of the electrostatic interaction between the uncharged His^{105} and M6P is predicted to facilitate dissociation of phosphorylated ligands at pH 7.4. In the acidic environment of the endosome, it is proposed that disruption, via protonation, of intermonomer electrostatic interactions that tie loop D of one monomer to the α-helix of the other monomer in the ligand bound conformation would "free" loop D to move into the binding pocket, resulting in the displacement of ligand. In addition, protonation of Glu^{133} that is located in the binding pocket is predicted to weaken its interaction with the 3- or 4-hydroxyl group of M6P and disrupt the electrostatic environment of the entire binding pocket, thereby enhancing the release of M6P. The repositioning of loop D into the binding pocket eliminates its intermonomer interaction with the N-terminal α-helix. This loss of intermonomer contact may trigger the

reorientation of the two monomers as the receptor changes its quaternary structure, adopting a more closed conformation in the unbound state (Dahms et al. 2008; Olson et al. 2008). These results allowed to suggest that the receptor regulates its ligand binding upon changes in pH; the pK_a of Glu^{133} appeared to be responsible for ligand release in the acidic environment of the late endosomal compartment, and the pK_a values of the sugar phosphate and His^{105} were accountable for its inability to bind ligand at the cell surface where the pH was about 7.4.

Sequence comparison between CD-MPR and CI-MPR shows that they are related. In fact, the extracytoplasmic domain of CD-MPR is homologous to the approximately 145 amino acid long repeating domains present in the CI-MPR with sequence identity ranging from 14% to 28%. These studies allowed to conclude that these two receptors located on different chromosomes (12p13 and 6q26, respectively) have diverged from a common ancestral gene (Dahms et al. 1987; Klier et al. 1991). This receptor is a 46 kDa single polypeptide chain that contains a putative signal sequence and a transmembrane domain. The CD-MPR is a single membrane-spanning domain, which separates a N-terminal extracytoplasmic region with five potential Asn-linked glycosylation sites, from a C-terminal cytoplasmic region without Asn-glycosylation sites. Sequence analysis of the bovine CD-MPR revealed that it consists of a 28 amino-acid residue N-terminal signal sequence, a 159 amino acid residue luminal domain, a 25 amino acid residue transmembrane domain and a 67 amino acid residue C-terminal cytoplasmic domain. It is highly conserved from mouse to human (93% homology). The CD-MPR appears to be a homodimer at the membrane (Stein et al. 1987a), and either a dimer or a tetramer in solution (Dahms et al. 1987; Tong and Kornfeld 1989; Stein et al. 1987b).

3.4.3 Carbohydrate Binding Sites in MPRs

Soluble acid hydrolases constitute a group of over 60 heterogeneous enzymes that differ in size, oligomeric state, number of *N*-linked oligosaccharides, extent of phosphorylation, and the position of the M6P moiety and its linkage to the penultimate mannose residue in the oligosaccharide chain (Dahms et al. 2008). The two MPRs have been shown to display different affinities and capacities for transport of various acid hydrolases, and both receptors are necessary for the efficient sorting of all lysosomal enzymes to the lysosome as neither MPR can fully compensate for the other (Dahms et al. 2008). These studies indicate that the two MPRs recognize distinct but overlapping populations of acid hydrolases. A proteomic analysis of serum from mutant mice deficient in either the CD-MPR or CI-MPR revealed that several lysosomal proteins are preferentially sorted by the CD-MPR (e.g., tripeptidyl peptidase I) or CI-MPR (e.g., cathepsin D) (Qian et al. 2008). Amine-activated glycans, covalently printed on N-hydroxysuccinimide-activated glass slides, interrogated with different concentrations of rCD-MPR or soluble CI-MPR. Neither receptor bound to non-phosphorylated glycans. The CD-MPR bound weakly or undetectably to the phosphodiester derivatives, but strongly to the phosphomonoester-containing glycans with the exception of a single Man7GlcNAc2-R isomer that contained a single M6P residue. By contrast, the CI-MPR bound with high affinity to glycans containing either phospho-mono- or -diesters although, like the CD-MPR, it differentially recognized isomers of phosphorylated Man7GlcNAc2-R. This differential recognition of phosphorylated glycans by the CI- and CD-MPRs has implications for understanding the biosynthesis and targeting of lysosomal hydrolases (Song et al. 2009). Future studies will shed light onto the functional significance of two distinct MPRs in a given cell type.

3.4.4 Similarities and Dis-similarities between two MPRs

The CD-MPR and CI-MPR share a number of similarities with respect to carbohydrate recognition. For example, both MPRs bind the M6P with essentially the same affinity ($7–8 \times 10^{-6}$ M). Mannose or glucose 6-phosphate interact poorly with the MPRs ($K_i = 1–5 \times 10^{-2}$ M) (Tong et al. 1989; Tong and Kornfeld 1989). Like mammalian MPRs, calotes MPR-300/IGF-IIR also binds IGF-II with high affinity (K_D ~12.02 nM). A number of synthetic analogs and those with the highest affinity to the CI-MPR were found to be isosteric to M6P. Several M6P analogues with phosphonate, carboxylate or malonate groups displayed a higher affinity and a stronger stability in human serum than M6P itself. These derivatives could be used to favour the delivery of specific therapeutic compounds to lysosomes, notably in enzyme replacement therapies of lysosomal diseases or in neoplastic drug targeting. Although analogues containing two negative charges were the best ligands, the presence of a phosphorous atom was not necessary for recognition (Gary-Bobo et al. 2007). In addition, linear mannose sequences which contain a terminal M6P linked α1, 2 to the penultimate mannose were shown to be the most potent inhibitors (Distler et al. 1991; Tomoda et al. 1991), suggesting that the MPRs bind an extended oliogosaccharide structure which includes the M6P α1,2 Man sequence. The crystal structure of CD-MPR complexed with α1, 2-linked phosphorylated trimannoside has revealed the site of penultimate and prepenultimate mannose rings in the binding pocket and their hydrogen bond interactions with the receptor (Olson et al. 2008). Furthermore, multivalent interactions

between the receptor and a lysosomal enzyme result in high affinity binding, of the order of 1–10 nM for both MPRs (Dahms et al. 2008; Watanabe et al. 1990). The two MPRs exhibit optimal ligand binding at ~pH 6.4 and no detectable binding below pH 5, which is in accordance with their function of releasing ligands in acidic environment of the endosome.

In contrast to these similarities, the two MPRs differ in their binding properties, which depend on pH, cations, and nature of phosphodiesters. The CI-MPR retains phosphomannosyl binding capabilities at neutral pH which corresponds well with the ability of this receptor to bind and internalize lysosomal enzymes at the cell surface. In contrast, the ligand binding ability of the CD-MPR is dramatically reduced at a pH $>$ 6.4 (Tong et al. 1989; Tong and Kornfeld 1989) which is consistent with its decreased ability to bind and internalize lysosomal enzymes at the cell surface (Stein et al. 1987a, b). The inability to purify the CD-MPR by phosphomannosyl affinity chromatography performed in the absence of cations led to its designation as a "cation-dependent" receptor (Hoflack and Kornfeld 1985a, b). However, the presence of cations increases the binding affinity of the CD-MPR towards M6P (Tong and Kornfeld 1989) and lysosomal enzymes only fourfold (Sun et al. 2005) but has no effect on the binding affinity of CI-MPR. This finding differentiates the CD-MPR from C-type lectins which have an absolute requirement for calcium to carry out their sugar binding activities. Mutagenesis studies (Sun et al. 2005) indicated that a conserved aspartic acid residue at position 103 of the CD-MPR, which is not present in the CI-MPR, necessitates the presence of a divalent cation in the binding pocket to obtain high affinity ligand binding by functioning to neutralize the negative charge of Asp^{103} juxtaposed to the phosphate oxygen of M6P. The CI-MPR, unlike the CD-MPR, is able to recognize MP-GlcNAc phosphodiesters as well as lysosomal enzymes derived from *Dictyostelium discoideum* which contain mannose 6-sulfate residues and small methyl phosphodiesters, M6P-OCH_3, but not phosphomonoesters (c/r Dahms et al. 2008).

3.5 Functional Mechanisms

3.5.1 Sorting of Cargo at TGN

At TGN, sorting of cargo to different destinations is regulated by several mechanisms: (1) biochemically distinct coats can specify protein sorting,; (2) cytosol-oriented sorting signals of cargo proteins direct them to the appropriate export site; (3) TGN might be organized into discrete subdomains dedicated to assemble specific coat population (Traub and Kornfeld 1997). The constitutive pathway in polarized epithelial cells (e.g. MDCK cells) includes the apical- and basolateral routes. Sorting to the basolateral pathway is mediated by cytoplasmic sorting signals of the cargo molecules. They include tyrosine residues, the dileucine motif, or "adders" that contains neither dileucine- or tyrosine-motifs (Keller and Simons 1997). The machinery responsible for the basolateral sorting is currently unknown while some data implicate that it could be mediated by AP-1 and clathrin (Futter et al. 1998). However, unequivocal evidence showing that the TGN contains sorting mechanisms able to discriminate between proteins traveling to apical and basolateral surfaces has not been obtained. Sorting signals involved in the targeting to selective pathway, among others, include the M6P residues in lysosomal hydrolases and tyrosine- and di-leucine-based sorting determinants in membrane proteins which direct them into CCVs and further into the endosomal/lysosomal system (Kirchhausen et al. 1997; Marks et al. 1997; Rohn et al. 2000). The membrane proteins are e.g. the lysosomal associated membrane protein (LAMP) and the lysosomal integral membrane protein (LIMP). A conformation-dependent motif is suggested to destine the proteins for secretion for a regulated secretory pathway by Keller and Simons (1997).

At TGN, the sorting and transport of a group of soluble proteins via a selective pathway to lysosomes relies on the existence of M6P residues on their oligosaccharides (Le Borgne and Hoflack 1998a, b). They serve as recognition signals for MPRs. Sorting of MPRs and their bound ligands to their destinations is mediated preferentially by the interaction of tyrosine- and dileucine-based sorting signals present in their tails with the adaptor protein complex AP-1 and by transport in CCVs (Le Borgne et al.1996; Le Borgne and Hoflack 1998a). The pinching off of the CCVs from the membranes is effected by dynamin (Jones et al. 1998; Kasai et al. 1999).

Though the CD-MPR and the CI-MPR deliver soluble acid hydrolases to lysosome in higher eukaryotic cells by binding with high affinity to M6P residues, found on N-linked oligosaccharides of their ligands, for many other transmembrane proteins, the MPRs contain multiple molecular sorting signals in their cytoplasmic domains that mediate their intracellular traffic between distinct membrane-bound compartments (Lobel et al. 1989). A schematic representation of the different vesicle transport pathways originating from TGN is given (Fig. 3.1).

3.5.1.1 Interaction of Phosphorylated Oligosaccharides and Lysosomal Enzymes with CD-MPR and CI-MPR

Oligosaccharides with phosphomonoesters interact with the CD-MPR, and molecules with two phosphomonoesters showed the best binding. Lysosomal enzymes with several oligosaccharides containing only one phosphomonoester had a higher affinity for the receptor than did the isolated

oligosaccharides, indicating the possible importance of multivalent interactions between weakly binding ligands and the receptor. The binding of phosphorylated lysosomal enzymes to the CD-MPR is markedly influenced by pH. At pH 6.3, almost all of the lysosomal enzymes bound to the receptor. Results indicated that at neutral pH the phosphorylated oligosaccharides on some lysosomal enzyme molecules are oriented in a manner which makes them inaccessible to the binding site of the CD-MPR. Since the same enzymes bind to the CI-MPR at neutral pH, at least a portion of the phosphomannosyl residues must be exposed. It appeared that small variations in the pH of the Golgi compartment where lysosomal enzymes bind to the receptors could potentially modulate the extent of binding to the two receptors (Hoflack et al. 1987).

Lysosomal enzymes bearing phosphomannosyl residues bind specifically to MPRs in the Golgi apparatus and the resulting receptor-ligand complex is transported to an acidic prelyosomal compartment where the low pH mediates the dissociation of the complex (Le Borgne and Hoflack 1998). The transport of proteins from the secretory to the endocytic pathway is mediated by carrier vesicles coated with the AP-1 Golgi assembly proteins and clathrin. The MPRs are segregated into these transport vesicles. Together with GTPase-ARF-1, these cargo proteins are essential components for the efficient translocation of cytosolic AP-1 onto membranes of the TGN, the first step of clathrin coat assembly. The transport of lysosomal enzymes to lysosomes requires two distinct determinants in the CD-MPR carboxyl-terminal domain, a casein kinase II phosphorylation site critical for the efficient interaction of AP-1 with its target membranes and the adjacent di-leucine motif which appears more important for a post AP-1 binding step in the CD-MPR cycling pathway.

3.5.2 TGN Exit Signal Uncovering Enzyme

According to Ghosh et al. (2003) dynamic fusion/fission between the late endosomal and lysosomal compartments results in selective delivery of the hydrolases to the lysosome. TIP47/Rab9 prevents the MPRs from reaching the lysosomes, in which they would otherwise be degraded. The return pathway from the early endosomal compartment to the Golgi is probably mediated by PACS-1-assisted packaging into AP1-containing CCVs, whereas that from the late endosomal (LE) compartments is mediated by TIP47 and Rab9. Some of the MPRs go to the cell surface either from early or late endosomes through the recycling endosome (RE), or from proximal TGN cisternae as a consequence of mis-sorting. The cell-surface receptors are internalized in AP2 CCVs and delivered back to the endosomes.

Nair et al. (2005) proposed that the human M6P uncovering enzyme participates in the uncovering of M6P recognition tag on lysosomal enzymes, a process that facilitates recognition of those enzymes by MPRs to ensure a delivery to lysosomes. Uncovering enzyme has been identified on TGN. The cytoplasmic tail of the uncovering enzyme does not possess any of the known canonical signal sequences for interaction with Golgi-associated γ-ear-containing adaptor proteins. The identification of a TGN exit signal in its cytoplasmic tail elucidates the trafficking pathway of uncovering enzyme, a crucial player in the process of lysosomal biogenesis (Nair et al. 2005). However, uncovered phosphates are not essential for optimal recognition by the phosphomannosyl receptor.

3.5.3 Association of Clathrin-Coated Vesicles with Adaptor Proteins

It is suggested that 46-kDa MPR contains multiple binding sites for clathrin adaptors (Honing et al. 1997). The Golgi-derived and plasma membrane-derived clathrin-coated vesicles can be distinguished by the nature of their underlying assembly proteins AP-1 and AP-2, two related heterotetrameric complexes (Robinson 1994). Localization studies are consistent with the notion that AP-1 is associated with TGN-derived vesicles, whereas AP-2 is found in plasma membrane-derived vesicles. In vitro studies have shown that the translocation of cytosolic AP-1 onto membranes requires ADP-ribosylation factor ARF-1 (Traub et al. 1993), a small GTPase also involved in coatomer binding and vesicular transport in early secretory pathway (Rothman 1994; Boman and Kahn 1995). The AP-1 complex interacts with sorting signals in the cytoplasmic tails of cargo molecules and targeted disruption of the mouse μ1A-adaptin gene causes embryonic lethality. Under normal conditions, MPRs are cargo molecules that exit the TGN via AP-1-clathrin-coated vesicles. But the steady-state distribution of MPR46 and MPR300 in μ1A-deficient cells is shifted to endosomes at the expense of TGN. Thus, MPR46 fails to recycle back from the endosome to the TGN, indicating that AP-1 is required for retrograde endosome to TGN transport of the receptor (Meyer et al. 2000).

Binding Sites in CI-MPR for AP-1

The trafficking of CI-MPR between the TGN and endosomes requires binding of sorting determinants in the cytoplasmic tail of the receptor to adaptor protein complex-1 (AP-1). A GST pull-down binding assay identified four binding motifs in the cytoplasmic tail of CI-MPR: a tyrosine-based motif ^{26}YSKV^{29}, an internal dileucine-based motif ^{39}ETEWLM^{44}, and two casein kinase 2 sites ^{84}DSEDE^{88} and ^{154}DDSDED^{159}. The YSKV motif mediated the strongest interaction with AP-1 and the two CK2 motifs bound AP-1 only when they were

phosphorylated. The COOH-terminal dileucines were not required for interaction with AP-1 (Ghosh and Kornfeld 2004).

AP-3 Adaptor Complex Defines a Novel Endosomal Exit Site

The AP-3 adaptor complex has been implicated in the transport of lysosomal membrane proteins. The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins (Le Borgne et al. 1998b). Electron microscopy showed that AP-3 is associated with budding profiles evolving from a tubular endosomal compartment that also exhibits budding profiles positive for AP-1. AP-3 colocalizes with clathrin, but to a lesser extent than does AP-1. The AP-3- and AP-1-bearing tubular compartments contain low amounts of the CI-MPR and the lysosome-associated membrane proteins (LAMPs) 1 and 2. AP-3 defines a novel pathway by which lysosomal membrane proteins are transported from tubular sorting endosomes to lysosomes. In an attempt to find the site of action of AP-3 Chapuy et al. (2008) showed that sorting of TRP-1 and CD-MPR was AP-1 dependent, while budding of tyrosinase and LAMP-1 required AP-3. Depletion of clathrin inhibited sorting of all four cargo proteins, suggesting that AP-1 and AP-3 are involved in the formation of distinct types of CCVs, each of which is characterized by the incorporation of specific cargo membrane proteins. Harasaki et al. (2005) indicated that three proteins: CI-MPR, carboxypeptidase D (CPD) and low-density lipoprotein receptor-related protein 1 (LRP1) have AP-dependent sorting signals, which may help to explain the relative abundance of AP complexes in CCVs.

AP-4 as a Component of the Clathrin Coat Machinery

AP-4, a protein complex related to clathrin adaptors (Dell'Angelica et al. 1999) is localized mainly in the Golgi complex, as well as on endosomes and transport vesicles. Interestingly, AP-4 is localized with the clathrin coat machinery in the Golgi complex and in the endocytic pathway. Moreover, AP-4 is localized with the CI-MPR, but not with the transferrin receptor, LAMP-2 or invariant chain. The difference in morphology between CI-MPR/AP-4-positive vesicles and CI-MPR/AP-1-positive vesicles raises the possibility that AP-4 acts at a location different from that of AP-1 in the intracellular trafficking pathway of CI-MPR (Barois and Bakke 2005).

3.5.4 Role of Di-leucine-based Motifs in Cytoplasmic Domains

Ludwig et al. (1993) indicated that CD-MPR is required for efficient intracellular targeting of multiple lysosomal enzymes, although homozygous mice lacking CD-MPR suggested that other targeting mechanisms could partially compensate for the loss of CD-MPR in vivo. The cytoplasmic domain of the M6P/IGF2R has two signals for lysosomal enzyme sorting in the Golgi, a di-leucine-based motif (LLHV sequence) and the tyrosine-based endocytosis motif (YKYSKV sequence) (Johnson and Kornfeld 1992), whereas a di-leucine-based motif near the carboxyl terminus of the CD-MPR (HLLPM sequence) in cytoplasmic domain is essential for efficient targeting of newly synthesized lysosomal enzymes (Johnson and Kornfeld 1992). Several other transmembrane proteins destined to the lysosomes also contain di-leucine-based motifs in their cytoplasmic domains that are essential for their proper delivery to lysosomes (Sandoval and Bakke 1994). In the light of these different results, it has been proposed that di-leucine-based motifs mediate sorting of membrane proteins in the TGN. In both MPRs, the di-leucine motifs are flanked by casein kinase II phosphorylation sites that are phosphorylated in vivo (Méresse et al. 1990; Hemer et al. 1993). Such a post-translational modification occurs when the M6P/IGF2R exits from the TGN and represents a major, albeit transient, modification (Méresse and Hoflack 1993). Thus far, the functional importance of the phosphorylation sites in the M6P/IGF2R trafficking has remained controversial (Johnson and Kornfeld 1992; Chen et al. 1993). Mouse L cells deficient in the M6P/IGF-IIR were transfected with normal bovine CD-MPR cDNA or cDNAs containing mutations in the 67-amino acid cytoplasmic tail and assayed for their ability to target the lysosomal enzyme cathepsin D to lysosomes. Mutant receptors with the carboxyl-terminal His-Leu-Leu-Pro-Met67 residues deleted or replaced with alanines sorted cathepsin D below the base-line value (Johnson and Kornfeld 1992). Of the eight amino acids mutated in bovine CD-MPR, four (Gln^{66}, Arg^{111}, Glu^{133}, and Tyr^{143}) were found to be essential for ligand binding. In addition, mutation of the single histidine residue, His^{105}, within the binding site diminished the binding of the receptor to ligand, but did not eliminate the ability of the CD-MPR to release ligand under acidic conditions (Olson et al. 1999a).

A casein kinase II (CK-II) phosphorylation site in the cytoplasmic tail of CD-MPR determines the interaction of AP-1 Golgi assembly proteins with membranes. Mauxion et al. (1996) demonstrated that the casein kinase II phosphorylation site in the CD-MPR cytoplasmic domain determines the high affinity of AP-1 for membranes and that mutations introduced independently in the tyrosine-based or the di-leucine-based motifs are not sufficient to modify these interactions. MPR-negative fibroblasts have a low capacity of recruiting AP-1 which can be restored by re-expressing the MPRs in these cells. This property helped to identify the protein motif of the CD-MPR

cytoplasmic domain that is essential for these interactions. It was found that the targeting of lysosomal enzymes requires the CD-PDR cytoplasmic domain that is different from tyrosine-based endocytosis motifs. The first is a casein kinase II phosphorylation site (ESEER) probably acts as a dominant determinant controlling CD-MPR sorting in the TGN. The second is the adjacent di-leucine motif (HLLPM), which, by itself, is not critical for AP-1 binding, but is absolutely required for a downstream sorting event (Mauxion et al. 1996).

Domain 5 of CD-MPR Preferentially Binds Phosphodiesters: Sequence alignment predicts that domain 5 contains four conserved residues (Gln, Arg, Glu, Tyr) which are essential for M6P binding by the CD-MPR and domains 1–3 and 9 of the CI-MPR. Surface plasmon resonance (SPR) analyses of constructs containing single amino acid substitutions showed that these conserved residues (Gln^{644}, Arg^{687}, Glu^{709}, Tyr^{714}) are critical for carbohydrate recognition by domain 5. Furthermore, the N-glycosylation site at position 711 of domain 5, which is predicted to be located near the binding pocket, has no influence on the carbohydrate binding affinity. Using endogenous ligands for the MPRs demonstrated that, unlike the CD-MPR or domain 9 of the CI-MPR, domain 5 exhibits a 14–18-fold higher affinity for MP-GlcNAc than M6P, implicating this region of the receptor in targeting phosphodiester-containing lysosomal enzymes to the lysosome (Chavez et al. 2007). Crystallographic studies have shown that at pH 6.5, the CD-MPR bound to M6P adopts a significantly different quaternary conformation than the CD-MPR in a ligand-unbound state, a feature unique among known lectin structures. Additional crystal structures of the available CD-MPR revealed the positional invariability of specific binding pocket residues which implicate intermonomer contact(s), as well as the protonation state of M6P, as regulators of pH-dependent carbohydrate binding (Olson et al. 2008).

Interaction of MPRs with GGA Proteins: The GGAs (Golgi-localizing, γ-adaptin ear homology domain, ARF-binding), the multidomain family of proteins have been implicated in protein trafficking between Golgi and endosomes. Evidence suggests that CI-MPR and CD-MPR bind specifically to VHS domains of GGAs through acidic cluster-dileucine motifs at the carboxyl ends of their cytoplasmic tails. However, the CD-MPR binds VHS domains more weakly than the CI-MPR. Alignment of C-terminal residues of two receptors revealed a number of non-conservative differences in the acidic cluster-dileucine motifs and the flanking residues. Studies indicate that GGAs participate in lysosomal enzyme sorting mediated by CD-MPR (Doray et al. 2002).

3.5.5 Sorting Signals in Endosomes

The endocytosis of cell surface proteins is mediated by tyrosine-based (Trowbridge et al. 1993) or di-leucine-based motifs (Sandoval and Bakke 1994). In case of CI-MPR/IGF2 receptor, its endocytosis requires a single YSKV sequence (Jadot et al. 1992), while that of CD-MPR requires two distinct motifs. The bovine CD-MPR cycles between TGN, endosomes and the plasma membrane. When the terminal 40 residues were deleted from the 67-amino acid cytoplasmic tail of the CD-MPR, the half-life of the receptor was drastically decreased and the mutant receptor was recovered in lysosomes; amino acids 34–39 being critical for avoidance of lysosomal degradation. Findings indicated that the cytoplasmic tail of the CD-MPR contains a signal that prevents the receptor from trafficking to lysosomes. The transmembrane domain of the CD-MPR also contributes to this function (Rohrer et al. 1995).

The 67-amino acid cytoplasmic tail of CD-MPR contains a signal(s) that prevents the receptor from entering lysosomes where it would be degraded. A receptor with a Trp^{19} –> Ala substitution in the cytoplasmic tail resulted into highly missorted to lysosomes whereas receptors with either Phe^{18} –> Ala or Phe^{13} –> Ala mutations were partially defective in avoiding transport to lysosomes. Results indicated that the di-aromatic motif (Phe^{18}-Trp^{19} with Trp^{19} as the key residue) in its cytoplasmic tail is required for the sorting of the receptor from late endosomes back to the Golgi apparatus. Because a diaromatic amino acid sequence is also present in the cytoplasmic tail of other receptors known to be internalized from the plasma membrane, this feature may be a general determinant for endosomal sorting (Schweizer et al. 1997). However, the CI-MPR lacks such a di-aromatic motif. Studies indicate that sorting of the CD-MPR in late endosomes requires a distinct di-aromatic motif with only limited possibilities for variations, in contrast to the CI-MPR, which seems to require a putative loop (Pro^{49}-Pro-Ala-Pro-Arg-Pro-Gly^{55}) along with additional hydrophobic residues in the cytoplasmic tail. This raises the possibility of two separate binding sites on Tip47 because both receptors require binding to Tip47 for endosomal sorting (Nair et al. 2003).

3.5.6 Palmitoylation of CD-MPR is Required for Correct Trafficking

Evasion of lysosomal degradation of the CD-MPR requires reversible palmitoylation of a cysteine residue in its cytoplasmic tail. Because palmitoylation is reversible and essential for correct trafficking, it presents a potential regulatory mechanism for the sorting signals within the cytoplasmic domain of

the CD-MPR. The two cysteine residues (Cys^{30} and Cys^{34}) in the cytoplasmic tail of the CD-MPR are palmitoylated via thioesters and Cys^{34} residue influences the biologic function of the receptor. Mutation of Cys^{34} to Ala resulted in the gradual accumulation of the receptor in dense lysosomes and the total loss of cathepsin D sorting function in the Golgi. A Cys^{30} to Ala mutation had no biologic consequences, showing the importance of Cys^{34}. Mutation of amino acids 35–39 to alanines impaired palmitoylation of Cys^{30} and Cys^{34} and resulted in abnormal receptor trafficking to lysosomes and loss of cathepsin D sorting. The palmitoylation of Cys^{30} and Cys^{34} leads to anchoring of this region of the cytoplasmic tail to the lipid bilayer. Thus, anchoring via Cys^{34} is essential for the normal trafficking and lysosomal enzyme sorting function of the receptor (Schweizer et al. 1996). The palmitoylation of the CD-MPR occurs enzymatically by a membrane-bound palmitoyltransferase, which cycles between endosomes and the plasma membrane. The localization of the palmitoyltransferase indicates it as a regulator of the intracellular trafficking of the CD-MPR and also affects the sorting/activity of other receptors cycling through endosomes (Stöckli and Rohrer 2004).

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P-Type Lectins: Cation-Independent Mannose-6-Phosphate Reeptors

4

G.S. Gupta

4.1 Cation-Independent Mannose 6-Phosphate Receptor (CD222)

4.1.1 Glycoprotein Receptors for Insulin and Insulin-like Growth Factors

Insulin and the insulin-like growth factors, IGF-I (IGF1) and –II (IGF2) are structurally related peptides that elicit a large number of similar biological effects in target cells. Three well-characterized receptor complexes bind one or more of these peptides with high affinity. Two of these receptors, denoted as type 1, are ligand-activated tyrosine kinases with similar heterotetrameric $\alpha 2\beta 2$ subunit structures which bind insulin or IGF-1, respectively, with highest affinity. Ligand-stimulated tyrosine autophosphorylation of these receptors further activates their intrinsic tyrosine kinase activities both in vitro and in intact cells. Rapid signal transduction follows such receptor autophosphorylation and tyrosine kinase activation, leading to increased serine phosphorylation of many cell proteins and decreased serine phosphorylation of several others. A third receptor in this group binds IGF-1 and -2, lacks kinase activity and is denoted as type II IGF receptor (IGF2R). The cell surface receptor for IGF2 also functions as a cation-independent M6PR. Therefore, cation-independent mannose 6-phosphate receptor (CI-MPR) is also referred as insulin-like growth factor 2 receptor (IGF2R) or IGF2/MPR. The CI-MPR/IGF2R is a single transmembrane domain glycoprotein that plays a major role in the trafficking of lysosomal enzymes from the trans-Golgi network (TGN) to the endosomal-lysosomal (EL) system. This CI-MPR/IGF2R has also a potential role in growth factor maturation and clearance, and mediates IGF2-activated signal transduction through a G-protein-coupled mechanism. The IGF2R/CI-MPR rapidly recycles between the cell surface membrane and intracellular membrane compartments, providing for the rapid uptake of both IGF2 and M6P-linked lysosomal enzymes. Insulin action markedly increases the proportion of receptors in the plasma membrane and the uptake of bound ligands. Embryonic development and normal growth require exquisite control of IGFs (Dahms et al. 2008; Gary-Bobo et al. 2007).

4.2 Characterization of CI-MPR/IGF2R

4.2.1 Primary Structures of Human CI-MPR and IGF2R Are Identical

The full-length cDNA of 9,104-nt for human CI-MPR contains 7,473 nt encoding a protein of 2,491 aa. The amino acid sequence includes a putative signal sequence of 40 aa, an extracytoplasmic domain consisting of 15 homologous repeat sequences of 134–167 aa, a transmembrane region of 23 aa, and a cytoplasmic domain of 164 aa. The predicted molecular size is greater than 270 kDa. Repeats 7–15 of the extracytoplasmic domain of the human receptor are highly homologous with the sequence of the bovine receptor (Lobel et al. 1987). The nucleotide sequence for the full-length cDNA and the deduced amino acid sequence for the CI-MPR has identity of 99.8% at nt level and 99.4% identity at aa level to the human IGF2R receptor from HepG2 hepatoma cells (Morgan et al. 1987). Kiess et al. (1988) also supported that the type II IGF receptor and the CI-MPR are the same protein, but the binding sites of IGF2 and M6P are distinct. The structural and biochemical features of the IGF2 receptor appeared to be identical to those of the CI-MPR (Kiess et al. 1988; Morgan et al. 1987; Oshima et al. 1988). Phenotypic changes in the transformed cell lines support the role of the CI-MPR in intracellular sorting and targeting of lysosomal enzymes (Oshima et al. 1988).

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4.2.2 Mouse IGF2R/CI-MPR Gene

The mouse IGF2R/CI-MPR gene is 93 kb long, comprising 48 exons, and codes for a predicted protein of 2,482 amino acids. The extracellular part of the receptor is encoded by exons 1–46, with each of 15 related repeating motifs being determined by parts of 3–5 exons. A single fibronectin type II-like element is found in exon 39. The transmembrane domain of MPR also is encoded by exon 46 and the cytoplasmic region by exons 46–48. The positions of exon-intron splice junctions are conserved between several of the repeats in IGF2R/CI-MPR and the homologous extracellular region of the gene for other known receptor, the CD-MPR (Szebenyi and Rotwein 1994).

Promoter Elements: The expression of CI-MPR is controlled by both epigenetic and tissue-specific factors. The 93-kb mouse gene has been characterized for its 48 exons. Transient transfection assays revealed that promoter gene of IGF2R/CI-MPR was orientation-specific and was maximal with a plasmid containing 266 bp of IGF2R/CI-MPR DNA. In vitro DNase I footprinting revealed an extended 54-bp footprint within the proximal promoter that contained two E-boxes and potential binding sites for transcription factors Sp1, NGF-IA, and related proteins. These results define a strong minimal IGF2R/CI-MPR promoter of no more than 266 bp and identify a 54-bp enhancer within this promoter fragment (Liu et al. 1995).

Imprinting of Mouse Igf2/Mpr or Igf2r Gene: *Igf2/Mpr* has been mapped to the mouse Tme locus and shown to be an imprinted gene, which further suggests a role in embryonic growth regulation. Igf2 is a general embryonic mitogen, and mice lacking *Igf2* are markedly reduced in size. *Igf2r* acts to fine tune the amount of growth factor, and embryos lacking this gene show overgrowth and die perinatally (Wang et al. 1994). Gametic imprinting is a developmental process that uses cis-acting epigenetic mechanisms to induce parental-specific expression in autosomal and X-linked genes. The biological function of imprinting in mammals is not fully understood. It has been proposed that CG rich sequences resembling CpG islands, which are associated with many imprinted genes and often subject to parental-specific methylation, could act as a common imprinting element. The link between imprinting and growth regulation is best exemplified by the *Igf2* and *Igf2r* genes. Both genes show parental-specific expression patterns in the embryo. The mouse *Igf2r* gene contains a CpG island known as region2, in the second intron. Region2 was proposed as the imprinting element of this gene because it inherited a methylation imprint from the female gamete that was maintained only on the maternal chromosome in diploid cells. Barlow and associates used yeast artificial chromosome transgenes carrying the complete Igf2r locus, to test if imprinting and parental-specific methylation of the mouse *Igf2r* gene is maintained when transferred to other chromosomal locations and to test whether imprinting is dependent on the intronic CpG island proposed as the imprinting element for this gene. Gametic imprints are epigenetic modifications which are imposed onto the gametic chromosomes and cause parental-specific differences in the expression of a small number of genes in the embryo. As a consequence, correct imposition of the imprints in the parental germlines is a prerequisite for successful development of mammals and any anomaly in the expression of imprinted genes is often accompanied by aberration of embryonic growth. The phenomenon of gametic imprinting is predicted to arise by an unusual regulation of the imposition and erasure of epigenetic modifications. One of events which are required for imprinting is that the imprint must be imposed in one of the gametes before fertilisation (Wutz and Barlow 1998; Ludwig et al. 1996).

Chromosomal Mapping of Gene for IGF2/CI-MPR: The gene for IGF2R that has been found to be identical to the CI-MPR has been mapped in the human and murine species. The genes are located in a region of other conserved syntenic genes on the long arm of human chromosome 6, region 6q25-q27, and mouse chromosome 17, region A-C. The CI-MPR/IGF2R locus in man is asyntenic with the genes encoding IGF2, the IGF-I receptor (IGF1R), and the CD-MPR (Laureys et al. 1988). The *Igf2r* gene in bovine is localized to BTA9 chromosome 9q27–28 (Friedl and Rottmann 1994).

4.2.3 Bovine CI-MPR

The 215-kDa CI-MPR from fetal calf liver has a carboxyl-terminal cytoplasmic domain of 163 amino acids that is rich in acidic residues, a 23-amino acid transmembrane segment, a 44-residue amino-terminal signal sequence, and an extracellular domain containing at least eight homologous repeats of approximately 145 amino acids. One of the repeats is similar to the type II repeat of fibronectin. Each repeat contains a highly conserved 13-amino acid unit bordered by cysteine residues that may be functionally important (Lobel et al. 1987). The large 2269-residue extracytoplasmic region is composed of 15 contiguous domains that display a similar size (147 residues) and distinctive pattern of 8 cysteine residues, and exhibit significant amino acid identity (14–38%) when compared to each other and to the CD-MPR, giving rise to the prediction that they have a similar

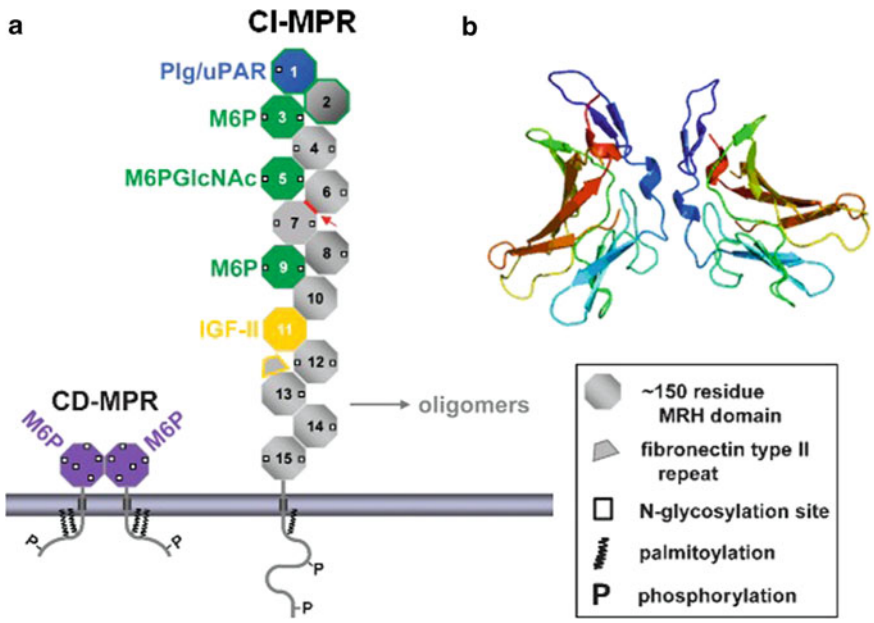

Fig. 4.1 (**a**) Domain organization of M6PRs: The 279-residue CD-MPR exists as a homodimer. The 2499-residue CI-MPR also undergoes oligomerization and most likely exists as a dimer. The M6P binding sites of the CD-MPR (*purple*) and CI-MPR (*green*) are indicated. Domains 1 and 2 are outlined in *green* since the presence of these two domains enhances the affinity of domain 3 for lysosomal enzymes by ~1,000-fold (Hancock et al. 2002b). The CD-MPR contains a single high affinity M6P binding site per polypeptide. In contrast, CI-MPR contains three carbohydrate recognition sites: two high affinity sites are localized to domains 1–3 and domain 9 and one low affinity site is contained within domain 5. The IGF2 (*gold*) and plasminogen (Plg)/uPAR (*blue*) binding sites are also indicated. The fibronectin type II repeat present in domain 13 is outlined in *yellow* since its presence increases the affinity of domain 11 for IGF2 by ~10-fold. The *red arrow* indicates the location of a proteolytically sensitive cleavage site between domains 6 and 7 (Westlund et al. 1991) (Adapted with permission from Dahms et al. 2008 © Oxford University Press). (**b**) The overall folding of CI-MPR is similar to that of CD-MPR, but unlike CD-MPR, CI-MPR is cation-independent. The CI-MPR (PDB ID: 1E6F) most likely exists as a dimmer (Dahms and Hancock 2002)

tertiary structure. This hypothesis has been confirmed, in part, by crystal structure determinations which show that the extracytoplasmic region of the CD-MPR and domains 1, 2, 3, 11, 12, 13, and 14 of the CI-MPR all exhibit the same fold (Olson et al. 1999a, 2004a, b; Brown et al. 2008) (see below). Except for domain 13 of the CI-MPR which has 12 cysteine residues due to a 43-residue insertion homologous to the type II repeat of fibronectin, each of the domains of the CI-MPR that has been crystallized contains eight cysteine residues that form four intramolecular disulfide bridges. The MPRs undergo several types of co- and posttranslational modifications, including N-glycosylation, palmitoylation, and phosphorylation (Fig. 4.1). Palmitoylation of the CD-MPR has been shown to prevent its degradation in lysosomes (Rohrer et al. 1995) while serine phosphorylation of both MPRs has been shown to influence their intracellular transport (Braulke 1999; Breuer et al. 1997). In addition, canonical sorting motifs (i.e., D/EXXLL and YXXφ) within the cytosolic regions of MPRs have been shown to be recognized by components of vesicular machinery that dictate the localization and intracellular trafficking of the receptors (Ghosh et al. 2003b; Bonifacino 2004). Alignment of the 15 domains and the extracytoplasmic domain of the CI-MPR shows that all have sequence similarities and suggests that all are homologous (Lobel et al. 1988). The bovine CI-MPR and the human IGF2R were shown to be 80% identical in their amino acid sequences as deduced from cDNA clones (Morgan et al. 1987). The CI-MPR (215 kDa) has been isolated from embryonic bovine tracheal cells and embryonic human skin fibroblasts. Results suggest the presence of amide-linked palmitic acid in the structure of the CI-MPR (Westcott and Rome 1988). Amino acid sequences deduced from rat c-DNA clones encoding the IGF2R closely resemble those of the bovine CI-MPR, suggesting they are identical structures. It is also shown that IGF2 receptors are adsorbed by immobilized pentamannosyl-6-phosphate and are specifically eluted with M6P. Results indicate that the type II IGF receptor contains cooperative, high-affinity binding sites for both IGF2 and M6P-containing proteins (MacDonald et al. 1988)

4.2.4 CI-MPR in Other Species

In contrast to the bovine, rat, and human CI-MPRs, which bind human IGF2 and IGF-I with nanomolar and micromolar affinities, respectively, the chicken receptor does not bind either radioligand at receptor concentrations as high as

1 μM. The bovine receptor binds chicken IGF2 with high affinity as compared to chicken receptor that binds this ligand with only low affinity (μM range). These data demonstrate that the chicken CI-MPR lacks the high affinity binding site for IGF2 (Canfield and Kornfeld 1989). Like chicken, frog CI-MPR also lacks high affinity IGF2-binding site.

Yang et al. (1991) demonstrated that chick embryo fibroblasts (CEFs) bind and internalize lysosomal enzymes in a M6P-inhibitable fashion, and possess a protein immunologically related to the mammalian IGF2/M6P receptor that binds lysosomal enzymes with M6P recognition markers but does not bind IGF2. The 8,767-bp full-length cDNA of chicken CI-MPR encodes a protein of 2,470 aa that includes a putative signal sequence, an extracytoplasmic domain consisting of 15 homologous repeat sequences, a 23-residue transmembrane sequence, and a 161-residue cytoplasmic domain. It shows 60% sequence identity with human and bovine CI-MPR homologs, and all but two of 122 cysteine residues are conserved (Zhou et al. 1995). American opossum (Didelphis virginiana) liver expressed both the CI-MPR and the CD-MPR. Both receptors contained Asn-linked oligosaccharides. In contrast to CD-MPRs isolated from other species, the opossum CD-MPR displayed heterogeneity with respect to the number of Asn-linked oligosaccharide chains it contains. The CI-MPR from opossum liver bound human recombinant IGF2. However, the opossum CI-MPR bound IGF2 with a lower affinity (K_D = 14.5 nM) than the bovine receptor (K_D = 0.2 nM) (Dahms et al. 1993a). The CI-MPR from the opossum binds bovine IGF2 with low affinity. The kangaroo CI-MPR has a lower affinity for IGF2 than its eutherian (placental mammal) counterparts. Furthermore, the kangaroo CI-MPR has a higher affinity for kangaroo IGF2 than for human IGF2. The cDNA sequence of the kangaroo CI-MPR indicates that there is considerable divergence in the area corresponding to the IGF2 binding site of the eutherian receptor

4.3 Structure of CI-MPR

4.3.1 Domain Characteristics of IGF2R/CI-MPR (M6P/IGF2R)

The IGF2R/CI-MPR is an ~300-kDa type I transmembrane glycoprotein that consists of an N-terminal signal sequence, a large extracytoplasmic (EC) region composed of 15 homologous repeating domains having ~145 amino acids each, a single transmembrane region, and a short C-terminal cytoplasmic domain (Fig. 4.1a), which lacks kinase activity (Ghosh et al. 2003a; Lobel et al. 1988; Oshima et al. 1988; Scott and Firth 2004). It appears to exist and function as a dimer (Fig. 4.1b). The ~46-kDa CD-MPR is a much smaller type I transmembrane glycoprotein that in some species requires divalent cations for optimal ligand binding (Tong et al. 1989a). Significantly, each of the 15 IGF2/MPR extracytoplasmic domains displays amino acid sequence identity (14–38%), similar size (~147 residues) (Ghosh et al. 2003a), and cysteine distribution to each other and to the single, EC domain of CD-MPR, giving rise to the suggestion that they exhibit similar disulfide bonding and tertiary structures (Lobel et al. 1988).

Domains 3, 5 and 9 of the IGF2R have been shown to contain M6P binding sites (Hancock et al. 2002a, b; Reddy et al. 2004) and the binding sites for other ligands, including the urokinase plasminogen activator receptor, plasminogen, TGFβ, and retinoic acid. A distinct IGF2 binding site is located on domain 11, and the additional presence of domain 13 is required for high affinity IGF2 binding equivalent to intact IGF2R (Dahms et al. 1994; Garmroudi and MacDonald 1994). Separate domains, expressed in *Pichia pastoris*, were tested for their ability to bind carbohydrate ligands and the carbohydrate modifications (mannose 6-sulfate and M6P methyl ester) found on *Dictyostelium discoideum* lysosomal enzymes. The carbohydrate binding sites of the IGF2/MPR were located to domains 3 and 9 of the extracytoplasmic region (Hancock et al. 2002b).

The crystal structures of one of the M6P binding sites (domains 1–3) (Olson et al. 2004a, b) and the IGF2 binding site (domains 11–14) (Brown et al. 2008) have been obtained. The structure of domains 1–3 is very compact and forms a triangular disk of 70 Å (each side) × 50 Å (thickness), with each corner of the triangle occupying one domain. In contrast, the relative orientations of domains 11–14 are very different. The structure of domains 11–14 is rather elongated (50 Å × 60 Å × 115 Å high) and resembles beads on a string, with each domain forming a bead. Combining these two modular structures, along with prediction that the remaining two A simplified representation of the entire extracellular portion (domains 1–15) of the CI-MPR is shown in Fig. 4.1. A similar model has been proposed by Brown et al. (2008). Consistent with the three-domain architecture for each M6P binding site is the presence of a proteolytically sensitive site between domains 6 and 7 (Westlund et al. 1991). The structure of the IGF2 binding site reveals that IGF2 binds in the same location as M6P, thus the same M6PR-H fold can function in protein–protein or protein–carbohydrate interactions. However, it is not clear how various ligand binding sites are oriented relative to each other and how conformation is influenced by pH or ligand binding. Studies are required to support the positioning of ligand binding sites on one face of the molecule as shown in model.

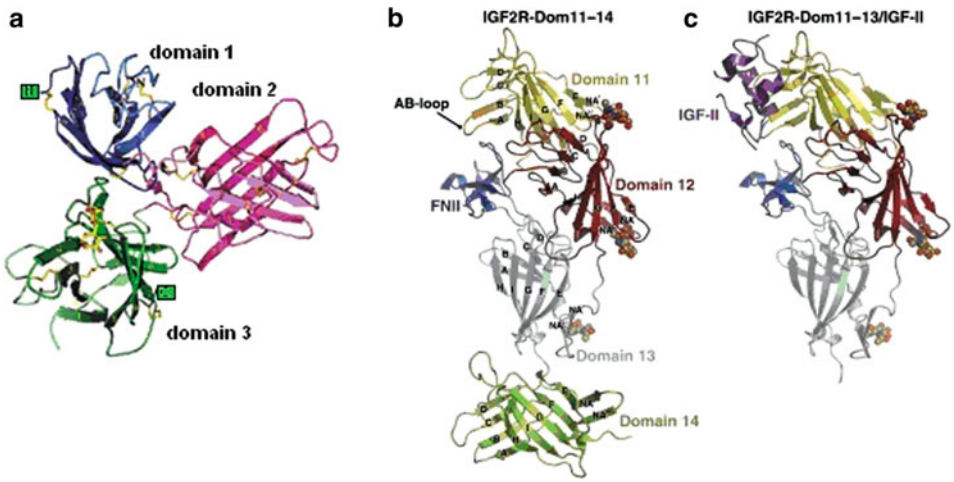

Fig. 4.2 Three-dimensional structure of domains 1–3 of bovine CI-MPR with M6P: (**a**) Stereo view of the M6P complex encompassing residues 7–432 of the bovine CI-MPR. The N and C terminus of protein encoding domain 1 (*blue*), domain 2 (*pink*), and domain 3 (*green*) are indicated, as are the disulfide bonds (*yellow*). β-Strands are labeled (see enlarged view), and the location of M6P (*gold ball-and-stick model*) is shown in green in domain 3 (see enlarged view) (PDB ID: 1SZ0). The CI-MPR contains multiple M6P binding sites that map to domains 3, 5, and 9 within its 15-domain extracytoplasmic region, functions as an efficient carrier of M6P-containing lysosomal enzymes (Olson et al. 2004a) (Adapted with permission from Olson et al. 2004a © American Society for Biochemistry and Molecular Biology). Figure (**b**) and (**c**) are representations of IGF2R fragment structures CI-MPR-Domain11–14 and the complex between CI-MPRDomain11–13 and IGF-II. Glycans are shown as spheres and strand labelling is given in panel B (Adapted by permission from Macmillan Publishers Ltd, EMBO J. Brown et al. © 2008)

4.3.2 Crystal Structure

Truncated forms of CD-MPR and CI-MPR: The CD-MPR has been crystallized under different pH conditions: in an unbound state at pH 6.5 and pH 4.8 (Olson et al. 2002, 2008), or at pH 7.4 in a bound state (Olson et al. 2008). The extracytoplasmic region of the CD-MPR has been studied with X-ray crystallography in complex form with a single sugar M6P (Roberts et al. 1998), or with various oligosaccharide structures (Olson et al. 1999b, 2008). Seven out of fifteen domains of the CI-MPR have also been crystallized: the N-terminal 432 residues (domains 1, 2, and 3), which house a high affinity M6P binding site, bound to either a mannose residue from a crystallographic neighbor or M6P (Olson et al. 2004a, b); the IGF2 binding site (domains 11–14) in the unbound state (Brown et al. 2002; Uson et al. 2003) or domains 11–13 in an IGF2 bound state (Brown et al. 2008). These studies provide a framework from which the mechanism of ligand binding by the MPRs can be inferred (Fig. 4.2).

The extracytoplasmic domain of CD-MPR has been crystallized as a dimer (Figs. 3.1 and 3.2; Chap. 3). Each polypeptide chain folds into an N-terminal α-helix followed by four anti-parallel β-strands, which together comprise the solvent-exposed front face. The dimer interface β-sheet (β5–β9), which accounts for ~20% of the surface area of the monomer, is composed of five anti-parallel β-strands, with strand 9 interjecting between strands 7 and 8. Subsequent determination of the structure of domains 1–3 (Fig. 4.2) and domains 11–14 of the CI-MPR showed that this overall topology is conserved with the exception of the N-terminal region: neither domains 1–3 nor 11–14 contain the α-helix; rather, this secondary structural element is replaced by two β-strands. The quaternary domain arrangement of CD-MPR is not conserved in the structure of domains 1–3 of the CI-MPR. The structure of the N-terminal region of the CI-MPR shows the three domains form a wedge with domains 1 and 2 oriented such that the four-stranded N-terminal sheet (β1–β4) of domain 1 and the five-stranded C-terminal sheet (β5–β9) of domain 2 form a continuous surface (Fig. 4.2b). In comparison to the CD-MPR in which extensive contacts exist between the two dimer interface β-sheets (β5–β9) (Fig. 3.1), the interaction between the three N-terminal domains of the CI-MPR is quite different and much less extensive; the contacts between the three domains are mediated mainly by residues within the linker regions and loops (Fig. 4.2). However, the contacts between domains 1, 2, and 3 are important for maintaining the integrity of the binding pocket housed within domain 3. The multiple interactions between residues of domains 1 and 2 with residues of loops C and D of domain 3 are likely to aid in the stabilization of the binding pocket and provide an explanation for the inability of a construct encoding domain 3 alone to generate a high affinity carbohydrate binding site (Hancock et al. 2002b).

A comparison of the sugar binding pocket of CD-MPR and domain 3 of CI-MPR reveals that residues which interact with the mannose ring (Gln, Arg, Glu, and Tyr) are located in a strikingly similar position in the base of the pocket and form the same contacts with the ligand. These four residues have been shown to be essential for M6P recognition by

mutagenesis and are conserved in all species and in the other two M6P binding sites of the CI-MPR (i.e., domains 5 and 9). The presence of this signature motif for phosphomannosyl binding (Gln, Arg, Glu and Tyr) along with conserved cysteine residues led to predict that domain 5 of the CI-MPR contains a M6P binding site, a hypothesis which was confirmed by Reddy et al. (2004). Mutagenic studies of Gln, Arg, Glu, and Tyr in domains 5 (Chavez et al. 2007) and 9 (Hancock et al. 2002a) demonstrate their essential role in carbohydrate recognition by these binding sites. Thus, the strict requirement for a terminal mannose residue by both receptors is reflected in the similarities in that region of the binding pocket responsible for sugar recognition (Dahms et al. 2008).

In contrast, the phosphate recognition region of the binding site appears to have the most variability both in amino acid composition and in structure. In both receptors the lid is formed by residues joining β-strands 6 and 7 (loop C). This lid region is larger in the CD-MPR and the positioning of the disulfide anchors the loop in a more closed position which translates into a more sterically confined binding region. The conformationally constrained lid may account for the inability of CD-MPR to bind phosphodiesters. Shortening of both loops C and D effectively makes the binding pocket of domains 1–3 more open than that of the CD-MPR, allowing for this region of the CI-MPR to bind a larger repertoire of ligands, including phosphomonoesters, mannose 6-sulfate, and phosphodiesters. Thus, the diversity in ligand recognition by the two receptors appears to be accomplished by alterations in the receptor binding site architecture surrounding the phosphate moiety.

Brown et al. (2008) reported crystal structures of IGF2R domains 11-12, 11-12-13-14 and domains 11-12-13/IGF-II complex. A distinctive juxtaposition of these domains provides the IGF-II-binding unit, with domain 11 directly interacting with IGF-II and domain 13 modulating binding site flexibility. The complex shows that Phe19 and Leu53 of IGF-II lock into a hydrophobic pocket unique to domain 11 of mammalian IGF2Rs. Mutagenesis analyses confirmed this IGF-II 'binding-hotspot', revealing that IGF-binding proteins and IGF2R have converged on the same high-affinity site (Brown et al. 2008) (Fig 4.2b, c).

4.4 Ligands of IGF2R/CI-MPR

4.4.1 Extracellular Ligands of IGF2R/CI-MPR

The M6P/IGF2R has been shown to bind at least two classes of ligands, the M6P-containing and the non-M6P-containing polypeptide ligands, all of which bind to sites within the EC region (Braulke 1999). Newly synthesized M6P-containing ligands such as lysosomal acid hydrolases bind to the M6P/IGF2R in the *trans*-Golgi network through M6P residues on their *N*-linked oligosaccharides. Through binding of a large class of ligands, the M6P/IGF2R mediates several important cellular functions, such as the endocytosis and/or targeting of acid hydrolases to lysosomes (Kornfeld 1992), the proteolytic activation of latent TGF-β1 (Dennis and Rifkin 1991; Ghahary et al. 1999), mediation of the migration and angiogenesis induced by proliferin (Groskopf et al. 1997), and the internalization of granzyme B (Motyka et al. 2000).

Proliferin is a prolactin-related glycoprotein secreted by proliferating mouse cell lines and by mouse placenta. The proliferin secreted by cultured cell binds to CI-MPRs and may be a lysosomal protein targeted to lysosomes. The activity of MPRs in murine fetal and maternal liver and in placenta is regulated during pregnancy (Lee and Nathans 1988). The angiogenic activity of proliferin depends on its interaction with CI-MPR. This recognition involves at least in part the carbohydrate moiety of proliferin which competes with M6P (Lee and Nathans 1988). Tumor growth depends on its vascularisation and needs micro-vessels development to expand its cell population and to become invasive (Folkman 2002). The expression of proliferin seems to increase when a tumor becomes more invasive and exhibits a strong angiogenic activity.

M6P-containing ligands, such as latent form of TGF-β, placental hormone proliferin, and granzyme B bind at the cell surface (Ghosh et al. 2003a; Dahms and Hancock 2002). The IGF2/MPR binding to mannose 6-phosphorylated latent form of TGF-β at the cell surface results in the proteolytic activation of this critical growth factor that regulates the cellular differentiation and proliferation of many cell types (Dennis and Rifkin 1991; Godár et al. 1999). The activation of latent TGF-β to active TGF-β is a critical step in wound healing. Cell surface recognition of M6P-modified proliferin by the IGF2/MPR is required for proliferin-induced angiogenesis (Jackson and Linzer 1997.). The precursor form of the renin (prorenin), a key enzyme of the cardiac renin-angiotensin system, contains M6P residues that enable IGF2/MPR binding and internalization of prorenin, resulting in its subsequent proteolytic activation in endosomal compartments (Saris et al. 2001; Nguyen and Contrepas 2008).

4.4.2 Binding Site for M6P in CI-MPR

The interactions of the bovine CD-MPR with monovalent and divalent ligands have been studied. This receptor appears to be a homodimer or a tetramer. Each mole of receptor monomer bound 1.2 mol of the monovalent ligands, M6P and pentamannose phosphate with K_D values of $8 \times {}^{-6}$ M and 6×10^{-6} M, respectively and 0.5 mol of the divalent ligand, a high mannose oligosaccharide with

two phosphomonoesters, with a K_D of 2×10^{-7} M. When Mn^{2+} was replaced by EDTA in the dialysis buffer, the K_D for pentamannose phosphate was 2.5×10^{-5} M (Tong et al. 1989b). The interactions of the bovine CI-MPR with a variety of phosphorylated ligands showed the K_D for M6P, pentamannose phosphate, bovine testes β-galactosidase, and a high mannose oligosaccharide with two phosphomonoesters as 7×10^{-6} M, 6×10^{-6} M, 2×10^{-8} M, and 2×10^{-9} M, and the mol of ligand bound/mol of receptor monomer as 2.17, 1.85, 0.9, and 1.0, respectively. Study indicates that the CI-MPR has two M6P-binding sites/polypeptide chain (Tong et al. 1989a).

The two high affinity binding sites are not functionally equivalent with respect to ligand preference, having distinct K_D for the multivalent M6P-ligand β-glucuronidase (2.0 versus 4.3 nM for repeats 3 and 9, respectively) (Marron-Terada et al. 1998). The pH optimum for carbohydrate binding was also more acidic for repeat 9 than repeat 3 (pH 6.4 versus pH 6.9, respectively), and the two sites differed in their ability to recognize distinctive modifications found on *Dictyostelium discoideum* glycoproteins, such as mannose 6-sulfate and M6P methyl esters (Marron-Terada et al. 2000). Additionally, repeat 9 alone could fold into a high affinity ligand binding domain, whereas repeat 3 was dependent on residues in adjacent repeats 1 and/or 2 for optimal ligand binding (Hancock et al. 2002b). Although it exhibits significant sequence homology with repeats 3 and 9, as well as sharing four conserved residues key for M6P binding, repeat 5 had an ~300-fold lower affinity for M6P than repeat 9 or repeats 1–3, possibly due to the absence of two half-cystines that form a stabilizing disulfide bond in repeats 3 and 9 (Reddy et al. 2004).

The MPRs differ in number of M6P binding sites contained within their polypeptide chains. While CD-MPR contains one (Tong and Kornfeld 1989b), the CI-MPR contains three M6P binding sites (Tong et al. 1989b; Reddy et al. 2004). Expression of recombinant truncated forms of the CI-MPR revealed its three carbohydrate binding sites: two high affinity sites (K_i = ~10 μM for M6P) map to domains 1–3 and domain 9 (Hancock et al. 2002b) while domain 5 houses a low affinity (K_i = ~5 mM for M6P) binding site (Reddy et al. 2004). A comparison of the binding properties of the individual carbohydrate recognition sites demonstrated that domain 9 of the CI-MPR exhibits optimal binding at pH 6.4–6.5, similar to that of the CD-MPR. In contrast, the N-terminal M6P binding site (i.e., domains 1–3) has a significantly higher optimal binding pH of 6.9–7.0 (Marron-Terada et al. 2000). This observation may not only explain the relatively broad pH range of ligand binding by the CI-MPR but likely is a main contributor to the ability of the CI-MPR, as opposed to CD-MPR, to internalize exogenous ligands at slightly alkaline pH 7.4 present at cell surface. Domain 9 of the CI-MPR, like the CD-MPR, is highly specific for phosphomonoesters (Chavez et al. 2007). In contrast, the N-terminal carbohydrate recognition site of the CI-MPR is promiscuous in that, in addition to M6P, it efficiently binds mannose 6-sulfate and the M6P-OCH_3 phosphodiester with only a 20-fold or 10-fold, respectively, lower affinity than M6P (Marron-Terada et al. 2000). Surface plasmon resonance analyses demonstrated that unlike CD-MPR and domain 9 of CI-MPR, domain 5 of CI-MPR exhibits a 14–18-fold higher affinity for MP-GlcNAc than M6P and implicates this region of the receptor in targeting phosphodiester-containing lysosomal enzymes (i.e., those acid hydrolases that escaped the action of the uncovering enzyme in TGN) to the lysosome (Chavez et al. 2007). The presence of three distinct carbohydrate recognition sites in the CI-MPR likely accounts for the ability of the CI-MPR to recognize a greater diversity of ligands than the CD-MPR, both in vitro and in vivo (Dahms et al. 2008; Bohnsack et al. 2009).

Residues Corresponding to the M6P Binding: Residues corresponding to the M6P binding site of the CD-MPR were mutated to Ala, and Tyr^{1542}, Thr^{1570}, Phe^{1567}, and Ile^{1572} within this hydrophobic patch were shown to be critical for IGF2 binding. Ile^{1572} had been identified as essential for IGF2 binding by Garmroudi and MacDonald (1994). Interestingly, mutation of Glu^{1544} (which lies at the edge of this binding site) to Ala or Lys leads to an increase in binding affinity (Zaccheo et al. 2006). Although the binding site on domain 11 has been mapped, it was not clear whether IGF2 contacts domain 13.

Alanine mutagenesis of structurally determined IGF2R showed that two hydrophobic residues in the CD loop (F1567 and I1572) are essential for binding, with a further non-hydrophobic residue (T1570) that slows the dissociation rate. These findings have implications in the molecular architecture and evolution of the domain 11 IGF2-binding site, and the potential interactions with other domains of IGF2R (Zaccheo et al. 2006). Brown et al. (2008) reported crystal structures of IGF2R domains 11-12, 11-12-13-14 and domains 11-12-13/IGF2 complex. A distinctive juxtaposition of these domains provides the IGF2-binding unit, with domain 11 directly interacting with IGF2 and domain 13 modulates binding site flexibility. This complex showed that Phe^{19} and Leu^{53} of IGF2 lock into a hydrophobic pocket unique to domain 11 of mammalian IGF2Rs. Mutagenesis analyses confirmed this IGF2 'binding-hotspot', revealing that IGF-binding proteins and IGF2R have converged on the same high-affinity site (Brown et al. 2008).

Substitution of Arg^{435} in domain 3 of the amino-terminal binding site and Arg^{1334} in domain 9 of the second binding site results in a dramatic loss of ligand binding activity. Earlier Dahms et al. (1993b) and Marron-Terada et al. (2000) had identified a single arginine residue in each of

the two IGF2/MPR M6P-binding sites, Arg^{435} in domain 3 and Arg^{1334} in domain 9, to be important for M6P binding by the IGF2/MPR. In comparison to CD-MPR, amino acid residues involved in carbohydrate recognition by the IGF2/MPR were studied by Hancock et al. (2002a). The three-dimensional structure of the EC region of the bovine CD-MPR complexed with M6P (Roberts et al. 1998) or pentamannosyl phosphate (Olson et al. 1999b) revealed the nature of carbohydrate recognition by the CD-MPR, and provided the framework from which to decipher the molecular basis of M6P recognition by the IGF2/MPR (Hancock et al. 2002a). Site-directed mutagenesis generated in soluble, truncated forms of the IGF2/MPR showed that residues of IGF2/MPR EC domains 3 and 9 predicted by a structure-based sequence alignment with the CD-MPR to be in the N- and C-terminal M6P-binding pockets of the IGF2/MPR, respectively, are essential for high affinity M6P binding. Pentamannosyl phosphate-agarose affinity chromatography revealed four amino acid residues (Gln-392, Ser-431, Glu-460, and Tyr-465) in domain 3 and four residues (Gln-1292, His-1329, Glu-1354, and Tyr-1360) in domain 9 as essential for carbohydrate recognition by the IGF2/MPR in addition to the previously determined Arg-435 (domain 3) and Arg-1334 (domain 9) residues. It is emphasized that the two IGF2/MPR M6P-binding sites utilize a mechanism similar to that of the CD-MPR for high affinity M6P binding and that the N- and C-terminal carbohydrate recognition domains of the IGF2/MPR are structurally similar to each other and to the CD-MPR.

A structure-based sequence alignment predicts that domain 5 contains four conserved key residues (Gln, Arg, Glu, and Tyr) identified as essential for carbohydrate recognition by CD-MPR and domains 3 and 9 of CI-MPR, but lacks two cysteine residues predicted to form a disulfide bond within the binding pocket. Reddy et al. (2004) demonstrated that the CI-MPR contains a third M6P recognition site that is located in domain 5 and that exhibits lower affinity than the carbohydrate-binding sites present in domains 1–3 and 9. While CI-MPR contains three distinct M6P binding sites located in domains 3, 5, and 9, only domain 5 exhibits a marked preference for phosphodiester-containing lysosomal enzymes. Although domain 5 contains only three of the four disulfide bonds found in the other seven domains whose structures have been determined to date, it adopts the same fold consisting of a flattened β-barrel. Structure determination of domain 5 bound to *N*-acetylglucosaminyl 6-phosphomethylmannoside, along with mutagenesis studies, revealed the residues involved in diester recognition, including Y679. These results show the mechanism by which the CI-MPR recognizes Man-P-GlcNAc-containing ligands and provides new avenues to investigate the role of phosphodiester-containing lysosomal enzymes in the biogenesis of lysosomes (Olson et al. 2010).

Recognition Sites of CI-MPR by X-ray Analysis: The crystal structure of the N-terminal 432 residues of the CI-MPR, encompassing domains 1–3, was solved in the presence of bound M6P. The structure reveals the unique architecture of this carbohydrate binding pocket and provides insight into the ability of this site to recognize a variety of mannose-containing sugars (Olson et al. 2004a). The three domains, which exhibit similar topology to each other and to the 46 kDa CD-MPR, assemble into a compact structure with the uPAR/plasminogen and the carbohydrate-binding sites situated on opposite faces of the molecule. Of the 15 IGF2R extracellular domains, domains 1–3 and 11 are known to have a conserved β-barrel structure similar to that of avidin and the CD-MPR, yet only domain 11 binds IGF2 with high specificity and affinity. The crystal structures for repeat 11 by Brown et al. (2002) and repeats 1–3 by Olson et al. (2004a, b) allowed these workers to propose different models for the overall structure of the EC domain of the IGF2R. The EC domain of the receptor shows considerable homology among repeats and the CD-MPR (16–38% identity) (Lobel et al. 1988). This high level of sequence identity accounts for structural similarities among domains, including conserved disulfide bond organization, random coil linker regions connecting the domains, and an overall core flattened β-barrel structure. The 1–3 triple-repeat crystal revealed a structure in which repeat 3 sits on top of repeats 1 and 2 (Olson et al. 2004b). Olson et al. (2004b) have proposed that the M6P/IGF2R forms distinct structural units for every three repeats of the EC region, producing five tri-repeat units that stack in a back-to-front manner. In this model, the IGF2-binding site is located on the opposite face of the structure relative to the M6P-binding sites.

Monomer Versus Oligomer: Traditionally thought to function as a monomer, the M6P/IGF2R is now considered to operate optimally in the membrane as an oligomer for high affinity M6P binding and efficient internalization of ligands (York et al. 1999; Byrd and MacDonald. 2000; Byrd et al. 2000). Intermolecular cross-linking of two M6P/IGF2R partners was shown to occur upon binding of the multivalent ligand, β-glucuronidase, resulting in increased rate of ligand internalization (York et al. 1999). The initial rate of internalization of β-glucuronidase was faster than for the monovalent ligand, IGF2, which showed that multivalent ligands enhance the rate of receptor movement, likely due to clustering of the M6P/IGF2R for improved interaction with the endocytic machinery in the formation of clathrin-coated pits (Le Borgne and Hoflack 1997, 1998; York et al. 1999). Further studies demonstrated that alignment of the M6P binding domains of monomeric partners of a receptor dimer is responsible for bivalent, high affinity binding, also supporting the importance of receptor oligomerization (Byrd and MacDonald 2000).

While studying the dimerization domain(s) of the M6P/IGF2R, Kreiling et al. (2005) hypothesized that one or more dimer interaction domains would be located at or near the ligand binding domains in the EC region of the receptor, and that these regions would contribute preferentially to receptor dimerization. It was concluded that a distinct dimerization domain for the M6P/IGF2R does not exist per se, but instead, interactions between monomeric receptor partners apparently occur all along the EC region of the receptor with special contribution made by repeat 12 (Kreiling et al. 2005).

Signal for Internalization of Bovine CI-MPR: The signal for the rapid internalization of bovine CI-MPR/IGF2R was localized to the inner half of the 163-amino acid cytoplasmic tail, including tyrosine 24 and tyrosine 26 (Lobel et al. 1989). Canfield et al. (1991) indicated that the sequence Tyr-Lys-Tyr-Ser-Lys-Val serves as the internalization signal for CI-MPR/IGF2 receptor. The crucial elements of this sequence are present in the cytoplasmic tails of a number of other membrane receptors and proteins known to undergo rapid internalization.

4.4.3 The Non-M6P-Containing Class of Ligands

The ability of the CI-MPR to interact with many different proteins and a lipophilic molecule is facilitated by the receptor's large (~2270 amino acids) extracytoplasmic region comprising 15 homologous domains, in which several distinct ligand-binding sites have been localized to individual domains. The CI-MPR interacts, in a M6P-independent fashion, on cell surface with IGF2 (Dahms et al. 1994), uPAR (Nykjaer et al. 1998), plasminogen (Godár et al. 1999), retinoic acid (Kang et al. 1997), serglycin (Lemansky et al. 2007), heparanase (Wood and Hulett 2008), latent TGFβ precursor, Granzyme B, growth factors, Herpes virus, and CD26 (Gary-Bobo et al. 2007). Limited studies on full-length (300 kDa) and truncated serum form of CI-MPR with retinoic acid indicated that the ~40 kDa C-terminal region of the receptor, which is absent in the serum form of the CI-MPR, is essential for retinoic acid binding (Kang et al. 1997). Among non-M6P-containing ligands, the interaction between CI-MPR and IGF2 has been studied extensively and as a result the CI-MPR is also referred to as the IGF2 receptor (IGF2-MPR). The IGF2 binding site has been located to domain 11 (Devi et al. 1998; Linnell et al. 2001). However, the nature of interaction between the CI-MPR and uPAR is unclear since serum form of CI-MPR binds uPAR in a M6P-dependent fashion whereas membrane-associated full-length CI-MPR binds uPAR in a M6P-independent manner (Kreiling et al. 2003). The uPAR and plasminogen binding sites have been localized to domain 1 (Leksa et al. 2002) (Fig. 4.1a). Although the interactions between the receptor and these ligands are protein–protein or protein–lipid mediated, the interaction between the CI-MPR and serglycin, a lysosomal soluble proteoglycan, appears to be carbohydrate-based via serglycin's chondroitin sulfate chains (Lemansky et al. 2007). Based on observation that domains 1–3, but not domain 9, of the CI-MPR can interact with Man-6-sulfate, it can be predicted that serglycin binds to the N-terminal region (i.e., domains 1–3) of the receptor (Marron-Terada et al. 2000).

IGF2 Binding: The underlying specificity of the interaction between IGF2 and IGF2R is not understood. IGF-I and IGF2 share >60% sequence identity, yet the IGF2R only binds IGF2 with high affinity. Residues Phe^{48}, Arg^{49}, and Ser^{50} have been implicated in IGF2R binding (Sakano et al. 1991). However, these residues are conserved between IGF-I and IGF2 and therefore would not drive the specificity of binding. Ala^{54} and Leu^{55} of IGF2 differ from the corresponding residues in IGF-I (Arg^{55}, Arg^{56}) and are important for IGF2R binding. Forbes et al. (2001) mutated residues Ala^{54} and Leu^{55} of IGF2 in the second A domain helix to arginine (found in the corresponding positions of IGF-I) and measured IGF2R binding. There is a 4- and 3.3-fold difference in dissociation constants for $Ala^{54}R$ IGF2 and $Leu^{55}R$ IGF2, respectively, and a 6.6-fold difference for $Ala^{54}R$ $Leu^{55}R$ IGF2 compared with IGF2. Hence, residues at positions 54 and 55 in IGF2 are important for and equally contribute to IGF2R binding. The IGF2-binding site has been mapped to repeat 11 of the EC region, with high affinity binding being conferred by residues contributed by the 13th repeat (Dahms et al. 1994; Garmroudi and MacDonald 1994; Devi et al. 1998).

The interaction of soluble forms of human CI-MPR/IGF2R with IGFs and mannosylated ligands was analyzed in real time. IGF2R proteins containing domains 1–15, 10–13, 11–13, or 11–12 were combined with rat CD4 domains 3 and 4. Results suggest that domain 13 acts as an enhancer of IGF2 affinity by slowing the rate of dissociation, but additional enhancement by domains other than 10–13 also occurs (Linnell et al. 2001). Structural analyses of repeat 11 identified the putative IGF2-binding site in a hydrophobic pocket at the end of a β-barrel structure (Brown et al. 2002).

In addition, IGF2 residues in the non-conserved C domain are important for IGF2R binding and play a role in conferring the specificity of binding (Roche et al. 2006). However, the effects of mutation on residue Ala^{54} or Leu^{55} or the C domain are insufficient to account for the complete lack of binding by IGF-I, suggesting that another determinant provides the IGF2 specificity. In addition to Thr^{16} as the major determinant on IGF2 responsible for the specificity of IGF2R binding, Delaine et al. (2007) revealed a hydrophobic

patch critical for IGF2R binding, which encompasses residues Phe^{19} and Leu^{53} and includes Thr^{16} and Asp^{52}. Thr^{16} was identified as playing a major role in determining why IGF2, but not IGF-I, binds with high affinity to the IGF2R. In nut-shell, a binding surface on IGF2, important for binding to domain 11 of the IGF2R, is achieved predominantly through hydrophobic interactions (Delaine et al. 2007). The structure of the IGF2R·IGF2 complex has not been solved.

Urokinase-Type Plasminogen Activator Receptor and Plasminogen: The other members of the non-M6P-containing ligands are urokinase-type plasminogen activator receptor (uPAR) and plasminogen, whose binding sites have been mapped to a peptide region within EC repeat 1 (Godár et al. 1999; Leksa et al. 2002). The uPAR plays an important role on the cell surface in mediating extracellular degradative processes and formation of active TGF-β, and in nonproteolytic events such as cell adhesion, migration, and transmembrane signaling. The uPAR binds to CI-MPR with an affinity in low μM range, but not to CD-MPR. The binding is not perturbed by uPA and appears to involve domains DII + DIII of the uPAR protein moiety. The binding occurs at site(s) on the CI-MPR different from those engaged in binding of M6P epitopes or IGF2 (Godár et al. 1999; Leksa et al. 2002; Nykjaer et al. 1998). The uPAR-M6P/IGF2R interaction appears to be weak, of low affinity, and confined to a small subpopulation of uPAR molecules (Kreiling et al. 2003), which calls into question the physiological relevance of this interaction.

Interactions with Other Proteins: The CI-MPR/IGF2R has been observed to bind to soluble forms of glycosyl-phosphatidylinositol-linked molecules, one of mammalian origin (rat Thy-1) and two of protozoan origins. Of the two phosphate groups found on the soluble forms of the protozoan glycosyl-phosphatidylinositol-linked molecules: (1) the internal M6P diester (which forms a part of the ethanolamine bridge) and (2) the inositol-1,2 cyclic phosphate group (which arises after cleavage of the membrane associated form with phosphatidylinositol-specific PLC); only the former appears to be recognized by CI-MPR/IGF2R (Green et al. 1995). Human hDNase binds to immobilized CD-MPR, with the strongest binding exhibited by the protein bearing diphosphorylated oligosaccharides (Cacia et al. 1998). CI-MPR interacts with cubilin and megalin, the renal apical brush-border membrane (BBM) endocytic receptors (Yammani et al. 2002). The proliferin secreted by cultured cell binds to CI-MPRs and therefore may be a lysosomal protein targeted to lysosomes. The activity of MPRs in murine fetal and maternal liver and in placenta is regulated during pregnancy (Lee and Nathans 1988). No specific receptor capable of binding α-L-fucosidase independent of M6P was demonstrable, despite published results that support the existence of a M6P independent trafficking mechanism in lymphoid cells for this enzyme (Dicioccio and Miller 1992). Retinoic acid, a unique ligand for the M6P/IGF2R, binds the cytoplasmic region and is thought to function by altering intracellular trafficking of the M6P/IGF2R and its cargo (Kang et al. 1997).

Observations indicate the role for CI-MPR in the adherence and invasion of *L. monocytogenes* of mammalian cells, perhaps in combination with known mechanisms (Gasanov et al. 2006). Mordue and Sibley (1997) characterized the intracellular fate of Toxoplasma in bone marrow-derived macrophages following two modes of uptake: phagocytosis versus active invasion. It appeared that Toxoplasma evades endocytic processing due to an absence of host regulatory proteins including CI-MPR necessary to drive endocytic fusion. This divergence from normal maturation occurs during the formation of primary vacuole. Varicella-zoster virus (VZV) remains a public health issue around the globe. The CI-MPR is critical to both entry and egress of enveloped VZV and in pathogenesis of varicella (Hambleton 2005). Entry into cells and transmission between cells of herpes simplex virus can be facilitated by IGF2R binding of M6P-modified herpes simplex viral glycoprotein D (Brunetti et al. 1995, 1998; Chen et al. 2004)

4.5 Complementary Functions of Two MPRS

Lysosomal membrane proteins and soluble lysosomal proteins are transported from the trans-Golgi network (TGN) to endosomes and lysosomes via coated-vesicles, which bud from the donor compartment and are transported to and fuse with the proper acceptor compartment. The MPRs, while transporting lysosomal enzymes as cargo to endosomal compartments, are recognized by the Golgi-localized, γ-ear-containing, ARF-binding proteins (GGAs) family of clathrin adaptor proteins and accumulate in forming clathrin-coated vesicles (CCV) (Ghosh et al. 2003b; Ghosh and Kornfeld 2004). They are trafficked to the early endosome where, in the relatively low pH environment of the endosome, the MPRs release their cargo. The MPRs are recycled back to the Golgi by way of interaction with Golgi-localized, γ-ear-containing, GGAs and vesicles. The cargo proteins are then trafficked to the lysosome via late endosome independently of the MPRs. The proteins forming the vesicle-coat bind to the cytoplasmic domains of the cargo proteins and recruit additional proteins like clathrin to the site of vesicle formation. These proteins are hence called adaptor proteins (AP) or adaptor-protein complexes and their subunits are called adaptins. The family of heterotetrameric adaptor-protein complexes consists of AP-1, AP-2, AP-3 and AP-4 and all four are required for lysosome biogenesis. They are ubiquitously expressed in mammals and

many of the adaptins also exist as tissue-specific isoforms encoded by different genes or generated by alternative splicing. Adaptor-protein complexes are compartment specific proteins and recruit their specific accessory proteins to the site of vesicle formation, which is believed to regulate vesicle budding and fission and vesicle transport (Fig. 3.4; Chap. 3) (Schu 2005). Endocytosed proteins destined for degradation in lysosomes are targeted mainly to early endosomes following uptake. Late endosomes are the major site for entry of newly synthesized lysosomal hydrolases via the CI-MPR into the degradative pathway. Immunoelectron microscopic data support a model in which early endosomes gradually mature into late endosomes (Stoorvogel et al. 1991). Runquist and Havel (1991) examined the distribution of CI-MPR in early and late endosomes and a receptor-recycling fraction isolated from livers of estradiol-treated rats.

4.5.1 Why Two MPRs

Two MPRs have been implicated in the M6P-dependent transport of lysosomal enzymes to lysosomes. It is not known why two different MPRs are present in most cell types. Ludwig et al. (1994), while studying targeting of lysosomal enzymes in mice that lack either or both MPRs, demonstrated that both receptors are required for efficient intracellular targeting of lysosomal enzymes. More importantly, comparison of phosphorylated proteins secreted by different cell types indicated that the two receptors may interact in vivo with different subgroups of hydrolases. This observation may provide a rational explanation for the existence of two distinct MPRs in mammalian cells (Ludwig et al. 1994). The fibroblast cell lines lacking either the CI-MPR or the CD-MPR, partially missort phosphorylated lysosomal enzymes and secrete, in a large part, different phosphorylated ligands. The analysis of the phosphorylated oligosaccharides showed that the ligands missorted in the absence of CD-MPR were slightly but significantly depleted in oligosaccharides with two M6P residues, when compared with those missorted in the absence of CI-MPR. While these results could explain some differences between the structure and the sorting function of the two MPRs, they strongly suggest the reason why cells express two different but related MPRs (Munier-Lehmann et al. 1996a, b).

Despite extensive investigation, the relative roles and specialized functions of each MPR in targeting of specific proteins remain questions of fundamental interest. One possibility is that most M6P-glycoproteins are transported by both MPRs, but there may be subsets that are preferentially transported by each. Proteomic approach of serum from mice lacking either of the MPRs revealed a number of proteins that appear specifically elevated in serum from each MPR-deficient mouse. M6P-glycoforms of cellular repressor of E1A-stimulated genes, tripeptidyl peptidase I, and heparanase were elevated in absence of the CD-MPR and M6P glycoforms of α-mannosidase B1, cathepsin D, and prosaposin were elevated in the absence of the CI-MPR. Qian et al. (2008) suggest that cellular targeting appears to be MPR-selective under physiological conditions. Mammalian CI-MPR also mediates endocytosis and clearance of IGF2. Mutant mice that lack the CD-MPR are viable. Mice that lack the CI-MPR accumulate high levels of IGF2 and usually die perinatally, whereas mice that lack both IGF2 and CI-MPR are viable. Thus, while lack of the CI-MPR appears to perturb lysosome function to a greater degree than lack of the CD-MPR, each MPR has distinct functions for the targeting of lysosomal enzymes in vivo (Sohar et al. 1998). Additionally the receptors mediate the secretion (CD-MPR) and the endocytosis (CI-MPR) of lysosomal enzymes. However, trafficking of acid hydrolases is only part of the story. Evidence is emerging that one of the receptors can regulate cell growth and motility, and that it functions as a tumor suppressor (Ghosh et al. 2003a).

Basolateral endocytic pathway converges with the autophagic pathway after the early endosome in pancreas. Tooze et al. (1990) identified three distinct classes of autophagic compartments, which were referred to as phagophores, Type I autophagic vacuoles, and Type II autophagic vacuoles. Phagophores, the earliest autophagic compartment, contained no CI-MPR and cathepsin D. Where as type I autophagic vacuoles contained only very low levels of cathepsin D and CI-MPR, type II autophagic vacuoles by contrast were enriched for lysosomal enzymes, and also enriched for CI-MPR. The lysosomal enzymes present in Type II autophagic vacuoles carry M6P monoester residues. Thus, type II autophagic vacuoles are a prelysosomal compartment in which the combined endocytic and autophagic pathways meet the delivery pathway of lysosomal enzymes. Studies in kidney on the intracellular polarity of the CI-MPR showed that the CI-MPR was present in basolateral early endosomes and in late endosomes but absent from apical early endosomes (Parton et al. 1989; Prydz et al. 1990).

Prelysosomal Endosome Compartment: Cells contain an intracellular compartment that serves as both the "prelysosomal" delivery site for newly synthesized lysosomal enzymes by the MPR and as a station along the endocytic pathway to lysosomes. The MPR structures are prelysosomes involved in lysosomal enzyme targeting in rat cardiac myocytes (Marjomäki et al. 1990). Cell fractionation studies indicated that prelysosomal endosome compartment (PLC) is the site of confluence of the endocytic and biosynthetic pathways to lysosomes (Park et al. 1991).

In NRK cells 90% of the labelling for the receptor was found in the PLC, with the rest distributed over the other

compartments. The PLC is the first structure along the endocytic pathway that gives a significant reaction for acid phosphatase. However, the PLC is clearly distinct from the MPR-negative lysosomes, which are also acid phosphatase-positive (Griffiths et al. 1990b).

The cytoplasmic tail of CI-MPR plays an important role in the receptor trafficking, while the role of the luminal domain is controversial. It was noticed that the peripheral distribution of GFP, fused to the transmembrane and cytoplasmic domains of CI-MPR (G-CI-MPR-tail), was distinct from that of endogenous CI-MPR or of GFP fused to the full-length CI-MPR (G-CI-MPR-full). The CI-MPR luminal domain appeared to be required for tight interaction with endocytic compartments, and retention by them, and that there are additional transport steps, in which the binding to M6P-ligands is involved (Waguri et al. 2006).

Rate of Internalization of the IGF2R: The CI-MPR/IGF2R undergoes constitutive endocytosis, mediating the internalization of two unrelated classes of ligands: M6P-containing acid hydrolases and IGF2. To determine the role of ligand valency in CI-MPR/IGF2receptor-mediated endocytosis, the internalization rates of two ligands, β-glucuronidase (a homotetramer bearing multiple M6P moieties) and IGF2 were measured. The β-glucuronidase entered the cell three- to fourfold faster than IGF2. Purified IGF2R was present as a monomer, but its association with β-glucuronidase generated a complex composed of two receptors and one β-glucuronidase. Neither IGF2 nor the synthetic peptide induced receptor dimerization. It was indicated that intermolecular cross-linking of the IGF2R receptor occurs upon binding of a multivalent ligand, resulting in an increased rate of internalization (York et al. 1999).

M6P-Independent Pathways: The CI-MPR is known to play a role in endocytic uptake of granzyme B, and cells lacking MPR are considered poor targets for CTL that mediate allograft rejection or tumor immune surveillance. However, Trapani et al. (2003) suggest that the uptake of granzyme B into target cells is independent of MPR. Contrary to previous findings, mouse tumor allografts that lack MPR expression were rejected as rapidly as tumors that over-express MPR. Entry of granzyme B into target cells and its intracellular trafficking to induce target cell death in the presence of perforin are therefore not critically dependent on MPR or clathrin/dynamin-dependent endocytosis (Trapani et al. 2003). The β-glucocerebrosi- dase, the enzyme defective in Gaucher disease, is targeted to the lysosome independently of M6PR. Reports suggest a role for LIMP-2, a supporting protein for M6P as the M6P-independent trafficking receptor for β-glucocerebrosidase (Reczek et al. 2007).

4.5.2 Cell Signaling Pathways

The rat IGF2R develops transmembrane signaling by directly coupling to a G protein having a 40-kDa α subunit, Gi-2. By using vesicles reconstituted with the clonal human CI-MPR and G proteins, Murayama et al. (1990) indicated that the CI-MPR could stimulate guanosine 5′-O-(3-thiotriphosphate) (GTPγS) binding and GTPase activities of Gi proteins in response to IGF2. Results suggest that the human CI-MPR has two distinct signaling functions that positively or negatively regulate the activity of Gi-2 in response to the binding of IGF2 or M6P. The PKA type II seems to be associated with the membranes of precisely those subcellular compartments that are active in endocytosis and recycling of cell surface receptors. It appears to be related to the well-established role of cyclic AMP in signal transduction. It was proposed that activation of PKA in endocytic compartments may contribute to regulation (via phosphorylation) of the subcellular distribution of internalized surface receptors or their functional coupling to effector systems involved in signal transduction (Griffiths et al. 1990a).

4.6 Functions of CI-MPR

The mammalian IGF2R/CI-MPR (IGF2R) has multiple functions due to its ability to interact with a wide variety of ligands. (1) The IGF2R plays a major role in targeting M6P-labeled proteins via TGN and from the cellular membrane to lysosomes for degradation (Ghosh et al. 2003a, b). (2) It binds and internalizes IGF2, thereby maintaining correct levels of IGF2 locally and in the circulation. These functions are central in the processes of embryonic development and normal growth. (3) Circulating levels of the leukemia inhibitory factor are modulated via IGF2R-mediated endocytosis and targeting of this M6P-containing protein for degradation in the lysosomes (Blanchard et al. 1999). (4) Entry into cells and transmission between cells of herpes simplex virus can be facilitated by IGF2R binding of M6P-modified herpes simplex viral glycoprotein D (Brunetti et al. 1994; Brunetti et al. 1995, 1998). (5) The IGF2R also functions as a death receptor by mediating uptake of mannose 6-phosphorylated granzyme B, a serine proteinase that is essential for the rapid induction of target cell apoptosis by cytotoxic T cells (Motyka et al. 2000). (6) Further more, internalization of the M6P-containing T cell activation

antigen, CD26, by the IGF2R plays an important role in CD26-mediated T cell costimulation (Ikushima et al. 2000). (7) The ability of the IGF2R to recognize with high affinity the M6P signal found on many functionally distinct ligands underscores the importance of the IGF2R and its involvement in a myriad of essential physiological pathways (Hancock et al. 2002b). (8) In addition, the IGF2R may also mediate transmembrane signal transduction in response to IGF2 binding under certain conditions. CI-MPR/IGF2R may also be functional in terms of supporting cell adhesion and proliferation of myeloma cells (Nishiura et al. 1996).

CI-MPR Blocks Apoptosis Induced by HSV 1 Mutants Lacking Glycoprotein D

The herpes simplex virus (HSV) glycoprotein D (gD) is essential for HSV entry into cells, it contains M6P on its Asn-linked oligosaccharides, and binds to CI-MPR. It was supposed that the interaction between gD and CI-MPR could play a role in some aspect of virus entry or egress (Brunetti et al.1994). The HSV1 mutants lacking the gene encoding glycoprotein D (gD) and the gD normally present in the envelope of the virus ($gD^{-/-}$ stocks) or mutants lacking the gD gene but containing trans-induced gD in their envelopes ($gD^{-/+}$) cause apoptosis in human SK-N-SH cells. The CI-MPR blocks apoptosis induced by HSV1 mutants lacking gD and is likely the target of antiapoptotic activity of glycoprotein. This conclusion is consistent with published reports that phosphorylated gD interacts with CI-MPR (Zhou and Roizman 2002). The domains of glycoprotein D required to block apoptosis induced by herpes simplex virus 1 are largely distinct from those involved in cell-cell fusion and binding to nectin1 (Zhou et al. 2003)

CI-MPR/IGF2R is a Death Receptor for Granzyme B During Cytotoxic T Cell-Induced Apoptosis

CTL and NK cells destroy target cells via directed exocytosis of lytic effector molecules such as perforin and granzymes. Whether the endocytic mechanism is nonspecific or is dependent on the CI-MPR is not certain since further experiments with Granzyme B (GzmB) indicated this was not essential. The GzmB, a serine protease of CTLs and NK cells, induces apoptosis by caspase activation after crossing the plasma membrane of target cells. The GzmB can enter cells by endocytosis and in a perforin-independent manner through CI-MPR/IGF2R. Inhibition of the GzmB-CI-MPR interaction prevented GzmB cell surface binding, uptake, and the induction of apoptosis. Significantly, expression of the CI-MPR was essential for cytotoxic T cell-mediated apoptosis of target cells in vitro and for the rejection of allogeneic cells in vivo (Motyka et al. 2000). Since granzyme B must access the substrates within the cell, the endocytosis through CI-MPR binding is a key step in the granule-mediated killing process (Veugelers et al. 2006).

Kun et al. (2008) investigated the effects of ω-3 polyunsaturated fatty acids on apoptosis and granzyme B, perforin, and CI-MPR expression of intestinal epithelial cells of chronic rejection after small intestinal transplantation. ω-3 polyunsaturated fatty acids can suppress the rejection to mucosal cells of allograft at the time of chronic rejection in small intestinal transplantation, which may be significant in increasing the surviving rate of allograft, delaying the chronic dysfunction, and prolonging the lifetime of both allograft and acceptor.

Loss of CI-MPR Expression Promotes the Accumulation of Lysobisphosphatidic Acid

The importance of lipid domains within endocytic organelles in the sorting and movement of integral membrane proteins has been highlighted. The role of the unusual phospholipid lysobisphosphatidic acid (LBPA), which appears to be involved in the trafficking of cholesterol and glycosphingolipids, and accumulates in a number of lysosomal storage disorders, has received attention. Disruption of LBPA function leads to mis-sorting of CI-MPRs. The converse is also true, since spontaneous loss of CI-MPRs from a rat fibroblast cell line led to the formation of aberrant late endocytic structures enriched in LBPA. Accumulation of LBPA was directly dependent upon the loss of the MPRs, and could be reversed by expression of bovine CI-MBP in the mutant cell line. Therefore, loss of such a protein may cause the accumulation of abarrant organelles. It appears that the formation of inclusion bodies in many lysosomal storage diseases is also due to an imbalance in membrane trafficking within the endocytic pathway (Reaves et al. 2000).

Hepatocyte is Direct Target for TGFβ Activation Via the IGF2R/CI-MPR

The activation of latent proTGFβ normally seen in cocultures of bovine aortic endothelial and bovine smooth muscle cells can be inhibited by coculturing the cells with either M6P or antibodies directed against CI-MPR. The binding to the CI-MPR/IFE2R appears to be a requirement for activation of proTGFβ (Dennis and Rifkin 1991). The CI-MPR, over-expressed in hepatocytes during liver regeneration, has been implicated in the maturation of latent proTGFβ. It appears that: (1) the induction of the CI-MPR gene during liver regeneration and hepatocyte culture occurs in mid G1 phase; and (2) the CI-MPR mediates latent proTGFβ activation and thus may act, by targeting TGFβ to hepatocytes, as a negative regulator of hepatocyte growth (Villevalois-Cam et al. 2003).

Regulation of MPRs During the Myogenic Development of C2 cells

The CD-MPR lacks an IGF2-binding site and participates only in the intracellular trafficking of lysosomal enzymes. During terminal differentiation of the myogenic C2 cell line there is

an increase in cell surface expression of the IGF2/CI-MPR in parallel with a rise in secretion of IGF2. IGF2/CI-MPR mRNA increased by more than 10-fold during the initial 48 h of C2 muscle differentiation, similar to the rise in IGF2 mRNA. Comparable levels of both mRNAs are expressed in C2 myotubes and in primary cultures of fetal muscle. The differential regulation of the two M6P receptors during muscle differentiation suggests that they may serve distinct functions in development (Szebenyi and Rotwein 1991).

CI-MPR Binds Heparanase to Promote Extracellular Matrix Degradation

Heparanase, a β-D-endoglucuronidase, cleaves heparan sulfate, a structural component of ECM and vascular basement membrane (BM). The cleavage of heparan sulfate by heparanase-expressing cells facilitates degradation of the ECM/BM to promote cell invasion associated with inflammation, tumor metastasis, and angiogenesis. Heparanase has also been shown to act as a cell adhesion and/or signaling molecule upon interaction with cell surfaces. Three hundred-kDa CI-MPR is a cell surface receptor for heparanase. Furthermore, the tethering of heparanase to the surface of cells via CI-MPR was found to increase their capacity to degrade an ECM or a reconstituted BM. These results indicate a role for CI-MPR in the cell surface presentation of enzymatically active heparanase for the efficient passage of T cells into an inflammatory site and have implications for the use of this mechanism by other cell types to enhance cell invasion (Wood and Hulett 2008).

4.7 Proteins Associated with Trafficking of CI-MPR

4.7.1 Adaptor Protein Complexes

The AP-1 and AP-2 complexes are the most abundant adaptors in CCVs, but clathrin-mediated trafficking can still occur in the absence of any detectable AP-1 or AP-2. Results indicate that three proteins: CI-MPR, carboxypeptidase D (CPD) and low-density lipoprotein receptor-related protein 1 (LRP1) have AP-dependent sorting signals, which may help to explain the relative abundance of AP complexes in CCVs (Harasaki et al. 2005).

Four Binding Sites in CI-MPR for AP-1: The trafficking of CI-MPR between the TGN and endosomes requires binding of sorting determinants in the cytoplasmic tail of the receptor to adaptor protein complex-1 (AP-1). A GST pull-down binding assay identified four binding motifs in the cytoplasmic tailof CI-MPR: a tyrosine-based motif ^{26}YSKV^{29}, an internal dileucine-based motif ^{39}ETEWLM^{44}, and two casein kinase 2 sites ^{84}DSEDE^{88} and ^{154}DDSDED^{159}. The YSKV motif mediated the strongest interaction with AP-1 and the two CK2 motifs bound AP-1 only when they were phosphorylated. The COOH-terminal dileucines were not required for interaction with AP-1 (Ghosh and Kornfeld 2004).

AP-3 Adaptor Complex Defines a Novel Endosomal Exit Site: The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins (Le Borgne et al. 1998)**.** Electron microscopy showed that AP-3 is associated with budding profiles evolving from a tubular endosomal compartment that also exhibits budding profiles positive for AP-1. AP-3 colocalizes with clathrin, but to a lesser extent than does AP-1. The AP-3- and AP-1-bearing tubular compartments contain low amounts of the CI-MPR and the lysosome-associated membrane proteins (LAMPs) 1 and 2. Chapuy et al. (2008) showed that sorting of TRP-1 and CD-MPR was AP-1 dependent, while budding of tyrosinase and Lamp-1 required AP-3. Depletion of clathrin inhibited sorting of all four cargo proteins, suggesting that AP-1 and AP-3 are involved in the formation of distinct types of CCVs, each of which is characterized by the incorporation of specific cargo membrane proteins.

AP-4 as a Component of the Clathrin Coat Machinery: AP-4 is localized mainly in the Golgi complex, as well as on endosomes and transport vesicles. Interestingly, AP-4 is localized with the clathrin coat machinery in the Golgi complex and in the endocytic pathway. Moreover, AP-4 is localized with the CI-MPR, but not with the transferrin receptor, LAMP-2 (lysosomal-associated membrane protein-2) or invariant chain. The difference in morphology between CI-MPR/AP-4-positive vesicles and CI-MPR/AP-1-positive vesicles raises the possibility that AP-4 acts at a location different from that of AP-1 in the intracellular trafficking pathway of CI-MPR (Barois and Bakke 2005).

Serines 2421 and 2492 Are the Targets of a CK II Associated to the Golgi-Derived HAI Adaptor Complex: A kinase activity of bovine brain CCV phosphorylates the bovine CI-MPR with high efficiency (Km ~ 50–100 nM). The kinase is part of the coat of Golgi-derived CCV. The kinase is associated to the 47-kDa subunit of the complex and exhibits properties similar to a casein kinase II. This posttranslational modification occurs on serines 2421 and 2492 of bovine CI-MPR precursor, residues which are located in typical casein-kinase II recognition sequences. The same serines are phosphorylated in vivo (Méresse et al. 1990). The phosphorylation of two serines in the CI-MPR cytoplasmic domain is associated with a single step of transport of its recycling pathways and occurs when this receptor is in the TGN and/or has left this compartment via clathrin-coated vesicles (Méresse and Hoflack 1993).

4.7.2 Mammalian TGN Golgins

Four mammalian golgins are specifically targeted to the TGN membranes via their C-terminal GRIP domains. The TGN golgins, p230/golgin-245 and golgin-97, are recruited via the GTPase Arl1, whereas the TGN golgin GCC185 is recruited independently of Arl1. Of the four TGN golgins, p230/golgin-245, golgin-97, GCC185, and GCC88, GCC88 defines a retrograde transport pathway from early endosomes (EE) to the TGN. Derby et al. (2007) suggest a dual role for the GCC185 golgin in the regulation of endosome-to-TGN membrane transport and in the organization of the Golgi apparatus.

The maintenance of MPRs that cycle between the TGN and endosomes requires the function of the mammalian Golgi-associated retrograde protein (GARP) complex. Depletion of any of the three GARP subunits, Vps52, Vps53, or Vps54, by RNAi impairs sorting of the precursor of the cathepsin D, to lysosomes and leads to its secretion into the culture medium. As a consequence, lysosomes become swollen, likely due to a buildup of undegraded materials. Results indicate that the mammalian GARP complex plays a general role in the delivery of retrograde cargo into the TGN. The Vps54 mutant protein in the Wobbler mouse strain being active in retrograde transport explains the viability of these mutant mice (Pérez-Victoria et al. 2008).

4.7.3 TIP47: A Cargo Selection Device for MPR Trafficking

MPRs transport newly synthesized lysosomal hydrolases from Golgi to prelysosomes and then return to the Golgi for another round of transport. A 47 kDa protein (TIP47 = tail-interacting protein of 47 kDa) that binds selectively to the cytoplasmic domains of CI-MPR and CD-MPR is present in cytosol and on endosomes and is required for MPR transport from endosomes to the TGN in vitro and in vivo. TIP47 recognizes a phenylalanine/tryptophan signal in the tail of the CD- MPR that is essential for its proper sorting within the endosomal pathway. Thus, TIP47 binds MPR cytoplasmic domains and facilitates their collection into transport vesicles destined for the Golgi (Díaz and Pfeffer 1998). Recombinant TIP47 binds more tightly to the CI-MPR (K_D = 1 μm) than to the CD-MPR (K_D = 3 μm). In addition, TIP47 fails to interact with the cytoplasmic domains of TGN38 and other proteins that are also transported from endosomes to the TGN. Thus, TIP47 recognizes a very select set of cargo molecules (Krise et al. 2000).

Recognition of the CI-MPR by TIP47: TIP47 interaction with the 163-residue CI-MPR cytoplasmic domain is conformation dependent and requires CI-MPR residues that are proximal to the membrane. CI-MPR cytoplasmic domain residues 1–47 are dispensable, whereas residues 48–74 are essential for high-affinity binding. However, residues 48–74 are not sufficient for high-affinity binding; residues 75–163 alone display weak affinity for TIP47, yet they contribute to the presentation of residues 48–74 in the intact protein. TIP47 binding is competed by the binding of the AP-2-clathrin adaptor at (and near) residues 24–29 but not by AP-1 binding at (and near) residues 160–161. Finally, TIP47 appears to recognize a putative loop generated by the sequence PPAPRPG and other hydrophobic residues in the membrane-proximal domain. These data provide structural basis for TIP47-CI-MPR association (Orsel et al. 2000). The MPRs, transported from endosomes to the TGN, require Rab9 and the cargo adaptor TIP4. The CI-MPRs are enriched in the Rab9 domain relative to the Rab7 domain. TIP47 is likely to be present in this domain because a TIP47 mutant disrupted endosome morphology and sequestered MPRs intracellularly.

4.7.4 Sorting Signals in GGA and MPRs at TGN

CI-MPR takes a complex route that involves multiple sorting steps in both early and late endosomes (Lin et al. 2004). Specific sorting signals direct transmembrane proteins to the compartments of the endosomal-lysosomal system. The Golgi-localizing, γ-adaptin ear homology domain, ADP ribosylation factor (Arf)-interacting (GGA) proteins constitute a family of clathrin adaptors that are mainly associated with the TGN and mediate the sorting of MPRs. MPRs and ADP-ribosylation factors cooperate for high affinity interaction of the AP-1 golgi assembly proteins with membranes (Le Borgne et al. 1996). Three mammalian GGAs, GGA1, 2, and 3 have been implicated in the sorting of MPR. This sorting is dependent on the interaction of the N-terminal of VHS (VPS-27, Hrs, and STAM) domain of the GGAs with acidic-cluster-dileucine (ACLL) signals in the cytosolic tails of the receptors msuch as the CI- and CD-MPRs. The GGA proteins have modular structures with an N-terminal VHS domain followed by a GAT (GGA and TOM1) domain, a connecting hinge segment, and a C-terminal GAE (γ-adaptin ear) domain.

Cytoplasmic Domain of the CI-MPR Contains Different Signals for Rapid Endocytosis: Mutant receptors with 40 and 89 residues deleted from the carboxyl terminus of the cytoplasmic tail functioned normally in endocytosis, but were partially impaired in sorting. Mutant receptors with larger deletions leaving only 7 and 20 residues of the cytoplasmic tail were defective in endocytosis and sorting.

A mutant receptor containing alanine instead of tyrosine residues at positions 24 and 26 was defective in endocytosis, and partially impaired in sorting. Receptors deficient in endocytosis accumulated at the cell surface. Results indicate that the cytoplasmic domain of the Cl-MPR contains different signals for rapid endocytosis and efficient lysosomal enzyme sorting (Lobel et al. 1989). The chimeric CI-MPRs containing bovine extracytoplasmic domain and the human or mouse trans-membrane and cytoplasmic domains function identically to the bovine receptor, thus demonstrating that sorting signals are conserved. Analysis of a series of truncation and alanine scanning mutants revealed that a consensus casein kinase II site followed by 2 leucines near the COOH terminus that has the sequence D^{10}DSDEDLL^{3} is important for receptor function in sorting of lysosomal enzymes (Chen et al. 1993).

Golgi-Localized, γ-ear-Containing, Arf-Binding Proteins (GGAs) Mediate Sorting of the MPRs at the TGN: The cytosolic tails of both receptors contain acidic-cluster-dileucine signals that direct sorting from the trans-Golgi network (TGN) to the endosomal-lysosomal system. The lysosomal trafficking of the M6P-receptor and sortilin require that the GGAs be recruited to Golgi membranes where they bind in the cytosolic tail of the receptors and recruit clathrin to form trafficking vesicles. GGA recruitment to membranes requires Arf1, a protein that cycle between a GDP-bound inactive state and GTP-bound active state. The guanine nucleotide exchange factors (GEFs) promote the formation of Arf-GTP, while the GTPase activating proteins induce hydrolysis of GTP to GDP. Evidences indicate that the GEF, GBF1, colocalizes with the GGAs and interacts with the GGAs. Depletion of GBF1 or expression of an inactive mutant prevents recruitment of the GGAs to Golgi membranes and results in the improper sorting of cargo. In short, GBF1 is required for GGA recruitment to Golgi membranes and plays a role in the proper processing and sorting of lysosomal cargo. The receptors and the GGAs leave the TGN on the same tubulo-vesicular carriers. A dominant-negative GGA mutant blocking the exit of the receptors from the TGN indicated that the GGAs mediate sorting of the M6P-receptors at the TGN (Lefrançois and McCormick 2007; Puertollano et al. 2001). Ghosh et al. (2003) indicated that the three mammalian GGAs cooperate to sort cargo and are required for maintenance of TGN structure.

Acidic Cluster/Dileucine Motif (ACLL) of MPRs in Lysosomal Enzyme Sorting: During endocytic trafficking of CI-MPR, a cluster of acidic amino acids followed by a dileucine (ACLL) motif in the cytoplasmic tail has been proposed to mediate receptor sorting from TGN to late endosomes. Mutations in this motif impair lysosomal enzyme sorting by preventing association of CI-MPR with coat proteins. Alanine cluster mutagenesis demonstrates that the major sorting determinant is a conserved casein kinase II site followed by a dileucine motif (D^{157}DSDEDLL^{164}). Small deletions or additions outside this region have severe to mild effects, indicating that context is important. Single residue mutagenesis indicates that cycles of serine phosphorylation/dephosphorylation are not obligatory for sorting. In addition, the two leucine residues and four of the five negatively charged residues can readily tolerate conservative substitutions. In contrast, aspartate 160 could not tolerate isoelectric or isosteric substitutions, implicating it as a critical component of the sorting signal. Thus, the ACLL motif of CI-MPR is critical for receptor sorting at early stages of intracellular transport following endocytosis (Chen et al. 1997; Tortorella et al. 2007).

The GGA1 binds to CD-MPR in the TGN and targets the receptor to clathrin-coated pits for transport from the TGN to endosomes. The motif of the CD-MPR that interacts with GGA1 was shown to be DXXLL. However, reports on increased affinity of cargo, when phosphorylated by CK2, to GGAs created interest on the effect of the CD-MPR CK2 site on binding to GGA1. It seems that Glu58 and Glu59 of the CK2 site are essential for high affinity GGA1 binding in vitro, whereas the phosphorylation of Ser57 of the CD-MPR has no influence on receptor binding to GGA1. In vivo interaction between GGA1 and CD-MPR was abolished when all residues, namely, Glu58, Glu59, Asp61, Leu64, and Leu65, involved in GGA1 binding were mutated. In contrast, AP-1 binding to CD-MPR required all the glutamates (namely, Glu55, Glu56, Glu58, and Glu59) that surround the phosphorylation site, but like GGA1 binding, was independent of the phosphorylation of Ser57 (Stöckli et al. 2004).

Autoinhibition of the Ligand-Binding Site of GGA1/3 VHS Domains by an Internal ACLL Motif: Isolated VHS domains bind specifically to ACLL motifs present in the cytoplasmic tails of the MPRs. Full-length cytoplasmic GGA1 and GGA3 but not GGA2 bind the CI-MPR very poorly because of autoinhibition. The inhibition depends on the phosphorylation of a serine located three residues upstream of the ACLL motif. Substitution of the GGA1 inhibitory sequence into the analogous location in GGA2, which lacks the ACLL motif, results in autoinhibition of the latter protein. Results indicate that the activity of GGA1 and GGA3 is regulated by cycles of phosphorylation/dephosphorylation (Doray et al. 2002). Crystallographic analyses demonstrated that the phosphoserine residue interacts electrostatically with two basic residues on the VHS domain of GGA3, thus providing an additional point of attachment of the ACLL signal to its recognition module (Kato et al. 2002).

PACS-1, GGA3 and CK2 Complex Regulates CI-MPR Trafficking: Cycling of CI-MPR between the TGN and early endosomes is mediated by GGA3, which directs TGN export, and PACS-1, which directs endosome-to-TGN retrieval. The acidic cluster on PACS-1, similar to acidic cluster motifs on cargo proteins, acts as an autoregulatory domain that controls PACS-1-directed sorting. Despite executing opposing sorting steps, GGA3 and PACS-1 bind to an overlapping CI-MPR trafficking motif and their sorting activity is controlled by the CK2 phosphorylation of their respective autoregulatory domains. Study suggests a CK2-controlled cascade regulating hydrolase trafficking and sorting of itinerant proteins in the TGN/endosomal system (Scott et al. 2003, 2006). The PACS-1 connects the clathrin adaptor AP-1 to acidic cluster sorting motifs contained in the cytoplasmic domain of cargo proteins such as furin and CI-MPR and in viral proteins such as HIV-1 Nef. The phosphorylation state of an autoregulatory domain controls PACS-1-directed protein traffic

VHS Domain of GGA2 Binds to Acidic Cluster-Dileucine Motif in the Cytoplasmic Tail of CI-MPR: Structural Basis for Sorting-Signal Recognition: Shiba et al. (2002) reported the X-ray structure of the GGA1 VHS domain alone, and in complex with the carboxy-terminal peptide of CI-MPRs containing an ACLL sequence. The VHS domain forms a super helix with eight α-helices, similar to the VHS domains of TOM1 and Hrs. Unidirectional movements of helices α6 and α8, and some of their side chains, create a set of electrostatic and hydrophobic interactions for correct recognition of the ACLL peptide. This recognition mechanism provides the basis for regulation of protein transport from the TGN to endosomes/lysosomes, which is shared by sortilin and low-density lipoprotein receptor-related protein (Shiba et al. 2002).

The VHS domain of GGA2 was shown to bind to the acidic cluster-dileucine motif in the cytoplasmic tail of CI-MPR by Zhu et al. (2001). Receptors with mutations in this motif were defective in lysosomal enzyme sorting. The hinge domain of GGA2 bound clathrin, suggesting that GGA2 could be a link between cargo molecules and clathrin-coated vesicle assembly. Thus, GGA2 binding to the CI-MPR is important for lysosomal enzyme targeting. Misra et al. (2002) reported the structures of the VHS domain of human GGA3 complexed with signals from both MPRs. The signals bind in an extended conformation to α-helices 6 and 8 of the VHS domain. The structures highlight an Asp residue separated by two residues from a dileucine sequence as critical recognition elements. The side chains of the Asp-X-X-Leu-Leu sequence interact with subsites consisting of one electropositive and two shallow hydrophobic pockets, respectively. The rigid spatial alignment of the three binding subsites leads to high specificity (Misra et al. 2002).

Interactions of GGA3 with Ubiquitin Sorting Machinery: Puertollano and Bonifacino (2004) demonstrated the existence of another population of GGAs that are associated with early endosomes. RNAi of GGA3 expression results in accumulation of the CI-MPR and internalized epidermal growth factor (EGF) within enlarged early endosomes. This impairs the degradation of internalized EGF, a process that is normally dependent on the sorting of ubiquitinated EGF receptors (EGFRs) to late endosomes. Protein interactions showed that the GGAs bind ubiquitin. The VHS and GAT domains of GGA3 were responsible for this binding, and with TSG101, a component of the ubiquitin-dependent sorting machinery. Thus, GGAs may have additional roles in sorting of ubiquitinated cargo.

4.8 Retrieval of CI-MPR from Endosome-TO-GOLGI

4.8.1 Endosome-to-Golgi Retrieval of CIMPR Requires Retromer Complex

After releasing the hydrolase precursors into the endosomal lumen, the unoccupied CI-MPR returns to the TGN for further rounds of sorting. The mammalian retromer complex participates in this retrieval pathway. Endosome-to-Golgi retrieval of the MPR is required for lysosome biogenesis. This pathway is poorly understood. Analyses in yeast identified a complex of proteins called “retromer” that is essential for endosome-to-Golgi retrieval of the carboxypeptidase Y receptor Vps10p. Retromer comprises five distinct proteins: Vps35p, 29p, 26p, 17p, and 5p, which are conserved in mammals. Mammalian retromer is localized to endosomes and comprises two distinct sub complexes: the vacuolar protein sorting Vps26 (vacuolar sorting protein 26), Vps29 and Vps35 proteins sub complex that binds cargo and the SNX-1/2 sub complex that tubulates endosomal membranes. The hVps35 subunit of retromer interacts with the cytosolic domain of the CI-MPR. This interaction occurs in an endosomal compartment, where most of the retromer is localized. In particular, retromer is associated with tubular-vesicular profiles that emanate from early endosomes or from intermediates in the maturation from early to late endosomes. Depletion of retromer by iRNA increases the lysosomal turnover of the CI-MPR, decreases cellular levels of lysosomal hydrolases, and causes swelling of lysosomes. These observations indicate that retromer prevents the

delivery of the CI-MPR to lysosomes, probably by sequestration into endosome-derived tubules from where the CIMPR returns to the TGN (Arighi et al. 2004; Damen et al. 2006).

Cells lacking mammalian Vps26 fail to retrieve the CI-MPR, resulting in either rapid degradation of or mislocalization to the plasma membrane. Study supports the hypothesis that retromer performs a selective function in endosome-to-Golgi transport, mediating retrieval of the CI-MPR, but not furin (Seaman 2004). A conserved aromatic-containing sorting motif is critical for the endosome-to-TGN retrieval of the CI-MPR and for the interaction with retromer and the clathrin adaptor AP-1 (Seaman 2007).

4.8.2 Retromer Complex and Sorting Nexins (SNX)

Retromer Complex

In yeast, retromer is composed of Vps5p (the orthologue of SNX-1), Vps17p (a related SNX) and a cargo selective subcomplex composed of Vps26p, Vps29p and Vps35p. With the exception of Vps17p, mammalian orthologues of all yeast retromer components have been identified. For Vps17p, one potential mammalian orthologue is SNX-2. Sorting nexins (SNXs) are phox homology (PX) domain-containing proteins thought to regulate endosomal sorting of internalized receptors. SNX-1 and SNX-2 through their PX domain bind phosphatidylinositol 3-monophosphate [PtdIns(3)P] and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P(2)], and possess a Bin/Amphiphysin/Rvs domain that endows SNX1 with the ability to form dimers and to sense membrane curvature. However, in contrast to SNX-1, SNX-2 could not induce membrane tubulation in vitro or in vivo. Functionally, endogenous SNX-1/-2 co-localise on high curvature tubular elements of the 3-phosphoinositide-enriched early endosome, and that suppression of SNX-2 does not perturb the degradative sorting of receptors for epidermal growth factor or transferrin, nor the steady-state distribution of the CI-MPR. However, suppression of SNX-2 results in a subtle alteration in the kinetics of CI-MPR retrieval.

SNX1 Defines an Early Endosomal Recycling Exit for Sortilin and MPRs: In addition to MPRs, the multi-ligand receptor sortilin has also been implicated in transport of lysosomal hydrolases from the TGN to endosomes. The transport carriers involved have been identified recently. Sortilin of HepG2 cells predominantly localized to the TGN and endosomes. Sortilin and MPRs recycle to the TGN in SNX1-dependent carriers, which were named endosome-to-TGN transport carriers (ETCs). The ETCs emerge from early endosomes (EE), lack recycling plasma membrane proteins and exhibit unique structural features. Hence, ETCs are distinct from hitherto described EE-derived membranes involved in recycling. This study emphasizes an important role of EEs in recycling to the TGN (Mari et al. 2008).

SNX1 is associated with early endosomes, from where regulates the degradation of internalized epidermal growth factor (EGF) receptors through modulating endosomal-to-lysosomal sorting. Through coincidence detection, the BAR and PX domains efficiently target SNX1 to a microdomain of early endosome defined by high curvature and the presence of 3-phosphoinositides. In addition, the BAR domain endows SNX1 with an ability to tubulate membranes in-vitro and drive the tubulation of the endosomal compartment in-vivo. Using RNAi, SNX1 did not demonstrate a role in EGF or transferrin receptor sorting; rather it specifically perturbed endosome-to-TGN transport of CI-MPR (Carlton et al. 2004). Studies support an evolutionarily conserved function for SNX1 from yeast to mammals and provide functional insight into the molecular mechanisms underlying lipid-mediated protein targeting and tubular-based protein sorting. It appears that through coincidence detection SNX1 associates with a microdomain of the early endosome-characterized by high membrane curvature and the presence of 3-phosphoinositides-from where it regulates tubular-based endosome-to-TGN retrieval of the CI-MPR (Carlton et al. 2005). Bujny et al. (2007) suggest a role for SNX1 in the endosome-to-TGN transport of Shiga toxin and are indicative for a fundamental difference between endosomal sorting of Shiga and cholera toxins into endosome-to-TGN retrograde transport pathways.

Genetic Targeting of Retromer Components Leads to Embryonic Lethality: Mammalian retromer components sorting SNX-1 and SNX-2 result in embryonic lethality when simultaneously targeted for deletion in mice, whereas others have shown that Hβ58 (also known as mVps26), another retromer component, results in similar lethality when targeted for deletion. Genetic interaction of these mammalian retromer components in mice reveals a functional interaction between Hβ58, SNX-1, and SNX-2. SNX-2 plays a more critical role than SNX1 in retromer activity during embryonic development. This supports the existence of retromer complexes containing SNX-1 and SNX-2; SNX-2 as an important mediator of retromer biology. Moreover, mammalian retromer complexes containing SNX-1 and SNX-2 have an essential role in embryonic development that is independent of CI-MPR trafficking (Griffin et al. 2005). Observations indicate that the mammalian retromer complex assembles by sequential

association of SNX1/2 and Vps26-Vps29-Vps35 subcomplexes on endosomal membranes and that SNX1 and SNX2 play interchangeable but essential roles in retromer structure and function (Rojas et al. 2007). However, Carlton et al. (2005) indicated that although SNX-2 may be a component of the retromer complex, its presence is not essential for the regulation of endosome-to-TGN retrieval of the CI-MPR (Carlton et al. 2005).

SNX5 and SNX6 in Retromer Regulate CI-MPR

In yeast, an additional sorting nexin-Vps17p-is a component of the membrane bound coat. Mammalian retromer may require a functional equivalent of Vps17p. Wassmer et al. (2007) identified two proteins, SNX5 and SNX6, that, when suppressed, induced a phenotype similar to that observed upon suppression of known retromer components. Whereas SNX5 and SNX6 colocalised with SNX1 on early endosomes, only SNX6 appeared to exist in a immune-complex with SNX1. Interestingly, suppression of SNX5 and/or SNX6 resulted in a significant loss of SNX1, an effect that seemed to result from post-translational regulation of the SNX1 level. Such data suggest that SNX1 and SNX6 exist in a stable, endosomally associated complex that is required for retromer-mediated retrieval of CI-MPR. SNX5 and SNX6 may therefore constitute functional equivalents of Vps17p in mammals. Proteomic profiles of endosomally enriched membranes from wild-type or retromer-deficient mouse cells showed Eps15 homology domain-containing protein-1 (EHD1) in endosomally enriched membrane fractions from retromer-deficient cells. EHD1 is localized to tubular and vesicular endosomes, partially colocalizes with retromer and is associated with retromer in vivo. The interaction between EHD1 and retromer and the requirement for EHD1 to stabilize SNX1-tubules establish EHD1 as a novel facilitating component of endosome-to-Golgi retrieval (Gokool et al. 2007).

DOCK180, a DOCK180-family guanine nucleotide exchange factor for small GTPases Rac1 and Cdc42, shares two conserved domains, called DOCK homology region (DHR)-1 and -2. In order to understand the function of DHR1, SNX-1, 2, 5, and 6 were searched as its binding partners which make up a multimeric protein complex mediating endosome-to-TGN retrograde transport of CI-MPR. Colocalization of DOCK180 with SNX5 at endosomes and the iRNA-mediated knockdowns of SNX5 and DOCK180, and the redistribution of CI-MPR from TGN to endosomes suggested that DOCK180 regulates CI-MPR trafficking via SNX5 and that this function was independent of its Rac1 (Hara et al. 2008).

OCRL1 Regulates Protein Trafficking Between Endosomes and TGN

Oculocerebrorenal syndrome of Lowe is caused by mutation of OCRL1, a phosphatidylinositol 4,5-bisphosphate 5-phosphatase localized at the Golgi apparatus. The OCRL1 is associated with clathrin-coated transport intermediates operating between TGN and endosomes. OCRL1 interacts directly with clathrin heavy chain and promotes clathrin assembly in vitro. Over-expression of OCRL1 results in redistribution of clathrin and the CI-MPR to enlarged endosomal structures that are defective in retrograde trafficking to the TGN. It seems that OCRL1 plays a role in clathrin-mediated trafficking of proteins from endosomes to the TGN and defects in this pathway might contribute to the Lowe syndrome phenotype (Choudhury et al. 2005).

Hrs and ESCRT Proteins Function at Different Stages in Endocytic Pathway: A ubiquitin-binding endosomal protein machinery is responsible for sorting endocytosed membrane proteins into intraluminal vesicles of multivesicular endosomes (MVEs) for subsequent degradation in lysosomes. The Hrs-STAM complex and endosomal sorting complex required for transport (ESCRT)-I, -II and -III are central components of this machinery. Raiborg et al. (2008) revealed that none of them was required for recycling of CI-MPRs from endosomes to TGN. It seemed that they function at distinct stages of endocytic pathway.

Cholesterol Requirement for CI-MPR Exit from Late Endosomes to Golgi

The regulation of endocytic traffic of receptors has central importance in the fine tuning of cell activities. Miwako et al. (2001) provided evidence that cholesterol is required for the exit of CI-MPR from the endosomal carrier vesicle/multivesicular bodies (ECV/MVBs) to the Golgi. A Chinese hamster ovary cell mutant, LEX2, exhibits arrested ECV/MVBs in which CI-MPR and lysosomal glycoprotein-B (lgp-B) are accumulated. Results suggest that cholesterol is required for ECV/MVB reorganization that drives the sorting/transport of materials destined for the Golgi out of the pathways towards lysosomes.

Cholesterol accumulated in late Endocytic Compartments Does Not Affect Distribution and Trafficking of CI-MPR: The treatment with 3β-[2-(diethylamino)ethoxy] androst-5-en-17-one (U18666A) is known to cause the accumulation of cholesterol in late endosomal/lysosomal compartments in BHK cells. The accumulation of cholesterol within late endosomes/lysosomes in Niemann-Pick type C (NPC) fibroblasts and U18666A-treated cells causes impairment of retrograde trafficking of CI-MPR/IGF2R from late endosomes to TGN, where as the accumulation of cholesterol in late endosomes/lysosomes did not affect the retrieval of CI-MPR from endosomes to the TGN. However, treatment of normal and NPC fibroblasts with chloroquine, which inhibits membrane traffic from early endosomes to the TGN, resulted in a redistribution of CI-MPR to EEA1 and internalized transferrin-positive, but LAMP-2-negative, early-recycling

endosomes. It was proposed that in normal and NPC fibroblasts, CI-MPR is exclusively targeted from the TGN to early endosomes, from where it rapidly recycles back to the TGN without being delivered to late endosomes (Umeda et al. 2003). In HeLa cells, it was suggested that U18666A treatment primarily suppresses the CI-MPR transport pathways to late endosomes and from transferrin-containing endosomes, both of which may be dependent on cholesterol function (Tomiyama et al. 2004).

4.8.3 Small GTPases in Lysosome Biogenesis and Transport

Abundant information is available about the subcellular distribution and function of some of the endocytosis-specific Rabs (e.g. Rab5, Rab4, Rab7, Rab15, Rab22). Soon after endocytosis, internalized material is sorted along different pathways in a process that requires the coordinated activity of several Rab proteins. The molecular machinery behind lysosome biogenesis and the maintenance of the perinuclear aggregate of late endocytic structures is not well understood. A likely candidate for being part of this machinery is the small GTPase Rab7. Rab GTPases belong to the Ras GTPase superfamily and are key regulators of membrane traffic. Rab7 also appears to play a fundamental role in controlling late endocytic membrane traffic (Vitelli et al. 1997). In contrast to the wild-type protein, a rab7 mutant with a reduced GTPase activity is in part associated with lysosomal membranes. Results implicated rab7 as a GTPase functioning on terminal endocytic structures in mammalian cells (Méresse et al. 1995). Bucci et al. (2000) demonstrated that Rab7, controlling aggregation and fusion of late endocytic structures/lysosomes, is essential for maintenance of the perinuclear lysosome compartment. Stable BHK cell lines inducibly expressing wild-type or dominant negative mutant forms of the rab7 GTPase were used to study the role of a rab7-regulated pathway in lysosome biogenesis. Expression of mutant rab7N125I protein induced a dramatic redistribution of CI-MPR from its normal perinuclear localization to large peripheral endosomes.

Rab22a associates with early and late endosomes. Conversely, overexpression of Rab22aQ64L which strongly affects the morphology of endosomes, did not inhibit bulk endocytosis. Over-expression of Rab22a hampers the transport between endosomes and the Golgi apparatus. Moreover, these cells accumulated the CI-MPR in endosomes. Observations indicate that Rab22a can affect the trafficking from endosomes to the Golgi apparatus probably by promoting fusion among endosomes and impairing the proper segregation of membrane domains required for targeting to the TGN (Mesa et al. 2001, 2005).

4.8.4 Role for Dynamin in Late Endosome Dynamics and Trafficking of CI-MPR

It is well established that dynamin is involved in clathrin-dependent endocytosis and the endogenous dynamin plays an important role in the molecular machinery behind the recycling of the CI-MPR from endosomes to the TGN. Nicoziani et al. (2000) proposed that dynamin is required for the final scission of vesicles budding from endosome tubules.

Granzyme B (GzmB) endocytosis is facilitated by dynamin in many endocytic pathways. Uptake of and killing by purified granzyme B occurred by both dynamin-dependent and -independent mechanisms. It was proposed that under physiological conditions serglycin-bound granzyme B is critically endocytosed by a MPR, and receptor binding is enhanced by cell surface heparan sulfate (Veugelers et al. 2006). The CI-MPR participates in lysosomal and granular targeting of serglycin and basic proteins such as lysozyme associated with the proteoglycan in hematopoietic cells (Lemansky et al. 2007). However, the role of the CI-MPR has been refuted and that membrane receptors for GzmB on target cells are not crucial for killer cell-mediated apoptosis (Kurschus et al. 2005).

4.9 CI-MPR/IGF2R System and Pathology

4.9.1 Deficiency of IGF2R/CI-MPR Induces Myocardial Hypertrophy

Mice lacking the IGF2R have increased levels of circulating IGF2, are born larger, and die soon after birth due to cardiac hyperplasia. This phenotype is overcome by concomitant deletion of the IGF2 gene (Ludwig et al. 1996). The IGF2R function in extra-cellular matrix remodeling is associated with TGF-β activation and plasmin in the proteolytic cleavage caused by the interaction between latent TGF-β and uPAR. IGF2 and IGF2R have also been correlated with the progression of hypertrophy remodeling following abdominal aorta ligation. IGF2R is expressed in myocardial infarction scars. While exploring the function of IGF2R and IGF2 in myocardial extra-cellular matrix remodeling, it was suggested that after binding with IGF2, IGF2R may trigger intracellular signaling cascades involved in the progression of cardiac hypertrophy. Using Leu^{27I}GF2, an analog of IGF2 which interacts selectively with the IGF2R, the binding of Leu^{27I}GF2 to IGF2R resulted into an increase in the phosphorylation of PKCα and calcium/calmodulin-dependent protein kinase II (CaMKII) in a $G_{\alpha q}$-dependent manner. By the inhibition of PKCα/CaMKII activity, the IGF2 and Leu^{27I}GF2-induced cell hypertrophy

and up-regulation of ANP and BNP were significantly suppressed (Chu et al. 2008). It was suggested that IGF2R signaling inhibition may have potential use in the development of therapies preventing heart fibrosis progression and a new insight into the effects of IGF2R and its downstream signaling in cardiac hypertrophy (Chang et al. 2008; Chu et al. 2008).

Transport of lysosomal enzymes is mediated by CD-MPR and a CI-MPR. To address the consequences of abnormalities of cellular morphology and function on CI-MPR subcellular localization, fibroblasts from Pompe disease patients with different genotypes and phenotypes have been studied. In these cells, which showed abnormalities of cellular morphology, CI-MPR is mislocalized and its availability at the plasma membrane is reduced. These abnormalities in CI-MPR distribution result in a less efficient uptake of rhGAA by Pompe disease fibroblasts. CI-MPR-mediated endocytosis of rhGAA is an important pathway by which the enzyme is delivered to the affected lysosomes of Pompe muscle cells (Chap. 46). In hepatocytes of MPR-deficient neonatal mice lysosomal storage occurs when both MPRs are lacking, whereas deficiency of CI-MPR only has no effect on the ultrastructure of the lysosomal system. Some structural features have been shown to be crucial for the binding of M6P to CI-MPR.

4.9.2 MPRs in Neuromuscular Diseases

M6PRs in heart muscle function in the endocytosis and transport of lysosomal enzymes in cardiomyocytes. In association, the activity of β-N-acetyl-glucosaminidase significantly increases in the muscles of patients with myopathies (polymyositis and muscular dystrophies) but not in those with neurogenic muscle atrophies (amyotrophic lateral sclerosis, polyneuropathy or other neurogenic muscle disease). The content of M6PRs correlated with the muscular activity of β-N-acetylglucosaminidase, muscle atrophy index, and serum creatine kinase activity (Salminen et al. 1988).

Alzheimer's Disease (AD): The expression of the CD-MPR is increased in Alzheimer's disease. Endosomal and lysosomal changes are invariant features of neurons in AD. These changes include increased levels of lysosomal hydrolases in early endosomes and increased expression of CD-MPR, which is partially localized to early endosomes. The redistribution of lysosomal hydrolases to early endocytic compartments mediated by increased expression of CD-MPR may represent a potentially pathogenic mechanism for accelerating amyloid beta (Aβ) generation in sporadic AD, where the mechanism of amyloidogenesis is unknown (Mathews et al. 2002). However, despite CD-MPR gene being located next to a region on chromosome 12 linked to AD, Kölsch et al. (2004) could not find an association of C/T polymorphism in CD-MPR with AD.

CI-MPR Trafficking in Batten disease: The neuronal ceroid lipofuscinoses (NCLs, Batten disease) are a group of inherited childhood-onset neurodegenerative disorders characterized by the lysosomal accumulation of undigested material within cells. Cause of dysfunction may be associated with trafficking of CI-MPR, which delivers the digestive enzymes to lysosomes. A common form of NCL is caused by mutations in CLN3, a multipass transmembrane protein of unknown function. The ablation of CLN3 causes accumulation of CI-MPR in the TGN, reflecting a 50% reduction in exit. This CI-MPR trafficking defect is accompanied by a fall in maturation and cellular activity of lysosomal cathepsins. CLN3 is therefore essential for trafficking along the route needed for delivery of lysosomal enzymes, and its loss thereby contributes to and may explain the lysosomal dysfunction underlying Batten disease (Metcalf et al. 2008).

4.9.3 CI-MPR in Fanconi syndrome

In renal Fanconi syndrome (FS), Norden et al. (2008) suggest that the underlying gene defects in FS may disrupt normal membrane trafficking of CI-MPR, leading to mistrafficking of lysosomal enzymes via a default pathway from the Golgi to the apical surface of proximal tubule cells rather than to lysosomes. Lysosomal enzymes are then secreted into the tubular fluid and excreted in the urine. This contrasts with the widely held view that cell necrosis is the cause of lysosomal enzymuria in renal disease. Moreover, cathepsin D in FS urine is M6P-tagged.

4.9.4 Tumor Suppressive Effect of CI-MPR/IGF2R

The M6P/IGF2R gene is considered a "candidate" tumor suppressor gene. The phenotypic consequences of loss of M6P/IGF2R through somatic mutation are potentially very complex since M6P/IGF2R has a number of roles in cellular physiology. Loss of function mutations in M6P/IGF2R gene could contribute to multi-step carcinogenesis (Hébert 2006; Kreiling et al. 2003). Mutation of M6P/IGF2R causes both diminished growth suppression and augmented growth stimulation. The M6P/IGF2R is a negative regulator of cell growth. The genetic alterations in hepatocarcinomas and a few breast cancers suggest that this receptor behaves as a tumor suppressor. Mutational and functional evidences are consistent with CI-MPR/IGF2R being a tumor suppressor in human colon, liver, lung, breast, and ovarian cancers.

Decreased levels of functional M6P/IGF2R directly contribute to the process of carcinogenesis and loss of IGF2R activity in many cancers has been associated with increased tumor cell growth and tumor progression. This can arise from the loss of heterozygosity or mutation of the receptor (De Souza et al. 1995; Devi et al. 1998; Killian et al. 2001), leading to an increase in bioavailable IGF2, which then acts via the type 1 IGF receptor (IGF-1R) to promote cancer growth (Alexia et al. 2004). As cancer cell proliferation can be abrogated by blocking mRNA or protein products of these genes, tumors with extensive involvement of the IGF2 pathway are candidates for the therapeutics strategies aimed at interference with this pathway.

Studies on Cancer Cells: Loss or mutation of the M6P/IGF2R has been found in breast cancer. Human breast cancer cells, MCF-7 cells with the adenovirus carrying a ribozyme targeting the CI-MPR/IGF2R mRNA dramatically reduced the level of transcripts and the functional activity of IGF2R and exhibited a higher growth rate and a lower apoptotic index than control cells. The decreased expression of IGF2R enhanced IGF2-induced proliferation and reduced cell susceptibility to TNF-induced apoptosis (Chen et al. 2002). Tumor suppressive effect of IGF2R has been displayed by 66cl4, a mouse mammary tumor cell line deficient in the receptor (Li and Sahagian 2004). Furthermore, patients with heterozygosity (LOH) are much more likely to have elevated plasma TGFβ, suggesting an inability to normally process this cytokine (Kong et al. 2001). Ionizing radiation induces the rapid expression of M6P/IGF2R in a dose-dependent manner in MCF7 cancer cells. This increase is mediated, at least in part, by a stabilization of M6P/IGF2R transcripts by radiation in both estrogen receptor (ER) positive (MCF7 and T47D) and ER negative (MDA-MB-231) breast cancer cell lines (Iwamoto and Barber 2007).

In opposing studies, MDA-MB-231 breast cancer cells stably transfected with M6P/IGF2R cDNA exhibited not only a greatly reduced ability to form tumors but also a markedly reduced growth rate in nude mice. In vitro, increased M6P/IGF2R expression resulted in twofold reduced uptake of IGF2 and was associated with reduced cellular invasiveness and motility (Lee et al. 2003). A frequent loss of heterozygosity (LOH) in the 6q27-qter region in ovarian carcinomas confirmed the role of *IGF2R* gene in ovarian carcinomas and breast- and ovarian-cancer cell lines. The 2491 amino-acid sequence of M6P/IGF2R was perfectly conserved in 9 out of 10 samples, including MCF7 and MDA-MB231 cells and five ovarian carcinomas with LOH. The only amino-acid change (Thr → Ala) was in BG1 ovarian-cancer cells, and was due to an A → G substitution on one allele at nucleotide 2561. Rey et al. (2000) proposed that, in breast and ovarian cancers, the frequent loss of one allele, associated with over-expression of some of its ligands, might be sufficient to saturate the receptor protein, displace the ligands to other sites, and consequently facilitate tumor progression.

Members of the IGF family are involved in the pathogenesis of gastric cancer, probably by autocrine/paracrine stimulation of cell growth. Such tumors might be excellent candidates for therapeutic strategies aimed at interference with this pathway (Pavelić et al. 2007). Decreased IGF2R expression could partly account for the increased growth of lymph node carcinoma of the prostate (LNCaP). It appears that the IGF2- and M6P-binding functions of the IGF2R have opposing activities, with respect to growth of prostate cancer cells (Schaffer et al. 2003). Pavelić et al. (2007) suggested that IGF1, IGF2 and their receptors were involved in the progression of endometrial adenocarcinomas. M6P/IGF2R is expressed in human lung-cancer cells (Bredin et al. 1999). Harper et al. (2006) showed rescue of the *Igf2*-dependent intestinal and adenoma phenotype. This evidence shows the functional potency of allelic dosage of an epigenetically regulated gene in cancer and supports the application of an IGF2 ligand-specific therapeutic intervention in colorectal cancer. The MPRs are expressed in a polarized fashion in human adenocarcinoma cell line Caco-2 and the IGF2R/CI-MPR present on apical membranes, unlike the IGF-2R/CI-MPR expressed on the basolateral surface, is not functional in endocytosing lysosomal enzymes (Dahms et al. 1996). Reduced supply of IGF2 is detrimental to tumor growth, and this suggests that gain of function of IGF2 is a molecular target for human cancer therapy (Chap. 46).

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Mannose-6-Phosphate Receptor Homologous Protein Family

5

G.S. Gupta

5.1 Recognition of High-Mannose Type N-Glycans in ERAD Pathways

Protein quality control in the endoplasmic reticulum (ER) is an elaborate process conserved from yeast to mammals, ensuring that only newly synthesized proteins with correct conformations in the ER are sorted further into the secretory pathway. The ER discriminates between native and nonnative protein conformations, selectively transporting properly folded proteins to their final destinations through the secretory pathway, or alternatively, retrotranslocating misfolded proteins to the cytosol to be degraded by proteasomes. In the quality control process, high-mannose type N-glycans play important roles in protein-folding events. Proteins that fail to achieve proper folding or proper assembly are degraded in a process known as ER-associated degradation (ERAD). The ERAD pathway comprises multiple steps including substrate recognition and targeting to the retro-translocation machinery, retrotranslocation from the ER into the cytosol, and proteasomal degradation through ubiquitination. The quality-control system also surveys the ER lumen for terminally misfolded proteins. Polypeptides singled out by this system are ultimately degraded by the cytosolic ubiquitin-proteasome pathway. Key components of both the ER quality-control system and the ERAD machinery have been identified, but a connection between the two systems has remained elusive (Yoshida and Tanaka 2010). Recent studies have documented the important roles of sugar-recognition (lectin-type) molecules for trimmed high-mannose type N-glycans and glycosidases in the ERAD pathways in both ER and cytosol. Since the ER is distributed throughout the cytosol, studies suggest that the cytosolic face of the ER membrane serves as a "platform" for degradation of misfolded cytosolic proteins (Metzger et al. 2008). The fundamental system that monitors glycoprotein folding in the ER and the unique roles of the sugar-recognizing ubiquitin ligase and peptide:N-glycanase (PNGase) in the cytosolic ERAD pathway has been reviewed (Yoshida and Tanaka 2010).

Glycosylation of asparagine residues in Asn-x-Ser/Thr motifs is a common covalent modification of proteins in the lumen of the ER. Protein glycosylation itself and processing of the glycan transferred play a key role in the folding and conformation discrimination of glycoproteins within the ER. By substantially contributing to the overall hydrophilicity of the polypeptide, pre-assembled core glycans inhibit possible aggregation caused by the inevitable exposure of hydrophobic patches on the as yet unstructured chains. Thereafter, N-glycans are modified by ER-resident enzymes glucosidase I (GI), glucosidase II (GII), UDP-glucose:glycoprotein glucosyltransferase (UGT) and mannosidase(s) and become functional appendices that determine the fate of the associated polypeptide. The glycan ($Glc_3Man_9GlcNAc_2$) transferred to Asn residues is first deglucosylated by glucosidase I, a type II membrane protein with a lumenal hydrolytic domain, which removes the outermost Glc of the glycan (Fig. 5.1). The $Glc_2Man_9GlcNAc_2$ (G2M9) thus produced is then deglucosylated by glucosidase II (GII) that successively generates $Glc_1Man_9GlcNAc_2$ (G1M9) and $Man_9GlcNAc_2$ (M9) upon cleavage of Glcα1,3Glc (cleavage 1) and Glcα1,3Man (cleavage 2) bonds. Both GII-mediated cleavages play a determining role in the quality control of glycoprotein folding in the ER. Monoglucosylated glycan-bearing glycoproteins may interact with calnexin (CNX) and/or calreticulin (CRT), two ER-resident lectin chaperones that enhance folding efficiency by preventing aggregation and facilitating correct disulfide bond formation through their interaction with ERp57, a protein disulfide isomerase (Chap. 2). Furthermore, the interaction of folding intermediates and misfolded glycoproteins and the lectin-chaperones prevent their exit from the ER to the Golgi. The second GII-mediated cleavage that generates M9 abolishes the glycoprotein-lectin-chaperone interaction, thus allowing glycoproteins to pursue their transit through the secretory

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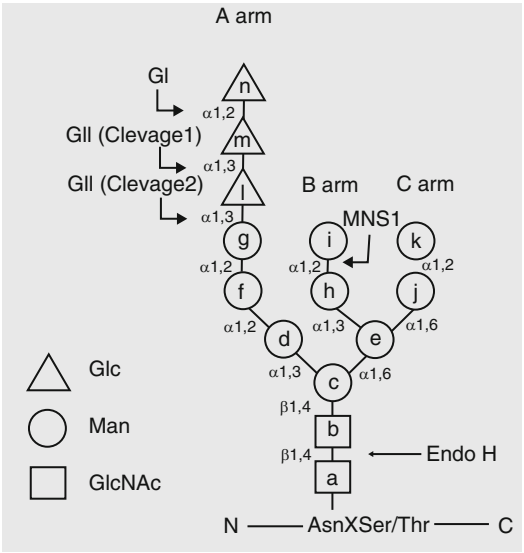

Fig. 5.1 Glycan structures: The structure depicted is that of the glycan transferred to Asn residues in *N*-glycosylation. Lettering (*a*, *b*, *c* ...) follows the order of addition of monosaccharides in the synthesis of the dolichol-P-P derivatives. Glucosidase I removes residue n and GII, residues *l* and *m*. GT re-adds residue *l*. ER mannosidase cleaves residue *i*. G2M9, G1M9, and M9 comprise residues *a*–*m*, *a*–*l*, and *a*–*k*, respectively, except when the glycans had been previously treated with Endo H. Because this enzyme cleaves the bond between residues *a* and *b*, those glycans are devoid of residue *a*. Jack bean α-mannosidase removes residues *h*–*k* and partially also residue *e* from the glucosylated glycans. Therefore, G2M4-5 comprise residues *b*–*d*, *f*, *g*, *l*, and *m* and partially also residue *e*. G1M4-5 is similar to G2M4-5 but lacks residue *m*. *Arm A* comprises residues *d*, *f*, *g*, and *l*–*n*; *arm B* comprises residues *h* and *i*; and *arm C* comprises residues *j* and *k* (Stigliano et al. 2009).

pathway. However, if not yet properly folded, glycoproteins may be reglucosylated by the uridine 5′-diphosphate (UDP)-Glc:glycoprotein glucosyltransferase (GT), an enzyme that specifically glucosylates nonnative conformers and regenerates monoglucosylated glycans. These, in turn, interact again with the lectin chaperones. Cycles of reglucosylation and deglucosylation catalyzed by the opposing activity of GT and GII continue until species acquire their proper tertiary structure (Trombetta and Parodi 2003).

Recent studies have improved our understanding of how the removal of terminal glucose residues from N-glycans allows newly synthesized proteins to access the calnexin chaperone system; how substrate retention in this specialized chaperone system is regulated by de-/re-glucosylation cycles catalyzed by GII and UGT1; and how acceleration of N-glycan dismantling upon induction of EDEM variants promotes ERAD under conditions of ER stress. In particular, characterization of cells lacking certain ER chaperones has revealed important new information on the mechanisms regulating protein folding and quality control. Tight regulation of N-glycan modifications is crucial to maintain protein quality control, to ensure the synthesis of functional polypeptides and to avoid constipation of the ER with folding-defective (Ruddock and Molinari 2006)

The mannose-6-phosphate (M6P) based system for targeting lysosomal hydrolases to lysosomes is conserved in mammals, birds, amphibians, and crustaceans but is absent in the unicellular protozoa *Trypanosoma* (Huete-Perez et al. 1999) and *Leishmania* (Clayton et al. 1995) (Chaps. 3, 4). *D. discoideum* and *Acanthamoeba castellani* both exhibit GlcNAc-phosphotransferase activity and can transfer GlcNAc-1-PO_4 to mannose residues (Lang et al. 1986). However, MPRs have not been identified in these species. The CD-MPR (Nadimpalli and von Figura 2002) and CI-MPR (Lakshmi et al. 1999; Nadimpalli et al. 2004) have also been reported in the invertebrate mollusc *Unio*. These studies demonstrate that numerous species in animal kingdom express *bone fide* MPRs that are capable of binding phosphomannosyl residues (Figs. 5.2, 5.3).

5.2 Proteins Containing M6PRH Domains

In recent years, several proteins (Mrl1, LERP, GlcNAc-1-phosphotransferase γ-subunit, ER glucosidase II β-subunit, OS-9, and XTP3-B/Erlectin) have been identified that contain mannose-6-phosphate receptor homology (MRH or M6PRH) domains (Dodd and Drickamer 2001; Munro 2001; Cruciat et al. 2006) and implicated in N-glycan recognition but none has been shown to bind M6P. Of these, only LERP has been shown to function in the delivery of lysosomal enzymes to the lysosome. In humans, none of them has transmembrane region (Fig. 5.2).

5.3 GlcNAc-Phosphotransferase

Lysosomal enzymes are targeted to the lysosome through binding to M6PRs because their glycans are modified with M6P. This modification is catalyzed by UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase), which transfers GlcNAc 1-phosphate from UDP-GlcNAc to mannose in high mannose-type glycans. The second step is removal of GlcNAc by *N*-acetylglucosamine-1-phosphodiester α-GlcNAcase (UCE) to expose mannose 6-phosphate. Bovine GlcNAc-phosphotransferase has been characterized as a multisubunit enzyme with the subunit structure $\alpha_2\beta_2\gamma_2$. The cDNA for the human γ-subunit was cloned and its gene has been localized to chromosome 16p. The α- and β-subunits have enzyme activity, whereas the γ-subunit (GNPTG), which contains the MRH domain, lacks catalytic activity and is believed to participate in substrate binding of the enzyme (Lee et al. 2007). However, the sugar-binding property of GNPTG is currently unknown. The α- and β -subunits have been cloned by Kudo et al. (2005). While the α- and β-subunits of human GlcNAc-phosphotransferase are encoded by a single cDNA as a

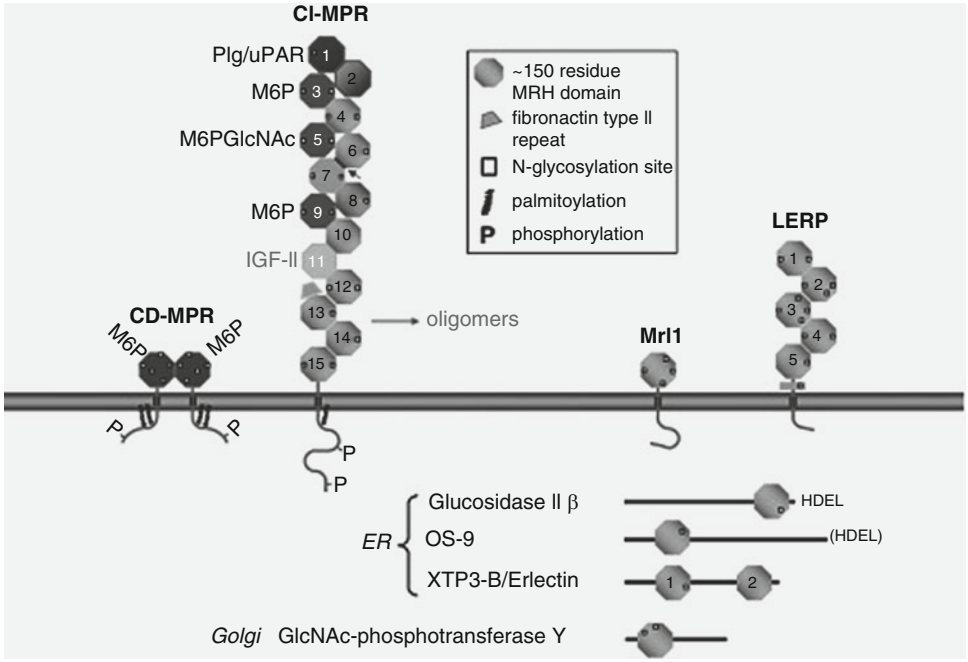

Fig. 5.2 Schematic diagram of M6PR-H domain containing proteins. The MPRs, 381-residue Mrl1, and 886-residue LERP are type I transmembrane glycoproteins. The 279-residue CD-MPR exists as a homodimer. The 2499-residue CI-MPR also undergoes oligomerization and most likely exists as a dimer. The M6P binding sites of the CD-MPR and CI-MPR are indicated. Domains 1 and 2 are outlined since the presence of these two domains enhances the affinity of domain 3 for lysosomal enzymes by ~1,000-fold (Hancock et al. 2002). The CD-MPR contains a single high affinity M6P binding site per polypeptide. In contrast, CI-MPR contains three carbohydrate recognition sites: two high affinity sites are localized to domains 1–3 and domain 9 and one low affinity site is contained within domain 5. The IGF-II and plasminogen (Plg)/uPAR binding sites are also indicated. The fibronectin type II repeat present in domain 13 increases the affinity of domain 11 for IGF-II by ~10-fold. The *arrow* indicates the location of a proteolytically sensitive cleavage site between domains 6 and 7 (Westlund et al. 1991). The 528-residue glucosidase II β subunit, 667-residue OS-9, and 483-residue XTP3-B/Erlectin are soluble resident ER proteins. Glucosidase II β subunit and the yeast ortholog of OS-9 contain a C-terminal ER retention signal (HDEL). The 305-residue N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) γ-subunit, in complex with the catalytic ∝/β subunits, is enriched in *cis*-Golgi cisternae (Adapted by permission from Dahms et al. 2008 © Oxford University Press)

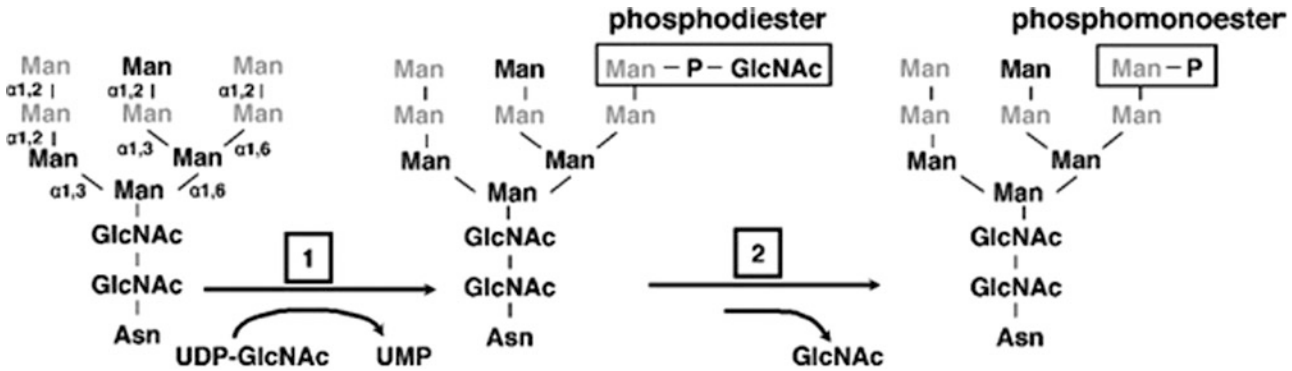

Fig. 5.3 Generation of the Man-6-P tag on *N*-linked oligosaccharides. Phosphorylation of mannose residues on *N*-linked oligosaccharides occurs in two steps. First, the GlcNAc-phosphotransferase transfers GlcNAc-1-phosphate from UDP-GlcNAc to the C-6 hydroxyl group of mannose to form the Man-P-GlcNAc phosphodiester intermediate. Second, the uncovering enzyme removes the GlcNAc moiety in the TGN, revealing the Man-6-P phosphomonoester. The five potential sites of phosphorylation are indicated (*gray*) (Reprinted by permission from Dahms et al. 2008 © Oxford University Press)

precursor and appear to be generated by a proteolytic cleavage at the Lys928-Asp929 bond, the γ-subunit is encoded by a second gene. The hydropathy plots of the deduced amino acid sequences revealed that the α- and β-subunits contain amino and carboxyl terminus transmembrane domains, respectively, whereas the γ-subunit does not. The mechanism responsible for the processing of the α/β-subunits precursor into the mature α- and β-subunits is not

yet known, but likely to be the result of protease(s) in the ER or *cis*-Golgi, where GlcNAc-phosphotransferase resides. Recombinant soluble GlcNAc-phosphotransferase exhibited specific activity and substrate preference similar to the wild type bovine GlcNAcphosphotransferase and was able to phosphorylate acid α-glucosidase in vitro (Kudo and Canfield 2006). The comparative domain organization of six human MRH-containing proteins has been presented in Fig. 5.2.

The M6PR-H domain is the only recognized domain in the γ-subunit of GlcNAc phosphotransferase. This enzyme catalyzes the first step in the biosynthesis of M6P sorting signals in the *cis*-Golgi (Fig. 5.3) and the M6PR-H domain may be involved in the recognition of substrate glycans. GlcNAc phoshotransferase is present only in organisms which also possess M6PRs, and raises the interesting possibility that the proteins involved in the synthesis and recognition of M6P evolved together from a common ancestor. The M6PRs and the GlcNAc-phosphotransferase, both essential components of the lysosomal enzyme targeting machinery, contain M6PR-H domains (Munro 2001). Lee et al. (2007), however, have shown that transgenic mice deficient in the γ-subunit of GlcNAc-phosphotransferase retain substantial activity toward acid hydrolases and that γ-subunit is not essential for substrate recognition. The α/β-subunits, in addition to their catalytic function, have some ability to recognize acid hydrolases as specific substrates. However, Lee et al. (2007) suggest that this specific recognition is somehow enhanced by the presence of γ-subunit. Clearly, detailed analysis of the putative carbohydrate binding properties of these M6PR-H-containing proteins is lacking. Alignment studies predict that M6PR-H domains containing three out of four conserved residues (Arg, Glu, Tyr) of M6PRs interact with 2-, 3-, and 4-hydroxyl groups of the mannose ring of M6P (Olson et al. 2004). It is likely that M6PR-H-containing proteins bind specifically to high mannose-type oligosaccharides, a concept in agreement with their proposed functions in ER -Golgi compartments (c/r Dahms et al. 2008; Dahms and Hancock 2002).

5.4 α-Glucosidase II

5.4.1 Function of α-Glucosidase II

The α-Glucosidase II (GII) is a soluble ER-resident heterodimer composed of two tightly but noncovalently bound α and β chains (GIIα and GIIβ) (Trombetta et al. 2001). GIIα is a 95–110 kDa protein that contains the consensus sequence (G/F)(L/I/V/M)WXDMNE) of the active site of family 31 glycosyl hydrolases and lacks the ER retention signal – XDEL at its C-terminus (Trombetta et al. 1996; D'Alessio et al. 1999). This subunit has a single active site but it has been proposed to have different kinetics for the first and second cleavages (Alonso et al. 1993), although recent work suggests that the differential trimming rates of both Glc units is not operative at the high protein concentrations occurring within the ER lumen (Totani et al. 2008).

Glucosidase II is responsible for the sequential removal of the two innermost glucose residues from the glycan G_3M_9 transferred to Asn residues in proteins. GII participates in the calnexin/calreticulin cycle; it removes the single glucose unit added to folding intermediates and misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. Trimming of the first α1,3-linked glucose from $Glc_2Man_9GlcNAc_2$ (G2M9) is important for a glycoprotein to interact with calnexin/calreticulin (CNX/CRT), and cleavage of the innermost glucose from $Glc_1Man_9GlcNAc_2$ (G1M9) sets glycoproteins free from the CNX/CRT cycle and allows them to proceed to the Golgi apparatus.

Using a genetic approach, the heterodimeric nature of GII was demonstrated in the fission yeast *Schizosaccharomyces pombe* and the microsomes from ΔGIIα and ΔGIIβ mutant cells were devoid of GII activity when using $Glc_1Man_9GlcNAc_2$ (G1M9) as substrate in the assays. Nevertheless, whereas *N*-glycans formed in intact ΔGIIα cells were identified as $Glc_2Man_9GlcNAc_2$ (G2M9), ΔGIIβ cells formed, in addition, small amounts of G1M9 (D'Alessio et al. 1999). It was suggested then that this last compound was formed either by GIIα transiently present in the ER in its way to secretion and/or by low amounts of GIIα that folded successfully in the absence of GIIβ. Moreover, ΔGIIβ cells presented the unfolding protein response as the BiP gene was induced in these mutant cells, thus showing that the subunit plays a key role in the efficient folding of glycoproteins. *S. pombe* is an organism that displays a glycoprotein folding quality control mechanism similar to that occurring in mammalian cells and it expresses an active GT (Fernández et al. 1994; D'Alessio et al. 1999). Furthermore, *S. pombe* GIIβ presents a high homology to its mammalian counterpart, including the presence of a consensus ER retention/retrieval sequence at its C terminus. Stigliano et al. (2009) reported that in the absence of GIIβ, the catalytic subunit GIIα of the fission yeast *S. pombe* folds to an active conformation able to hydrolyze *p*-nitrophenyl α-D-glucopyranoside. However, the heterodimer is required to efficiently deglucosylate the physiological substrates $Glc_2Man_9GlcNAc_2$ (G_2M_9) and $Glc_1Man_9GlcNAc_2$ (G_1M_9) (Fig. 5.1). The interaction of the mannose 6-phosphate receptor homologous domain present in GIIβ and mannoses in the B and/or C arms of the glycans mediates glycan

hydrolysis enhancement. Evidence indicates that also in mammalian cells GIIβ modulates G_2M_9 and G_1M_9 trimming (Stigliano et al. 2009).

The roles of GIIβ are controversial and have been object of growing interest in the last years, as autosomal dominant polycystic liver disease may develop in individuals carrying mutations in GIIβ gene (Drenth et al. 2005; Davila et al. 2004) and GIIβ is induced in differentiating neuritic rat progenitor cells and in response to the glial cell-derived neurotrophic factor (Hoffrogge et al. 2007). GIIβ is a 50- to 60-kDa subunit that has been suggested to be responsible for GIIα maturation to an active conformation in mammals (Pelletier et al. 2000; Treml et al. 2000) as well as for its presence in the ER as GIIβ displays an ER retention/retrieval consensus sequence at its C-terminus (Trombetta et al. 1996; D'Alessio et al. 1999). In contrast, the GIIβ subunit of the budding yeast *S. cerevisiae* does not display a consensus ER retention/retrieval sequence. Furthermore, GIIα was also retained in the ER of GIIβ null mutants and G_1M_9 was the *N*-glycan formed in these last cells, indicating that GIIβ is required for the second but not for the first cleavage (Wilkinson et al. 2006). It is to be noted that this microorganism lacks a classical CNX/CRT cycle because it does not express an active GT (Fernández et al. 1994).

5.4.2 M6PRH Domain in GIIβ

The β subunit of ER GII contains an M6PR-H domain preceded by two regions of coiled-coil and two complement-like repeats. A study has suggested a possible involvement of the M6PR-H domain of GIIβ (GIIβ-M6PR-H) in the glucose trimming process via its putative sugar-binding activity. Human GIIβ-M6PR-H binds to high-mannose-type glycans most strongly to the α1,2-linked mannobiose structure. In absence of GIIβ, the catalytic subunit GIIα of fission yeast *S. pombe* folds to an active conformation able to hydrolyze p-nitrophenyl α-d-glucopyranoside. Deletion of the homologue of the noncatalytic β-subunit in *S. pombe* drastically reduces glucosidase II activity. However, the heterodimer is required to efficiently deglucosylate the physiological substrates G_2M_9 and G_1M_9. The interaction of the M6PR-H domain present in GIIβ and mannoses in the B and/or C arms of the glycans mediates glycan hydrolysis enhancement. Results demonstrate the capacity of the GIIβ-M6PR-H to bind high-mannose-type glycans and its importance in efficient glucose trimming of N-glycans (Hu et al. 2009; Stigliano et al. 2009). As in GlcNAc phosphotransferase, the M6PR-H domain may serve to recognize the substrate glycan. Through its C-terminal HDEL signal, the β-subunit of GIIβ may retain the complete α1β1 complex in the ER (Trombetta et al. 2001).

5.4.3 Two Distinct Domains of β-Subunit of GII Interact with α-Subunit

The β-subunit, which contains a C-terminal His-Asp-Glu-Leu (HDEL) motif, may function to link the catalytic subunit to the KDEL receptor as a retrieval mechanism. DNA sequencing has revealed that both subunits are encoded by gene products that undergo alternative splicing in T lymphocytes. The catalytic α-subunit possesses two variably expressed segments, box A-1, consisting of 22 amino acids located proximal to the amino-terminus, and box A-2, composed of 9 amino acids situated between the amino-terminus and the putative catalytic site in the central region of the molecule. Box B1, a variably expressed seven amino acid segment in the β-subunit of glucosidase II, is located immediately downstream of an acidic stretch near the carboxyl-terminus. Screening of reverse transcribed RNA by PCR confirms the variable inclusion of each of these segments in transcripts obtained from a panel of T-lymphocyte cell lines. Thus, distinct isoforms of glucosidase II exist that may perform specialized functions (Arendt et al. 1999).

The human cDNA sequences of the α-subunit predicted a soluble protein (104 kDa) devoid of known signals for residence in the ER. It showed homology with several other glucosidases but not with glucosidase I. The α- subunit was the functional catalytic subunit of glucosidase II. The sequence of the β subunit (58 kDa) showed no sequence homology with other known proteins. It encoded a soluble protein rich in glutamic and aspartic acid with a putative ER retention signal (HDEL) at the C terminus. This suggested that the β subunit is responsible for the ER localization of the enzyme (Trombetta et al. 1996). The two subunits form a defined complex, composed of one catalytic subunit and one accessory subunit $\alpha_1\beta_1$ with a molecular mass of 161 kDa. The complex had an s value of 6.3 S, indicative of a highly nonglobular shape. The β subunit could be proteolytically removed from $\alpha_1\beta_1$ complex without affecting catalysis, demonstrating that it is not required for glucosidase II activity in vitro. It was suggested that the catalytic core of glucosidase II resides in a globular domain of the α-subunit, which can function independently of β subunit, while the complete α- and β subunits assemble in a defined heterodimeric complex with a highly extended conformation, which may favor interaction with other proteins in ER. Through its C-terminal HDEL signal, the β subunit may retain the complete $\alpha_1\beta_1$ complex in the ER (Trombetta et al. 2001).

Arendt and Ostergaard (2000) mapped the regions of the mouse β-subunit protein responsible for mediating the association with the α-subunit and identified two non-overlapping interaction domains (ID1 and ID2) within the β-subunit. ID1 encompasses 118 amino acids at the N-terminus of the mature polypeptide, spanning the cysteine-rich element in this region. ID2, located near the C-terminus, is contained within amino

acids 273–400, a region occupied in part by a stretch of acidic residues. Based on experimental facts it was postulated that the catalytic subunit of glucosidase II binds synergistically to ID1 and ID2, explaining the high associative stability of the enzyme complex (Arendt and Ostergaard 2000). *S. cerevisiae* gene *GTB1* encodes, a polypeptide with 21% sequence similarity to the β-subunit of human glucosidase II. The Gtb1 protein was shown to be a soluble glycoprotein (96–102 kDa) localized to the ER lumen where it was present in a complex together with the yeast α-subunit homologue Gls2p. Surprisingly, Δgtb1 mutant cells were specifically defective in the processing of monoglucosylated glycans. Thus, although Gls2p is sufficient for cleavage of the penultimate glucose residue, Gtb1p is essential for cleavage of the final glucose. Data demonstrate that Gtb1p is required for normal glycoprotein biogenesis and reveal that the final two glucose-trimming steps in N-glycan processing are mechanistically distinct (Wilkinson et al. 2006).

Watanabe et al. (2009) reported an in vivo enzymatic analysis using gene disruptants lacking either the G-II α- or β-subunit in the filamentous fungus *Aspergillus oryzae*. The fraction lacking the β-subunit retained hydrolytic activity toward p-nitrophenyl α-D-glucopyranoside but was inactive toward both $Glc_2Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$. When the fraction containing the β-subunit was added to the one including the α-subunit, the glucosidase activity was restored. These results suggested that the β-subunit confers the substrate specificity toward di- and monoglucosylated glycans on the glucose-trimming activity of the α-subunit (Watanabe et al. 2009). In order to further dissect these activities Quinn et al. (2009) mutagenized a number of conserved residues across the protein. Both the M6PRH and G2B domains of Gtb1p contribute to the Glc2 trimming event but the M6PR-H domain is essential for Glc1 trimming (Quinn et al. 2009).

5.4.4 Polycystic Liver Disease (PCLD) and β-Subunit of Glucosidase II

Autosomal-dominant polycystic liver disease (PCLD) is a rare disorder that is characterized by the progressive development of fluid-filled biliary epithelial cysts spread throughout the liver parenchyma and characterized by an increased liver volume due to many (>20) fluid-filled cysts of biliary origin. Positional cloning has identified two genes that are mutated in patients with polycystic liver disease, *PRKCSH and SEC63*, which encode the hepatocystin (β-subunit of glucosidase II) and Sec63, respectively. Both proteins are components of the molecular machinery involved in the translocation, folding and quality control of newly synthesized glycoproteins in the ER (Drenth et al. 2003). Most mutations are truncating and probably lead to a complete loss of the corresponding proteins and the defective processing of a key regulator of biliary cell growth. The finding that PCLD is caused by proteins involved in oligosaccharide processing was unexpected and implicates a new avenue for research into neocystogenesis, and might ultimately result in the identification of novel therapeutic drugs (Davila et al. 2004; Drenth et al. 2005) (see Chap. 45).

Hepatocystin is a protein kinase C substrate, a component of a cytosolic signal transduction complex, a receptor for advanced glycation end products, a vacuolar protein, and the β subunit of ER glucosidase II. The exact localization and cellular function of hepatocystin remain unclear. Normal hepatocystin localizes to the ER, where it assembles with the glucosidase II α-subunit. The 1338–2A—>G truncating mutation in hepatocystin observed in some polycystic liver disease patients produces a protein that is not retained in the ER but is secreted into the medium. This mutant protein fails to assemble with the glucosidase II α-subunit. As a consequence, mutant hepatocystin is undetectable in liver cysts. In addition, levels of normal hepatocystin and of the glucosidase II α-subunit are substantially reduced in liver and Epstein-Barr virus-immortalized B lymphoblasts from patients with polycystic liver disease. These findings are consistent with a role of hepatocystin in carbohydrate processing and quality control of newly synthesized glycoproteins in the ER. Therefore, altered ER processing of some key regulator of cell proliferation may underlie polycystic liver disease (Drenth et al. 2004).

Disease causing mutations in *PRKCSH* or *SEC63* are found in approximately 25% of the PCLD patients. Both gene products function in the ER, however, the molecular mechanism behind cyst formation remains to be elucidated. As part of the translocon complex, SEC63 plays a role in protein import into the ER and is implicated in the export of unfolded proteins to the cytoplasm during ERAD. *PRKCSH* codes for the β-subunit of glucosidase II (hepatocystin), which cleaves two glucose residues of $Glc_3Man_9GlcNAc_2$ N-glycans on proteins. Hepatocystin is thereby directly involved in the protein folding process by regulating protein binding to calnexin/calreticulin in the ER. A separate group of genetic diseases affecting protein N-glycosylation in the ER is formed by the congenital disorders of glycosylation (CDG). In distinct subtypes of this autosomal recessive multisystem disease specific liver symptoms have been reported that overlap with PCLD. Recent research revealed novel insights in PCLD disease pathology such as the absence of hepatocystin from cyst epithelia indicating a two-hit model for PCLD cystogenesis. This opens the way to speculate about a recessive mechanism for PCLD

pathophysiology and shared molecular pathways between CDG and PCLD (Janssen et al. 2010).

PCLD is often asymptomatic, but if symptoms arise, they are usually due to the mass effect of cysts. The phenotype is more severe in females and correlates with the number of pregnancies or estrogen use. The gene for PCLD has been assigned to chromosome 19p13.2–13.1. Mutations found in *PRKCSH* introduce stopcodons in the m-RNA, resulting in premature termination of translation to protein. The protein, designated as hepatocystin, is predicted to be localised in the ER (Jansen et al. 2009).

The PCLD is a distinct clinical and genetic entity that can occur independently from autosomal dominant polycystic kidney disease (PCKD) (Chap. 45). In contrast to PKD1, PKD2, and PKHD1, normal PRKCSH encodes a human protein termed "protein kinase C substrate 80 K-H" or "noncatalytic β-subunit of glucosidase II also designated as hepatocystin (Jansen et al. 2009)," which is highly conserved, expressed in all tissues, and contains a leader sequence, an LDLα domain, two EF-hand domains, and a conserved C-terminal HDEL sequence. Its function may be dependent on calcium binding, and its putative actions include the regulation of N-glycosylation of proteins and signal transduction via fibroblast growth-factor receptor. In light of the focal nature of liver cysts in PCLD, the apparent loss-of-function mutations in PRKCSH, and the two-hit mechanism operational in dominant polycystic kidney disease, PCLD may also occur by a two-hit mechanism (Li et al. 2003).

5.5 Osteosarcomas-9 (OS-9)

5.5.1 The Protein

OS-9 is a *N*-glycosylated protein expressed in two splice variants in the ER lumen. Transcription of both OS-9 variants is enhanced upon activation of Ire1/Xbp1 pathway in cells exposed to acute ER stress. OS-9 variants do not associate with folding-competent proteins, but form non-covalent complexes with misfolded ones. OS-9 association prevents secretion from the ER of misfolded NHK conformers and facilitates NHK disposal. OS-9 variants play a crucial role in maintaining the tightness of retention-based ER quality control and in promoting disposal of misfolded proteins from the mammalian ER. The OS-9 gene maps to a region (q13–15) of chromosome 12 that is highly amplified in human osteosarcomas and encodes a protein of unknown function. Su et al. (1996) identified two genes (OS-9 and OS-4) within 12q13–15, a region frequently amplified in human cancers. The full-length OS-9 cDNA sequence consists of 2,785 bp with an ORF of 667 amino-acids. The predicted polypeptide was water soluble and acidic. The OS-9 gene encoded a 2.8-kb mRNA transcribed, ubiquitously expressed in human tissues and revealed significant similarities with two ORFs from genomic sequences in *C. elegans* and *S. cerevisiae*. The ORF region comprises a functional domain present in a novel evolutionarily conserved gene family defined by OS-9. The gene spanned ~30.4 kbp and had 15 exons. The 1,010 bp sequence of the 5′ upstream region contains binding-sequence motifs TATA and CCAAT for general transcription. Primer extension analysis revealed two putative transcription start sites (Kimura et al. 1997).

Three isoforms of OS-9 cDNA were found in a myeloid leukemia HL-60 cDNA library. Isoform 1 consisted of 2,700 bp from which a 667 amino acid sequence was found to be identical with that of OS-9 cDNA from osteosarcoma cells. Isoform 2 cDNA lacked a 165 nt sequence in the coding region. Isoform 3 cDNA had an additional 45 bp deletion in coding region. Isoforms 2 and 3 encode 612 and 597 amino acid polypeptides, respectively. These three isoforms were found to be splice variants. Isoform 2 mRNA expressed predominantly in myeloid leukemia HL-60 cells, osteosarcoma OsA-CL cells and rhabdomyosarcoma Rh30 cells and in various tumor cell lines of sarcoma cells, carcinoma cells and myeloid leukemia cells, but 3–4 times higher expression in OsA-CL cells and Rh30 cells containing a homogeneously staining region of 12q13-15 (Kimura et al. 1998).

OS-9 is conserved in eukaryotes and functions together with M-type lectins in ERAD pathway used to get rid of irreversibly misfolded glycoproteins. OS-9 appears to identify glycoprotein targets through recognition of both N-linked glycans and other determinants.

5.5.2 Requirement of HRD1, SEL1L, and OS-9/XTP3-B for Disposal of ERAD-Substrates

In the ER, lectins and processing enzymes are involved in quality control of newly synthesized proteins for productive folding as well as in ERAD of misfolded proteins. Misfolded glycoproteins are translocated from the ER into the cytoplasm for proteasome-mediated degradation. While Htm1/EDEM protein has been proposed to act as a "degradation lectin" for ERAD of misfolded glycoproteins, OS-9 and XTP3-B/Erlectin are ER-resident glycoproteins that bind to ERAD substrates and, through the SEL1L adaptor, to the ER-membrane-embedded ubiquitin ligase HRD1. OS-9 protein is thought to participate in ERAD. Both proteins contain conserved mannose 6-phosphate receptor homology (MRH) domains, which are required for interaction with SEL1L, but

not with substrate. OS-9 associates with ER chaperone GRP94 which, together with HRD1 and SEL1L, is required for the degradation of an ERAD substrate, mutant α1-antitrypsin. It is suggested that XTP3-B and OS-9 are components of distinct, partially redundant, quality control surveillance pathways that coordinate protein folding with membrane dislocation and ubiquitin conjugation in mammalian cells (Christianson et al. 2008; Tamura et al. 2008; Bernasconi et al. 2010). Surprisingly, however, OS-9 is not required for ubiquitination or degradation of this nonglycosylated ERAD substrate. In a model, OS-9 recognises terminally misfolded proteins via polypeptide-based rather than glycan-based signals, but is only required for transferring those bearing N-glycans to the ubiquitination machinery (Alcock and Swanton 2009).

5.5.3 OS-9 Recognizes Mannose-Trimmed N-Glycans

Mannose trimming from the N-glycans plays an important role in targeting of misfolded glycoproteins for ERAD. It was demonstrated that the recombinant hOS-9 M6P-R homology domain specifically binds N-glycans lacking the terminal mannose from the C branch in vitro. The ability of hOS-9 to enhance glycoprotein ERAD depended on the N-glycan structures on NHK. Hosokawa et al. (2009) proposed a model for mannose trimming and the requirement for hOS-9 lectin activity in glycoprotein ERAD in which N-glycans lacking the terminal mannose from C branch are recognized by hOS-9 and targeted for degradation (Hosokawa et al. 2009) (Fig. 5.4). Results suggest that trimming of the outermost α1,2-linked mannose on the C-arm is a critical process for misfolded proteins to enter ERAD (Hosokawa et al. 2009; Mikami et al. 2010; Riemer et al. 2009).

5.5.4 Dual Task for Xbp1-Responsive OS-9 Variants

Alternative splicing products of OS-9 gene, OS-9.1 and OS-9.2, are ubiquitously expressed in human tissues and are amplified in tumors. They are transcriptionally induced upon activation of the Ire1/Xbp1 ER-stress pathway. OS-9 variants do not associate with folding-competent proteins. Rather, they selectively bind folding-defective ones thereby inhibiting transport of non-native conformers through the secretory pathway. The intralumenal level of OS-9.1 and OS-9.2 inversely correlates with the fraction of a folding-defective glycoprotein, the Null(hong kong) (NHK) variant of α1-antitrypsin that escapes retention-based ER quality control. OS-9 up-regulation does not affect NHK disposal, but reduction of the intralumenal level of OS-9.1 and OS-9.2 substantially delays disposal of this model substrate. OS-9.1 and OS-9.2 also associate transiently with non-glycosylated folding-defective proteins, but association is unproductive. Finally, OS-9 activity does not require an intact mannose 6-P homology domain. Thus, OS-9.1 and OS-9.2 play a dual role in mammalian ER quality control: first as crucial retention factors for misfolded conformers, and second as promoters of protein disposal from the ER lumen (Bernasconi et al. 2008). Vourvouhaki et al. (2007) suggested that OS-9 promotes cell viability and confers resistance to apoptosis, potentially implicating OS-9 in the survival of cancer cells.

5.5.5 Interactions of OS-9

N-copine is a two C2 domain protein that shows Ca^{2+}-dependent phospholipid binding and membrane association. OS-9 is capable of interacting with N-copine. The second C2 domain of N-copine binds with the carboxy-terminal region of OS-9 in Ca^{2+}-dependent manner. N-copine and OS-9 are co-expressed in the same regions of human brain (Nakayama et al. 1999). OS-9 interacts with the membrane proteinase meprin β found in brush border membranes of kidney and small intestine. This cytoplasmic region is indispensable for the maturation of meprin β, which included an ER-to-Golgi translocation. OS-9 associates with meprin β only transiently, coinciding with ER-to-Golgi transport of meprin β. The OS-9-binding site in the cytoplasmic domain of meprin β overlaps the region essential for this transport. The alternatively spliced forms of rat and mouse OS-9 have been characterized, but that only the non-spliced form of OS-9 binds to meprin β. It indicated that OS-9 may be involved in the ER-to-Golgi transport of meprin β (Litovchick et al. 2002). While the presence of a single *N*-linked oligosaccharide in the middle of the M6PR-H domain of OS-9 would inhibit the ability of the M6PR-H domain to function in stabilizing a multiprotein complex (Bernasconi et al. 2008), arguments of Dahms et al. (2008) suggest additional studies to be performed to test the hypothesis that the presence of an *N*-glycan chain inhibits the lectin activity of OS-9 and/or XTP3-B/Erlectin.

The presence of structural lesions in the luminal, transmembrane, or cytosolic domains determines the classification of misfolded polypeptides as ERAD-L, -M, or -C substrates and results in selection of distinct degradation pathways. It was shown that disposal of soluble (nontransmembrane) polypeptides with luminal lesions (ERAD-L_S substrates) is strictly dependent on the E3 ubiquitin ligase HRD1, the associated cargo receptor SEL1L, and two interchangeable ERAD lectins, OS-9 and XTP3-B. These

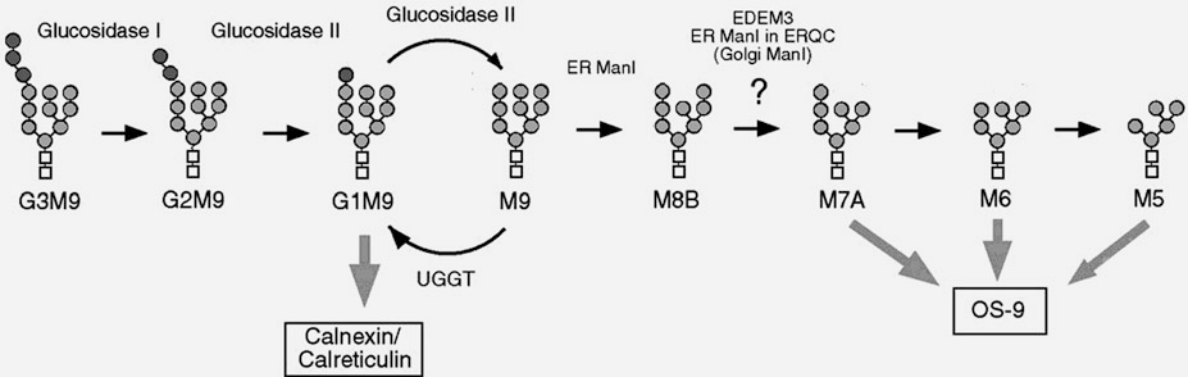

Fig. 5.4 *N*-glycan processing and recognition in mammals. Calnexin and calreticulin bind to monoglucosylated *N*-glycans and assist in the folding of glycoproteins. These lectins dissociate from the glycoprotein upon removal of the terminal glucose on monoglucosylated *N*-glycans by glucosidase II. In mammals, UDP-glucose:glycoprotein glucosyltransferase (*UGGT*), which is absent from the yeast genome, adds a glucose back onto the *N*-glycan if the glycoprotein has still not acquired the native conformation, thus forming the calnexin/calreticulin or monoglucose cycle. ER ManI generates M8B, followed by further mannose trimming to produce M7A ($Man_7GlcNAc_2$ isomer A), M6 ($Man6GlcNAc_2$) and M5 ($Man5GlcNAc_2$) *N*-glycans, which are recognized by OS-9. The candidate α1,2-mannosidases responsible for this process include EDEM3, ER ManI concentrated in the ER quality control compartment (*ERQC*), and Golgi α1,2-mannosidases for the ERAD substrates that may recycle between the ER and Golgi prior to degradation (Adapted by permission from Hosokawa et al. 2010 © Oxford University Press)

ERAD factors become dispensable for degradation of the same polypeptides when membrane tethered (ERAD-L_M substrates). Studies reveal that, in contrast to budding yeast, tethering of mammalian ERAD-L substrates to the membrane changes selection of the degradation pathway (Bernasconi et al. 2010). Several *N*-glycans containing terminal ∝1,6-linked mannose in the Man∝1,6(Man∝1,3) Man∝1,6(Man∝1,3)Man structure are good ligands for $OS\text{-}9^{M6PRH}$, having K_a values of approximately 10^4 M^{-1} and that trimming of either an ∝1,6-linked mannose from the C-arm or an ∝1,3-linked mannose from the B-arm abrogated binding to $OS\text{-}9^{M6PRH}$. An immunoprecipitation experiment demonstrated that the ∝1-antitrypsin variant $null^{Hong\ Kong}$, but not wild-type ∝1-antitrypsin, selectively interacted with OS-9 in the cells in a sugar-dependent manner. These results suggest that trimming of the outermost ∝1,2-linked mannose on the C-arm is a critical process for misfolded proteins to enter ERAD (Mikami et al. 2010).

An Auxiliary Protein for TRPV4 Maturation: Transient receptor potential (TRP) proteins constitute a family of cation-permeable channels that are formed by homo- or heteromeric assembly of four subunits. However, the mechanisms governing TRP channel assembly and biogenesis in general, remain largely unknown. OS-9 interacts with the cytosolic N-terminal tail of TRPV4. The underlying mechanisms revealed that OS-9 preferably binds TRPV4 monomers and other ER- variants of TRPV4 and attenuates their polyubiquitination. Thus, OS-9 regulates the secretory transport of TRPV4 and appears to protect TRPV4 subunits from the precocious ubiquitination and ER-associated degradation. Data suggest that OS-9 functions as an auxiliary protein for TRPV4 maturation (Wang et al. 2007).

OS9 Interacts with DC-STAMP: Dendritic cell-specific transmembrane protein (DC-STAMP) has been identified in a cDNA library of human monocyte-derived DCs. DC-STAMP is a multimembrane spanning protein that has been implicated in skewing haematopoietic differentiation of bone marrow cells towards the myeloid lineage, and in cell fusion during osteoclastogenesis and giant cell formation. (Jansen et al. 2009) reported that amplified OS9 physically interacts with DC-STAMP, and that both proteins co-localize in the ER in various cell lines, including immature DC. The TLR-induced maturation of DC leads to the translocation of DC-STAMP from the ER to the Golgi while OS9 localization is unaffected. Collectively, results indicate that OS9 is critically involved in the modulation of ER-to-Golgi transport of DC-STAMP in response to TLR triggering, suggesting a novel role for OS9 in myeloid differentiation and cell fusion (Jansen et al. 2009, 2010).

Interaction of OS9 with HIF-1: Hypoxia-inducible factor 1 (HIF-1) functions as a master regulator of oxygen homeostasis in metazoan. HIF-1 mediates changes in gene transcription in response to changes in cellular oxygenation. OS-9 interacts with both HIF-1α and HIF-1α prolyl hydroxylases. OS-9 gain-of-function promotes HIF-1α hydroxylation, VHL binding, proteasomal degradation of HIF-1α, and inhibition of HIF-1-mediated transcription. The loss of function of OS-9 by iRNA increases HIF-1α protein levels, HIF-1-mediated transcription, and vascular endothelial growth factor

(VEGF) mRNA expression under nonhypoxic conditions. These data indicate that OS-9 is an essential component of a multiprotein complex that regulates HIF-1α levels in an O_2-dependent manner (Baek et al. 2005). HIF-1α is believed to promote tumor growth and metastasis, and many efforts have been made to develop new anticancer agents based on HIF-1α inhibition. YC-1 is a widely used HIF-1α inhibitor both in vitro and in vivo, and is being developed as a novel class of anticancer drug (Kim et al. 2006).

5.6 YOS9 from *S. cerevisiae*

Due to easy manipulating of yeast genome, many aspects and components of ERAD have been discovered in *S. cerevisiae* (Brodsky 2007; Molinari et al. 2007). Studies on the involvement of mammalian ortholog OS-9 in ERAD have been hampered by data showing that OS-9 is a cytosolic protein (Litovchick et al. 2002). Studies were followed by a series of publications in which experimental design and interpretation of the data were based on the assumption that OS-9 is a cytosolic protein (Baek et al. 2005; Flashman et al. 2005; Wang et al. 2007). A homolog, Yos9, from *S. cerevisiae* is a membrane-associated glycoprotein that localizes to ER. Reports revealed that Yos9p is required for disposal of substrates with luminal folding defects, whereas it is dispensable for disposal of proteins with defects in the transmembrane and cytosolic domains (Carvalho et al. 2006; Denic et al. 2006; Szathmary et al. 2005). In yeast, the key pathway of ER quality control consists of a two-lectin receptor system made of Yos9p and Htm1/Mnl1p that recognizes N-linked glycan signals embedded in substrates. Szathmaryet al. (2005) provided genetic and biochemical evidence that Yos9 protein in *S. cerevisiae* is essential for efficient degradation of mutant glycoproteins. Yos9 is a member of the OS-9 protein family, which is conserved among eukaryotes and shows similarities with M6PRs. The amino acids conserved among OS-9 family members and M6PRs are essential for Yos9 protein function. Immunoprecipitation studies showed that Yos9 specifically associates with misfolded carboxypeptidase Y (CPY*), an ERAD substrate, but only when it carried $Man_8GlcNAc_2$ or $Man_5GlcNAc_2$ N-glycans. Experiments further suggested that Yos9 acts in the same pathway as Htm1/EDEM. Yos9 protein is important for glycoprotein degradation and may act via its M6PRH domain as a degradation lectin-like protein in the glycoprotein degradation pathway. Yos9p forms a complex with substrates and has a sugar binding pocket that is essential for its ERAD function. Nonetheless, substrate recognition persists even when the sugar binding site is mutated or CPY* is unglycosylated (Bhamidipati et al. 2005). However, Yos9p binds directly to substrates to discriminate misfolded from folded proteins. Substrates displaying cytosolic determinants can be degraded independently of this system. Studies suggest that mechanistically divergent systems collaborate to guard against passage and accumulation of misfolded proteins in secretory pathway. These and other considerations suggest that Yos9p plays a critical role in the bipartite recognition of terminally misfolded glycoproteins (Bhamidipati et al. 2005; Kim et al. 2005; Cormier et al. 2005).

YOS9 gene interacts with genes involved in ER-Golgi transport, particularly SEC34, whose temperature-sensitive mutant is rescued by *YOS9* over-expression. Interestingly, Yos9 appears to play a direct role in the transport of glycosylphosphatidylinositol (GPI)-anchored proteins to the Golgi apparatus. Yos9 binds directly to Gas1 and Mkc7 and accelerates Gas1 transport and processing in cells over-expressing. As Yos9 is not a component of the Emp24 complex, it may act as a novel escort factor for GPI-anchored proteins in ER-Golgi transport in yeast and possibly in mammals (Friedmann et al. 2002; Vigneron et al. 2002). Gauss et al. (2006a) reported an association between Yos9p and Hrd3p, a component of the ubiquitin-proteasome system that links these pathways. These workers identified designated regions in the luminal domain of Hrd3p that interact with Yos9p and the ubiquitin ligase Hrd1p. Binding of misfolded proteins occurs through Hrd3p, suggesting that Hrd3p recognizes proteins that deviate from their native conformation, whereas Yos9p ensures that only terminally misfolded polypeptides are degraded. Key components of ERAD are ER membrane-bound ubiquitin ligases. These ligases associate with the cytoplasmic AAA-ATPase Cdc48p/p97, which is thought to support the release of malfolded proteins from the ER. Gauss et al. (2006b) characterized a yeast protein complex containing ubiquitin ligase Hrd1p and the ER membrane proteins Hrd3p and Der1p. Hrd3p binds mis-folded proteins in ER lumen enabling their delivery to downstream components. Therefore, it was proposed that Hrd3p acts as a substrate recruitment factor for the Hrd1p ligase complex. Hrd3p function is also required for the association of Cdc48p with Hrd1p. Moreover, results demonstrated that recruitment of Cdc48p depends on substrate processing by Hrd1p ligase complex. Thus, Hrd1p ligase complex unites substrate selection in ER lumen and polyubiquitination in the cytoplasm and links these processes to the release of ER proteins via Cdc48p complex (Gauss et al. 2006b). Thus, Yos9p associates with the membrane-embedded ubiquitin ligase complex, Hrd1p-Hrd3p, and provides a proofreading mechanism for ERAD. The mammalian homologues of Yos9p, OS-9 and XTP3-B are also ER resident proteins that associate with the HRD1-SEL1L ubiquitin ligase complex and are important for the regulation of ERAD. Recent studies have also identified the N-glycan species with which both yeast Yos9p and mammalian OS-9 associate as M7A, a $Man_7GlcNAc_2$ isomer that lacks the

α1,2-linked terminal mannose from both the B and C branches. M7A has been known since then as a degradation signal in both yeast and mammals (Hosokawa et al. 2010). Taken together, a clear picture has not yet emerged concerning the role of M6PR-H domains in Yos9, OS-9, and XTP3-B/Erlectin.

5.7 Erlectin/XTP3-B

Erlectin, also referred to as XTP3-B, is a luminal ER-resident protein first characterized in *Xenopus*. In *Xenopus* XTP3-B has been shown to be involved in the regulation of glycoprotein trafficking and to be essential during development. It is present in vertebrate and invertebrate animals. XTP3-B is a soluble ER-resident protein that contains two M6PR-H domains in its sequence. XTP3-B interacts with a membrane-associated ubiquitin ligase complex, and is thought to participate in ERAD. Kremen1 and 2 (Krm1/2) are co-receptors for Dickkopf1 (Dkk1), an antagonist of Wnt/β-catenin signaling, and play a role in head induction during early *Xenopus* development. Erlectin (XTP3-B) containing M6PR-H- or PRKCSH- domains is implicated in N-glycan binding. Like other members of M6PR-H family, Erlectin is essential for Krm2 binding, and this interaction is abolished by Krm2 deglycosylation. Results indicate that Erlectin functions in N-glycan recognition in the endoplasmic reticulum, suggesting that it may regulate glycoprotein traffic (Cruciat et al. 2006).

Human XTP3-B (hXTP3-B) has two transcriptional variants, and both isoforms retard ERAD of the human α1-antitrypsin variant null Hong Kong (NHK), a terminally misfolded glycoprotein. The hXTP3-B long isoform strongly inhibited ERAD of NHK-QQQ, which lacks all of the N-glycosylation sites of NHK, but the short transcriptional variant of hXTP3-B had no effect. Examination revealed that the hXTP3-B long isoform associates with the HRD1-SEL1L membrane-anchored ubiquitin ligase complex and BiP and forms a 27 s ER quality control scaffold complex. The hXTP3-B short isoform, however, is excluded from scaffold formation. Another MRH domain-containing ER lectin, hOS-9, is incorporated into this large complex, but gp78, another mammalian homolog of the yeast ubiquitin ligase Hrd1p, is not (Hosokawa et al. 2008). XTP3-B specifically binds to AT (NHK) via a C-terminal M6PR-H domain in a glycan-dependent manner (Yamaguchi et al. 2010). These results indicate that the large ER quality control scaffold complex, containing ER lectins, a chaperone, and a ubiquitin ligase, provides a platform for the recognition and sorting of misfolded glycoproteins as well as nonglycosylated proteins prior to retrotranslocation into the cytoplasm for degradation (Hosokawa et al. 2008).

5.8 *Drosophila* Lysosomal Enzyme Receptor Protein (LERP)

A type I transmembrane protein termed lysosomal enzyme receptor protein (LERP) with partial homology to the mammalian M6PR-300 encoded by gene CG31072 was identified in *Drosophila* (Dennes et al. 2005). LERP contains 5 lumenal repeats that share homology to the 15 lumenal repeats found in all M6PR-300. Four of the repeats display the P-lectin type pattern of conserved cysteine residues. However, the arginine residues identified to be essential for M6P binding are not conserved. The LERP cytoplasmic domain shows highly conserved interactions with *Drosophila* and mammalian GGA adaptors known to mediate Golgi-endosome traffic of M6PRs and other transmembrane cargo. Moreover, LERP rescues missorting of soluble lysosomal enzymes in MPR-deficient cells, giving strong evidence for a function that is equivalent to the mammalian counterpart. However, unlike the mammalian M6PRs, LERP did not bind to the multimeric M6P ligand phosphomannan. LERP plays important role in biogenesis of *Drosophila* lysosomes; the GGA functions in the receptor-mediated lysosomal transport system in the fruit fly (Dennes et al. 2005). Authors showed that LERP, which is able to interact with *Drosophila* and mammalian Golgi-localized, Gamma-ear-containing, ADP-ribosylation factor-binding (GGA) adaptors that have been shown to sort M6PRs in clathrin-coated vesicles at the TGN (Bonifacino 2004; Ghosh and Kornfeld 2004), mediates lysosomal enzyme targeting and rescues the missorting of lysosomal enzymes that occurs in MPR-deficient mammalian cells. The protein-sorting machinery in fly cells is well conserved relative to that in mammals, enabling the use of fly cells to dissect CCV biogenesis and clathrin-dependent protein trafficking at the TGN of higher eukaryotes (Kametaka et al. 2010).

5.9 MRL1

In yeast, the mechanism by which soluble hydrolases, such as carboxypeptidase Y and proteinase A, reach the vacuole (functional equivalent of the lysosome) is very similar to that found in mammalian cells except that the vacuolar sorting signal is not carbohydrate-based, but rather resides in the propeptide region of hydrolase and is recognized by a receptor, Vps10, that cycles between the Golgi and endosomal compartments (Ni et al. 2006). The yeast genome contains an ORF that encodes a membrane protein that is distantly related to mammalian M6PRs. The protein encoded by this gene (which was termed MRL1) cycles through late endosome (Whyte and Munro 2001). MRL1 is a type I membrane glycoprotein that contains a single M6PR-H

domain like CD-MPR and co-localizes with Vps10 in *S. cerevisiae*. The vacuolar hydrolases of yeast *S. cerevisiae* do not contain 6-phosphate monoesters on their N-glycans, but contain the product of VPS10 gene as receptor for hydrolases. MRL1 also cycles between the Golgi and late endosome, but is unrelated to vertebrate M6PRs, and recognizes a specific amino acid sequence of carboxypeptidase Y. The delivery of carboxypeptidase Y or proteinase A was not affected in *S. cerevisiae* strains lacking MRL1. Moreover, there is a strong synergistic effect on the maturation of proteinases A and B when both MRL1 and VPS10 are deleted, which suggests that MRL1 may serve as a sorting receptor in the delivery of vacuolar hydrolases.

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Lectins of ERAD Pathway: F-Box Proteins and M-Type Lectins

6

G.S. Gupta

6.1 Intracellular Functions of N-Linked Glycans in Quality Control

Glycoprotein folding and degradation in endoplasmic reticulum (ER) is mediated by ER quality control system. Quality control in ER ensures that only properly folded proteins are retained in the cell through mechanisms that recognize and discard misfolded or unassembled proteins in a process called endoplasmic reticulum-associated degradation (ERAD). The ERAD pathway comprises multiple steps including substrate recognition and targeting to the retro-translocation machinery, retrotranslocation from the ER into the cytosol, and proteasomal degradation through ubiquitination. Roles of lectin-type molecules for trimmed high-mannose type N-glycans and glycosidases in the ERAD pathways in both ER and cytosol have been documented in recent years. Yoshida and Tanaka (2010) reviewed the fundamental system that monitors glycoprotein folding in the ER and the unique roles of sugar-recognizing ubiquitin ligase and peptide:N-glycanase (PNGase) in the cytosolic ERAD pathway. Mannose trimming plays an important role by forming specific *N*-glycans that permit the recognition and sorting of terminally misfolded conformers for ERAD. The EDEM (ER degradation enhancing α-mannosidase-like protein) subgroup of proteins belonging to Class I α1,2-mannosidase family (glycosylhydrolase family 47) has been shown to enhance ERAD. However, the mechanisms of substrate recognition and sorting to the ERAD pathway are poorly defined.

Post-translational modification of proteins regulates many cellular processes. For example, the attachment of *N*-linked glycans to nascent proteins in ER facilitates proper folding, whereas retention of high mannose glycans on misfolded glycoproteins serves as a signal for retrotranslocation and ubiquitin-mediated proteasomal degradation. N-glycosylation of proteins in ER has a central role in protein quality control. In ER and in early secretory pathway, where the repertoire of oligosaccharide structures is still small, the glycans play a pivotal role in protein folding, oligomerization, quality control, sorting, and transport. The glycans not only promote folding directly by stabilizing polypeptide structures but also indirectly by serving as recognition "tags" that allow glycoproteins to interact with a variety of lectins, glycosidases, and glycosyltranferases. Some of these (such as glucosidases I and II, calnexin, and calreticulin) have a central role in folding and retention, while others (such as α-mannosidases and EDEM) target unsalvageable glycoproteins for ER-associated degradation. Each residue in the core oligosaccharide and each step in the modification program have significance for the fate of newly synthesized glycoproteins. In the Golgi complex, the glycans acquire more complex structures and a new set of functions. The division of synthesis and processing between the ER and the Golgi complex represents an evolutionary adaptation that allows efficient exploitation of the potential of oligosaccharides (Helenius and Aebi 2004).

Most secretory and membrane proteins present outside cells are glycosylated. The oligosaccharides attached covalently to asparagine (N-linked) or serine/threonine (O-linked) of mature proteins have highly diverse structures and are implicated in many extracellular processes (1). This diversity of oligosaccharides is provided by hundreds of glycosyltransferases in Golgi complex. On the other hand, the N-linked oligosaccharides attached to newly synthesized polypeptides in ER are homogenous, relatively simple high-mannose oligosaccharides. High-mannose oligosaccharides are deeply involved in promoting protein folding and quality control, and in the trafficking and sorting of glycoproteins in that they are recognized by specific lectin-type molecules (2). Of these intracellular events, information on the role of high mannose oligosaccharides as tags for the quality control of proteins has been accumulated. The ER through a quality control mechanism discriminates correctly folded proteins from incorrectly folded or incompletely assembled

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non-functional proteins. (3) Protein folding and oligomer formation are assisted by ER chaperone proteins, and only correctly folded proteins are transported out of ER to Golgi complex. Any misfolded proteins are retained in the ER, and they are retro-translocated into the cytosol when misfolding persists, where they are degraded by the proteasome through ubiquitylation. This mechanism is known as ER-associated degradation (ERAD). (4) Recent studies indicate that various ER-resident high mannose oligosaccharide recognition molecules are involved in the several processes of the quality control system, such as individual steps of ER-retention, selection, and targeting of ERAD substrates. In this process (ERAD) the role of group of lectins has started to be appreciated. As an illustration, the F-box protein-1 (Fbs1) that recognizes sugar chains, specifically binds to the high-mannose oligosaccharides uniquely at the innermost position (5). Unlike other intracellular oligosaccharide recognition molecules, Fbs1 is present in the cytosol, where glycoproteins rarely exist. Although N-linked glycans are attached in the lumen of the ER or Golgi and are present on the outside of cells or inside vesicular compartments, it has been found that glycosylated molecules become detectable in the cytosol when cytosolic peptide: N-glycanase (PNGase) is knocked down by siRNA, suggesting that glycoproteins are deglycosylated after relocation from the ER into the cytosol (6). Fbs1 forms the SCF-type E3 ubiquitin ligase complex, and SCF-Fbs1 is probably responsible for the ubiquitylation of N-linked glycoproteins through the ERAD pathway. N-glycan serves as a signal for degradation by the Skp1-Cullin1-Fbx2-Roc1 (SCF-Fbx2) ubiquitin ligase complex. Like Fbs1, F-box protein Fbx2 binds specifically to proteins attached to N-linked high-mannose oligosaccharides and subsequently contributes to ubiquitination of N-glycosylated proteins. Reports indicate that SCF-Fbx2 ubiquitinates N-glycosylated proteins that are translocated from the ER to the cytosol by the quality control mechanism (Yoshida et al. 2002; Tai 2006).

6.2 The Degradation Pathway for Misfolded Glycoproteins

Eukaryotic cells have two main systems for intracellular protein degradation. Autophagy is an intracellular bulk degradation process and less selective. On the other hand, ubiquitin-proteasome system is responsible for most of the selective protein degradation for determination of life span of each intracellular protein. As stated above, in ubiquitin-proteasome system, a series of enzymes, E3 ubiquitin-ligases, play the most important role in the selection of target proteins.

6.2.1 Endoplasmic Reticulum-Associated Degradation (ERAD)

The quality control system includes the calnexin-calreticulin cycle, a unique chaperone system that recognizes $Glc_1Man_{9-6}GlcNAc_2$ and assists refolding of misfolded or unfolded proteins. When the improperly folded or incompletely assembled proteins fail to restore their functional states, they are degraded by ERAD system, which involves a retrograde transfer of proteins from the ER to the cytosol followed by degradation by 26S proteasome, a 2 MDa multicatalytic protease complex (Tsai et al. 2002). Precisely how they are selected for ERAD remains unclear, but what is clear is that the trimming of N-glycans plays a key role in the selection process. Studies have demonstrated that $Man_8GlcNAc_2$ structures serve as part of the signal needed for ERAD and that a lectin for $Man_8GlcNAc_2$ in ER accelerates the turnover rate of the misfolded glycoprotein (Hosokawa et al. 2001; Jakob et al. 2001; Nakatsukasa et al. 2001). It has been reported that many E3s are involved in the ERAD pathway, such as ER-embedded Hrd1 and Doa10, which have overlapping functions in yeast, and gp78, CHIP and Parkin, which ubiquitylate ER membrane proteins such as cystic fibrosis transmembrane conductance regulator (CFTR) and the Pael receptor in mammals (c/r Yoshida et al. 2003). In addition, the ERAD-linked E3 family, SCF^{Fbx2} participates in ERAD for selective elimination of glycoproteins (Yoshida et al. 2002). Misfolding or misassembly might be the general feature of all substrates; however, Fbx2 is expressed mainly in neuronal cells in the adult brain. Winston et al. (1999) and Ilyin et al. (2002) reported that several F-box proteins, including Fbx2, contain a conserved motif F-box-associated (FBA) domain or G-domain (sharing similarity with bacterial protein ApaG) in their C termini. Thus, SCF contains three core subunits, and a number of less critical components:

6.2.2 Ubiquitin-Mediated Proteolysis

Ubiquitin (Ub)-mediated proteolysis has a regulatory function in many diverse cellular processes. The ubiquitylation of a specific protein is carried out by the sequential activities of three enzymes, an activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin-ligase (E3) (Fig. 6.3a). In ubiquitin pathway, E3 is responsible for the selection of target proteins. In ubiquitin ligase system E1 activates ubiquitin, E2 transfers ubiquitin on to target proteins, and E3 selects proteins for ubiquitination. E3s are believed to exist as molecules with a large diversity, presumably in more than hundreds of species, which are classified into many subfamilies. Five types of E3s have

been identified that differ according to their subunit organization and or mechanism of Ub transfer. Ubiquitin-ligases E3s, identified in the ERAD pathway, include Hrd1 (Bays et al. 2001) and Doa10 (Swanson et al. 2001) in yeast, and gp78 (Fang et al. 2001), CHIP (Meacham et al. 2001) and Parkin (Imai et al. 2001) in mammals. In addition, a member of the ERAD-linked E3 family, SCF^{Fbs} which participates in ERAD for selective elimination of glycoproteins (Yoshida et al. 2002, 2003) has been identified. Whereas Hrd1, Doa10 and gp78 are localized in the ER, SCF^{Fbs} complexes are localized in the cytosol similar to CHIP and Parkin.

6.2.3 SCF Complex

One of the best characterized E3 families is the Skp1-Cullin1-F-box protein-Roc1 (SCF) complex (Deshaies 1999). In yeast (*Saccharomyces cerevisiae*), E3 type SCF complex is composed of Cullin1/Cdc53, Skp1, Roc1/Rbx1, and one member of family of F-box proteins, which are involved in trapping target proteins (Deshaies 1999; Ho et al. 2006). The cullin1, Rbx1, and Skp1 subunits appear to form the core ligase activity, with Rbx1 recruiting the E2 bearing an activated Ub (Hershko and Ciechanover 1998; Skowyra et al. 1999). In SCF complex, F-box protein is a variable component among 100 different members. F-box proteins in human are thought to allow the specific ubiquitylation of a wide range of substrates. In many instances, the F-box proteins serve as receptors for substrates that have various covalent modifications (phosporylation seems to be the predominant signal).

- Skp1—Bridging protein, forms part of the horseshoe-shaped complex, along with cullin (cul1). Skp1 is essential in the recognition and binding of the F-box (Figs. 6.1, 6.2a).
- Cullin (CUL1) forms the major structural scaffold of the SCF complex, linking the skp1 domain with the Rbx1 domain.
- Rbx1—Rbx1 contains a small zinc-binding domain called the RING Finger, to which the E2-ubiquitin conjugate binds, allowing the transferral of the ubiquitin to a lysine residue on the target protein.
- F-Box protein - As shown in Fig. 6.2b, Fbs1 has an N-terminal P domain whose function is not determined, an F-box domain that is required for Skp1 binding, and a substrate-binding domain that recognizes N-glycans. F box motif consisting of ~50 amino acid residues and a C-terminal region that interacts with the substrate and, thereby, the function of F-box protein is to trap target proteins. Fbs1 is named as the *F*-*b*ox protein that recognizes *s*ugar chains (Fig. 6.2c). Thus, F-box proteins perform the crucial role of delivering appropriate targets to the complex. These proteins all containing a N-terminal motif (F-box) interact with the rest of the SCF

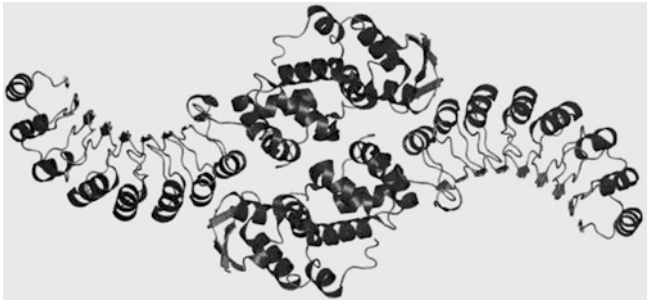

Fig. 6.1 Crystal structure of the human F-box protein Skp2 bound to Skp1 or (Skp1-Skp2) as horseshoe-shaped complex (Schulman et al. 2000) (PDB ID: 1FS2). Skp1 recruits the F-box protein through a bipartite interface involving both the F-box and the substrate-recognition domain. The structure raises the possibility that different Skp1 family members evolved to function with different subsets of F-box proteins, and suggests that the F-box protein may not only recruit substrate, but may also position it optimally for the ubiquitination reaction

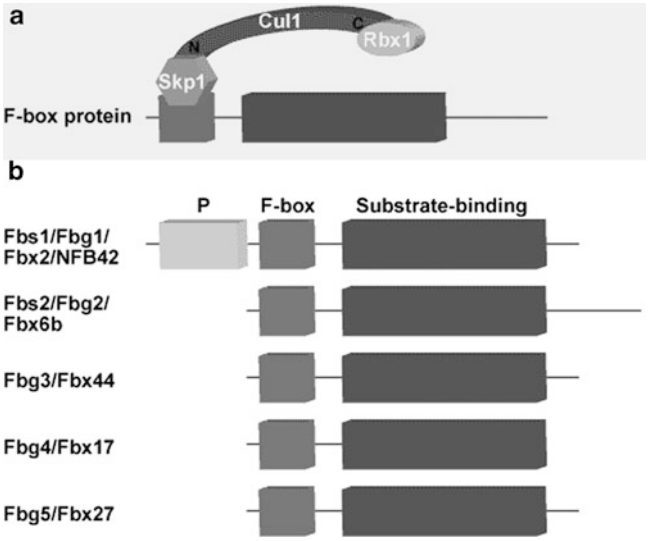

Fig. 6.2 The Fbs Family: (a) The SCF complex; (b) the domain structure of the Fbs family. In ubiquitin-proteasome system, E3 ubiquitin-ligases play important role in the selection of target proteins. E3s exist in hundreds of species. The largest known family of E3s is the SCF complex consisting of Skp1, Cul1, Rbx1/Roc1, and F-box proteins. In SCF complex, only F-box proteins are variable components, and at least 100 different members of F-box proteins in human are thought to allow for the specific ubiquitylation of a wide range of substrates. Fbs1 has an N-terminal pest (*P*) domain whose function is not determined, an F-box domain that is required for Skp1 binding, and a substrate-binding domain that recognizes *N*-glycans. Fbs1 might bind to denatured glycoproteins which have exposed inner chitobiose in *N*-glycans. Improperly folded proteins in the ER are retro-grade translocated into the cytosol where they are destroyed by the ubiquitin-proteasome system through ERAD. It has been reported that Fbs1 has at least four F-box proteins that show high homology in the substrate-binding domain. Among these proteins, Fbs1/Fgb1/Fbx2/NFB42, Fbs2/Fbg2/Fbx6b, Fbg3/Fbx44, Fbg4/Fbx17, and Fbg5/Fbx27, only Fbs2 and Fbs1 have the ability to interact with *N*-glycans, but the *N*-glycan binding activities of other proteins are not known (Adapted by permission from Yoshida 2007 © Japan Society for Bioscience, Biotechnology, and Agrochemistry)

complex by binding to the Skp subunit. The C-terminal portions of F-box proteins typically contain a variable protein-interaction domain that binds the target and thus confers specificity to the SCF complex presumably participate in substrate recognition.

Regulated protein degradation is a key recurring theme in multiple aspects of cell-cycle regulation. Progression of eukaryotic cell cycle is regulated through synthesis-degradation and phosphorylation-dephosohorylation of cell cycle regulating proteins. Two ubiquitin ligases are crucial in cell cycle. The anaphase-promoting complex or cyclosome (APC/C) controls metaphase-anaphase transition with its activator Cdc20. The APC/C is a ubiquitin-protein ligase required for the completion of mitosis in all eukaryotes. Mechanistic studies reveal how this remarkable enzyme combines specificity in substrate binding with flexibility in Ub transfer, thereby allowing the modification of multiple lysines on the substrate as well as specific lysines on ubiquitin itself (Matyskiela et al. 2009; Vodermaier 2004; Nakayama et al. 2005). Its activity is required for sister chromatids separation. APC/C with another activator, Cdh1, is also active in G1 phase and controls levels of mitosis regulating proteins

6.2.4 F-Box Proteins: Recognition of Target Proteins by Protein-Protein Interactions

F box proteins typically have a bipartite structure with an N terminal F box motif consisting of ~50 amino acid residues (Fig. 6.2c) and a C-terminal region that interacts with the substrate and, thereby, the function of F-box protein is to trap target proteins (Winston et al. 1999; Kipreos and Pagano 2000). However, it remains unknown how E3s accurately recognize target proteins. Studies suggest that phosphorylation of target proteins is a prerequisite for their recognition by SCF complexes (Hershko and Ciechanover 1998; Deshaies 1999; Kipreos and Pagano 2000). In addition, proline hydroxylation of transcription factor hypoxia-induced factor 1α (HIF1α) serves as a signal for ubiquitylation by the SCF-like Cullin2-based VBC ubiquitin-ligase (Ivan et al. 2001; Jaakkola et al. 2001). The F-box component of the SCF machineries is responsible for recognizing different substrates for ubiquitination. Interaction with components of the SCF complex is mediated through the F-box motif of the F-box protein while it associates with phosphorylated substrates through its second protein-protein interaction motif such as Trp-Asp (WD) repeats or leucine-rich repeats (LRRs). By targeting diverse substrates, F-box proteins exert controls over stability of proteins and regulate the mechanisms for a wide-range of cellular processes. F-box proteins play important function in various cellular settings such as tissue development, cell proliferation, and cell death as demonstrated in the modeling organism *Drosophila* (Ho et al. 2006). On the other hand, Fbx2 forms an SCF^{Fbx2} ubiquitin ligase complex that targets sugar chains in N-linked glycoproteins for ubiquitylation (Yoshida et al. 2002). Thus, Fbx2 is an example of F-box proteins that have evolved to recognize protein modifications other than phosphorylation and hydroxylation.

Winston et al. (1999) reported the identification of a family of 33 novel mammalian F-box proteins. The large number of these proteins in mammals suggests that the SCF system controls a correspondingly large number of regulatory pathways in vertebrates. Four of these proteins contain a novel conserved motif, the F-box-associated (FBA) domain, which may represent a new protein-protein interaction motif. The identification of these genes will help uncover pathways controlled by ubiquitin-mediated proteolysis in mammals. In *S. cerevisiae*, the F-box proteins Cdc4, Grr1 and Met^{30} target cyclin-dependent kinase inhibitors, G1 cyclins and transcriptional regulators for ubiquitination and reviewed in (Kipreos and Pagano 2000). Presently more than 700 F-box proteins that act as recognition modules to specifically target their dedicated substrates for ubiquitylation are known. Some F-box proteins function as phytohormone or light receptors, which directly perceive signals and facilitate specific target-protein degradation to regulate downstream pathways. If this new connection between ligand-regulated proteolysis and signaling proves to be more extensive, an entirely new way of understanding the control of signal transduction is in the offing (Somers and Fujiwara 2009).

The F-box protein family is the largest protein superfamily known, the total number of members ranging from approx. 20 in yeasts to several hundreds in higher eukaryotes. F-box proteins exhibit a typical bipartite structure. The N-terminal conserved F-box domain of 40 amino acid residues is required for direct interaction with Skp1 in the SCF complex (Feldman et al. 1997). With its C-terminal domain, the F-box protein recruits the target protein into the SCF complex, mostly by protein–protein interactions. Depending on the substrate-specific motifs at the C-terminus, F-box proteins are classified into three groups (Jin et al. 2004). The two classes of binding domains are WD40 and leucine-rich repeats, and hence they are named the Fbw (or FBXW) and Fbl (or FBXL) families respectively. The third class of F-box proteins is the Fbx (or FBXO) family, which does not contain any WD40 repeats or leucine-rich repeats. The Fbx (or FBXO) comprises of all F-box proteins with other C-terminal domains such as Kelch domains, Armadillo and tetratricopeptide repeats, zinc fingers and proline-rich domains.

Recently, a small subfamily of the Fbx family that comprises F-box proteins with a C-terminal SBD (sugar binding domain) was identified in mammals. Biochemical and structural studies demonstrated that these sugar-binding F-box proteins (or Fbs proteins) specifically interact with N-linked glycans (mostly with Man3−9GlcNAc2 structures) present on target glycoproteins and are assumed to play a key role in glycoprotein homoeostasis (Jin et al. 2004; Winston et al. 1999; Ilyin et al. 2002). Most substrates are targeted to SCF E3 ligases after they are covalently modified. While phosphorylation targets numerous substrates to the SCF complex, high-mannose oligosaccharide modification is required for substrate binding by Fbs1 in the SCF complex. Fbs1 belongs to a subfamily consisting of at least five homologous (FBXO2, FBXO6, FBXO17, FBXO27 and FBXO44) F-box proteins that contain a conserved F-box-associated (FBA) motif at their C-termini (Ilyin et al. 2002). All five proteins can co-precipitate with components of the SCF complex, and have been proposed to target misfolded glycoproteins for degradation by the Ub/proteasome system. Among these, mammalian FBXO2 and FBXO6 (also called Fbs1 and Fbs2 respectively) recognize high-mannose oligosaccharides to form SCF-type ubiquitin ligases.

In yeast and animals, a number of F-box proteins are present, easily classified by the nature of this interaction domain. In yeast, for example, the F-box protein Grr1p contains C-terminal leucine-rich repeats (LRRs) that recruit the phosphorylated forms of the cyclins Cln1p and Cln2p to the SCFGrr1 complex, whereas the Cdc4 F-box protein contains WD-40 repeats that recruit the phosphorylated form of the cyclin-dependent kinase inhibitor Sic1 to the SCFCdc4 complex. Studies in *Arabidopsis thaliana* indicate that F-box proteins and SCF complexes play critical roles in various aspects of plant growth and development. ASK1, one of the 19 Skp proteins in *Arabidopsis*, is involved in male gametogenesis and floral organ identity. Gagne et al. (2002) identified 694 potential F-box genes in *Arabidopsis*, making this gene superfamily one of the largest currently known in plants. Most of the encoded proteins contain interaction domains C-terminal to the F-box that presumably participate in substrate recognition.

6.3 F-Box Proteins with a C-Terminal Sugar-Binding Domain (SBD)

F-box protein is present in ubiquitin-ligase E3 and is responsible for substrate selection. Each F-box protein contains a conserved F-box domain, which interacts with other subunits in E3, and a substrate recognition domain, which determines the target specificity of the E3 complex. Many F-box proteins play a determining role in the substrate specificity of this degradation pathway. In most cases, selective recognition of the target proteins relies on protein–protein interactions mediated by the C-terminal domain of the F-box proteins. In mammals, the occurrence of F-box proteins with a C-terminal SBD (sugar-binding domain) that specifically interacts with high-mannose N-glycans on target glycoproteins has been documented. The identification and characterization of sugar-binding F-box proteins demonstrated that F-box proteins do not exclusively use protein–protein interactions but also protein–carbohydrate interactions in Ub/proteasome pathway. F-box protein (Such as Cdc4) contributes to the specificity of SCF by aggregating to target proteins independently of the complex and then binding to the Skp1 component, thus allowing the protein to be brought into proximity with the functional E2 protein. The F-box is also essential in regulating SCF activity during the course of the cell-cycle. SCF levels are thought to remain constant throughout the cell-cycle. Instead, F-box affinity for protein substrates is regulated through cdk/cyclin mediated phosphorylation of target proteins (Mizushima et al. 2007).

6.3.1 Diversity in SCF Complex due to Lectin Activity of F-Box Proteins

N-glycosylation acts as a targeting signal to eliminate intracellular glycoproteins by Fbx2-dependent ubiquitylation and subsequent proteasomal degradation. N-glycosylation of the proteins occurs when newly synthesized proteins enter the ER through translocation channel "translocon." N-glycans play an important role in glycoprotein transport and sorting (Ellgaard et al. 1999; Helenius and Aebi 2004), in particular at the initial step of secretion that occurs in ER compartment (Ellgaard et al. 1999; Helenius and Aebi 2004). N-linked glycoproteins are subjected to "quality control" in which aberrant proteins are distinguished from properly folded proteins and retained in ER (Helenius and Aebi 2004).

Ilyin et al. (2000) characterized 10 mammalian F-box proteins. Five of them (FBL3 to FBL7) share structural similarities with Skp2 and contain C-terminal leucine-rich repeats. The other five proteins have different putative protein-protein interaction motifs. Specifically, FBS and FBWD4 proteins contain Sec7 and WD40-repeat domains, respectively. The C-terminal region of FBA shares similarity with bacterial protein ApaG while FBG2 shows homology with the F-box protein NFB42. The marked differences in -F-box gene expression in human tissues suggest their distinct role in ubiquitin-dependent protein degradation. The cDNAs encoding FBG3, FBG4 and FBG5 display similarity with NFB42 (FBX2) and FBG2 (FBX6) proteins.

All five proteins are characterized by an approximately 180-amino-acid (aa) conserved C-terminal domain and thus constitute a third subfamily of mammalian F-box proteins. Genomic organization of the five FBG genes revealed that all of them consist of six exons and five introns. FBG1, FBG2 and FBG3 genes are located in tandem on chromosome 1p36, and FBG4 and FBG5 are mapped to chromosome 19q13. FBG genes are expressed in a limited number of human tissues including kidney, liver, brain and muscle tissues. Specifically, FBG2 mRNA was expressed in foetal liver, decreased after birth and re-accumulated in adult liver (Ilyin et al. 2002). Glenn et al. (2008) examined the substrate specificity of the only family of ubiquitin ligase subunits thought to target glycoproteins through their attached glycans. Five proteins comprising this FBA family (FBXO2, FBXO6, FBXO17, FBXO27, and FBXO44) contain a conserved G domain that mediates substrate binding. Glenn et al. (2008) showed that each family member has differing specificity for glycosylated substrates and suggested that the F-box proteins in the FBA family bind high mannose and sulfated glycoproteins, with one FBA protein, FBX044, failing to bind any glycans on the tested arrays. Differences in substrate recognition, SCF complex formation, and tissue distribution suggested that FBA proteins play diverse roles in glycoprotein quality control.

6.3.2 Fbs Family

6.3.2.1 Fbs1 and Fbs2 in ERAD Pathway

Studies suggest that Fbx6b/FBG2 binds several glycoproteins, though other F-box proteins failed to bind any of the glycoproteins tested. Based on these functional studies, Fbx2/FBG1 and Fbx6b/FBG2 were named as Fbs1 (F-box protein that recognizes sugar chains 1) and Fbs2, respectively. Fbs2 is widely distributed in a variety of mouse tissues, differing from the restricted expression of Fbs1 in adult brain and testis. Furthermore, a dominant negative Fbs2 mutant suppressed degradation of a typical ERAD substrate, the T cell receptor α subunit (TCRα). The human genome contains about 70 genes for F-box proteins, and at least five homologous F-box proteins containing a conserved motif in their C-termini are thought to recognize sugar chain of N-linked glycoproteins. Among these, Fbs1 and Fbs2 are involved in ERAD pathway and have the ability to bind to proteins modified with high-mannose oligosaccharides, whose modification occurs in the ER. Yoshida (2007) reviewed the in vivo function of Fbs1 and homologous proteins, intracellular oligosaccharide recognition molecules involved in the quality control system. Screening for proteins bound to various glycoproteins led to the identification of Fbs1 from mouse brain extracts (Yoshida et al. 2002). Similar to the ubiquitously expressed Fbs2, Fbs1 recognizes N-glycans at the innermost position as a signal for unfolded glycoproteins, probably in the ERAD pathway. Majority of Fbs1 is present as Fbs1-Skp1 heterodimers or Fbs1 monomers but not SCF(Fbs1) complex in situ. In vitro, Fbs1 prevented the aggregation of the glycoprotein through N-terminal unique sequence of Fbs1. Results suggest that Fbs1 assists clearance of aberrant glycoproteins in neuronal cells by suppressing aggregates formation, independent of ubiquitin ligase activity, and thus functions as a unique chaperone for those proteins (Yoshida et al. 2007) (Fig. 6.3).

Fbs1 can form an SCF complex specific to N-linked glycoproteins and can bind to proteins modified with high-mannose oligosaccharides, which occur in ER. N-linked glycoproteins are normally not accessible to the ubiquitylation machinery in the cytosol, because they reside within the lumen of the ER and other compartments of the secretory pathway. One of the identified Fbs1 substrates is pre-integrin β1 modified with high mannose oligosaccharides, and their physical association was detected in the cytosol in the presence of the proteasome inhibitor (Yoshida et al. 2002). Moreover, overexpression of the dominant-negative form Fbs1 lacking the F-box domain essential to the formation of the SCF complex led to inhibition of the degradation of ERAD substrates, suggesting that SCF(Fbs1) is involved in the ERAD pathway (Fig. 6.3).

6.3.2.2 X-Ray Crystallographic Structure of Fbs1

X-ray crystallographic and nuclear magnetic resonance (NMR) studies of the substrate-binding domain of Fbs1 have revealed that Fbs1 interacts with the inner chitobiose in *N*-glycans of glycoproteins by a small hydrophobic pocket located at the top of the β-sandwich. The intramolecular interactions of the innermost chitobiose and the polypeptide moiety generally hamper the intermolecular binding of macromolecules such as proteins. Therefore, Fbs1 might bind to denatured glycoproteins which have exposed inner chitobiose in N-glycans. Improperly folded proteins in the ER are retro-grade translocated into the cytosol where they are destroyed by the ubiquitin-proteasome system, a disposal system, called ER-association degradation (ERAD). The reason why Fbs1 interacts with internal chitobiose of N-glycans is because Fbs1 recognizes the innermost position of N-glycans as the signal for unfolded glycoproteins in the ERAD pathway. Hypothetically, it is suggested that although Fbs1 cannot access the inner chitobiose protected by polypeptide moieties in folded glycoproteins, Fbs1 can bind to exposed inner chitobiose by denaturation (Mizushima et al. 2004). The CRD in the murine F-box protein Fbs1 (Fbx2) bound to GlcNAc disaccharide is

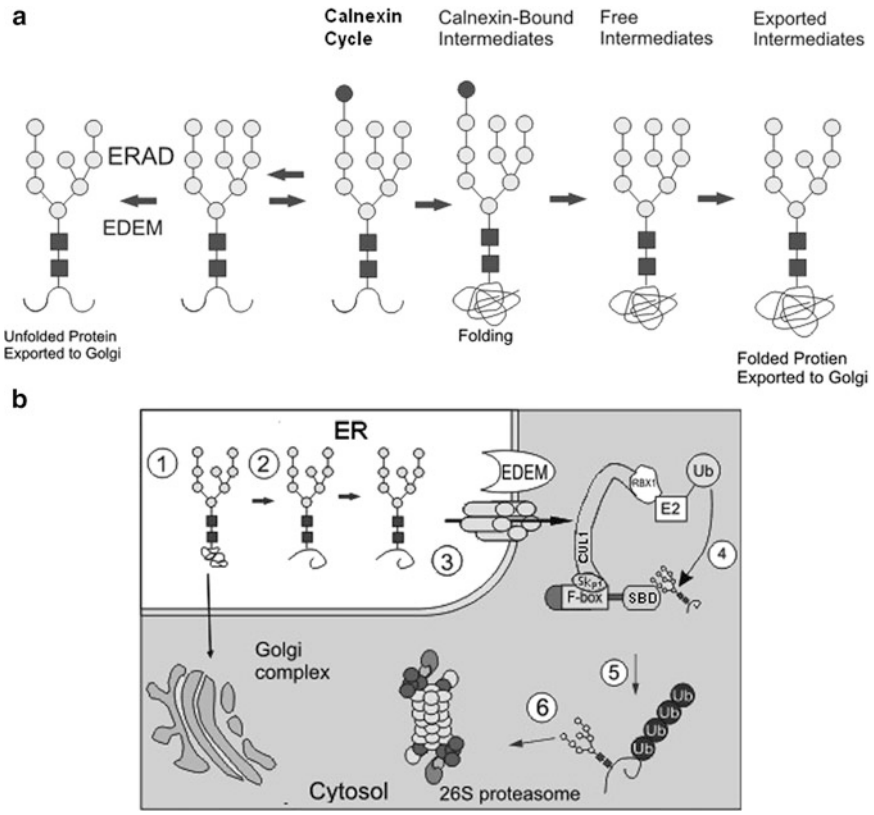

Fig. 6.3 (**a**) **N-linked glycan structures in ERAD.** (**b**) **Protein–carbohydrate interactions in the ERAD pathway:** Proper folding of newly synthesized glycoproteins occurs in the ER. Correctly folded glycoproteins (with $Man_9GlcNAc_2$ glycan) can exit the ER via vesicles to enter the secretory pathway *1*. Misfolded or unassembled glycoproteins (with $Man_8GlcNAc_2$ glycan) *2* are recognized by an ER-residing lectin, EDEM *3*, and retro-translocated from the ER into the cytosol where they are assembled in an active SCF complex through binding of their N-glycan with the SBD of an Fbs protein *4*. After conjugation with Ub *5*, the target proteins are recognized and degraded by the 26S proteasome *6*. *Circles* represent mannose and *squares* represent GlcNAc

shown in Fig. 6.4. Homologous domains present in F-box proteins from a range of animals may also function as CRDs.

6.3.2.3 Fbs1 and Fbs2 Homologous Proteins

Other glycoproteins modified with high-mannose oligosaccharides in the brain (Murai-Takebe et al. 2004; Kato et al. 2005) are also degraded in the ERAD pathway, mediated by SCFFbs1. SHP substrate-1 (SHPS-1), a transmembrane glycoprotein that regulates cytoskeletal reorganization and cell–cell communication, is particularly abundant in CNS and is a physiological substrate for SCF(NFB42) E3 ubiquitin ligase. Ectopic expression of Fbs1 resulted in elimination of misfolded SHPS-1 molecules from the ER and led to substantial up-regulation of SHPS-1 expression to the cell surface (Murai-Takebe 2004). Fbs1 mediated neuronal activity-dependent degradation of NR1, one of the NMDA subunits (Kato et al. 2005). Since the sugar moieties on the cell-surface NR1 are high-mannose, it appears possible that the mechanism of Fbs1 regulation of NMDA receptors is due not only to the ERAD pathway but also to retrograde transfer of endocytosed protein. It appears likely that Fbs1 assists in the clearance of aberrant glycoproteins in neuronal cells by suppressing aggregate formation, independent of ubiquitin ligase activity. Hence, Fbs1 has an additional function as a unique chaperone for those proteins.

Besides Fbs1**,** at least four F-box proteins that show high homology in SBD have been reported (Winston et al. 1999; Ilyin et al. 2002). These F-box proteins are composed of a highly homologous F-box domain, a substrate-binding domain, and a low homologous short linker sequence between F-box and SBD (Fig. 6.5). Although the homologies between Fbs2 and Fbg3 and between Fbg4 and Fbg5 in the short linker sequence are high, the linker sequence of Fbs1 shows no homology to any of them. It has been reported that the inefficient SCF complex formation of Fbs1 and the restricted presence of SCF^{Fbs1} bound to the ER membrane are due to the short linker sequence. The Fbs1, Fbs2, and Fbg3 genes are localized on human chromosome1p36.11–36.23 and on mouse chromosome 4E2, and the Fbg4 and Fbg5 genes are on human 19q13.2 and mouse 7B1. In the NCBI database Entrez gene, Fbs1, Fbs2, and Fbg5 encode single proteins, but Fbg3 and Fbx17 encode two proteins. Although Fbx17

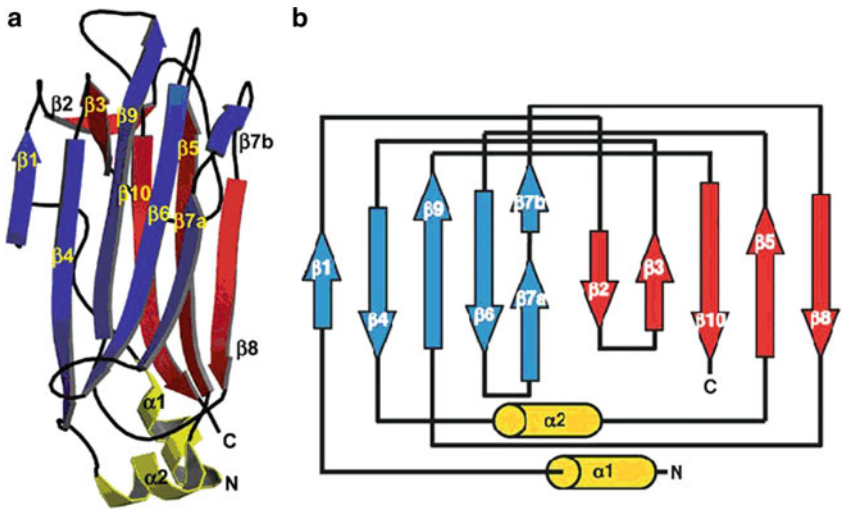

Fig. 6.4 Tertiary structure of SBD in Fbs1. (**a**) Overall structure of SBD of Fbs1 shown as a ribbon diagram (PDB ID: 1UMH; Mizushima et al. 2004). The SBD is composed of a ten-stranded antiparallel β-sandwich. Strands β1, β4, β6, β7 and β9 form one β-sheet, whereas other β-sheet consists of strands β2, β3, β5, β8 and β10. These two sheets are referred to as S1 and S2 sheets. β-strands belonging to S1 and S2 are *blue* and *red*, respectively. Loops and helices are *black* and *yellow*, respectively. (**b**) A topology diagram of SBD. The α-helices are *yellow cylinders* labeled α1 and α2. The β-strands are *arrows* labeled β1−β10. The left and right forms of β-strands correspond to S1 and S2, respectively, as in (**a**). *N* and *C* indicate N and C termini, respectively (Adapted by permission from Mizushima et al. 2004 © Macmillan Publishers Ltd)

encodes two proteins whose differences lie only in their N-terminal 9 amino acids by the different use of the first exon, Fbg3 encodes proteins non-homologous in their SBDs (isoform1 and isoform2).

The Fbg3 isoform1 protein is translated without skipping exons and shows high identity with Fbs2, as shown in Fig. 6.5. On the other hand, Fbg3 isoform2 is synthesized by skipping the fourth exon, and the resulting protein contains the 255 amino-acid region, which is different from C-terminal half of isoform1 (after residue-123). The expression of Fbs1, Fbg4, and Fbg5 is tissue specific, but that of Fbs2 and Fbg3 is relatively ubiquitous (Ilyin et al. 2002; Yoshida et al. 2003). Fbs1 is strongly expressed in neural cells in the adult brain and weakly in the testis. Fbg4 expression is predominant in the kidney and weak in the liver. Fbg5 is detected in the brain, heart, kidney, and liver.

Among five homologous F-box proteins (Ilyin et al. 2002), the FBG3 protein exhibits 75% identity with Fbs2, and the identity of Fbs1 and Fbs2 is similar to that of Fbs1 and FBG3. Interestingly, *Fbs1, FBG3*, and *Fbs2* genes are located in tandem on chromosome, and the expression of FBG3 is observed ubiquitously but strongly in the brain and testis (Yoshida et al. 2003). It is anticipated that FBG3 can recognize high mannose *N*-glycans because of their high homology. However, there was no sugar-binding activity for FBG3 despite several assay systems were used.

Despite the high homology between Fbs2 and Fbg3, no sugar-binding activity of Fbg3 has been detected. Of the Fbs family members, Fbs1 has the ability to bind strongly to the high-mannose oligosaccharides-modified proteins, while Fbs2 and Fbg5 bind them more weakly (Yoshida et al. 2002, 2003; Nelson et al. 2006), but Yoshida (2007) did not detect any sugar-binding activity for Fbg3 and Fbg4. X-ray crystallographic study of the substrate-binding domain of Fbs1 revealed that Fbs1 interacts with the innermost chitobiose of glycoproteins by Phe177, Tyr279, Try280, and Lys281 on the loop structures in mouse Fbs1 and in human Fbs1, Phe173, Tyr278, Trp279, and Lys280, as shown in Fig. 6.5 (Mizushima et al. 2004) Among these residues, the Phe177Ala, Tyr2798Ala, or Trp280Ala mutants lost the ability to interact with glycoproteins. The alignment of human Fbs1 and its homologs shows that these residues, except for Lys280, are conserved in Fbs1, Fbs2, Fbg3, and Fbg5. It is anticipated that Fbg4 does not interact with the chitobiose moiety, because the residue corresponding to Tyr278 of human Fbs1 is serine in Fbg4, but these four residues are conserved in Fbs2 and Fbg3. Further analysis of the structure of the substrate-binding domain of Fbs2 and Fbg3 is needed to elucidate the mechanism by which Fbg3 fails to bind to chitobiose.

Although Fbs1, Fbs2, and Fbg5 have similar abilities to bind to glycoproteins modified with high-mannose oligosaccharide, they have different potencies of binding to glycoproteins. To gain insight into the differences in their mechanisms for oligosaccharide recognition related to their cellular functions, further structural and biological analysis is required. Moreover, it would be intriguing to identify the substrates for Fbg3 and Fbg4, which are not capable of

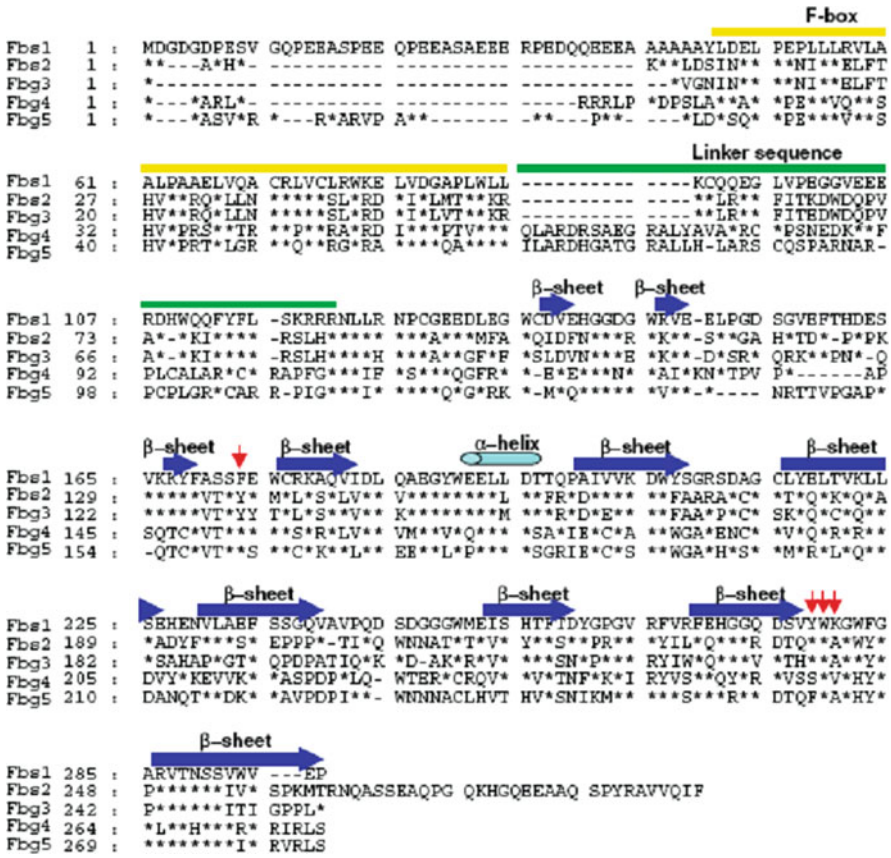

Fig. 6.5 Alignment of Human Fbs Family Proteins. Identical residues with Fbs1 are indicated by *asterisks*. F-box domain and the linker sequence are indicated by *yellow* and *green bars* respectively. Secondary structure elements of SBD in Fbs1 are shown above the amino acid sequence. Chitobiose binding residues are marked by *red arrows* (Adapted by permission from Yoshida 2007 © Japan Society for Bioscience, Biotechnology, and Agrochemistry)

binding to glycoproteins. Such analysis is clearly necessary for a better understanding of the overall functions of Fbs family proteins in vivo. There is little known at present about the organization of the genes encoding F-box proteins, with most studies focusing on the protein products (Kipreos and Pagano 2000).

How the SCF(Fbs1 and -2) complexes interact with unfolded glycoproteins? The SCF(Fbs1) complex was found associated with p97/VCP AAA ATPase and bound to integrin-β1, one of the SCF(Fbs1) substrates, in the cytosol in a manner dependent on p97 ATPase activity. Both Fbs1 and Fbs2 proteins interacted with denatured glycoproteins, which were modified with not only high-mannose but also complex-type oligosaccharides, more efficiently than native proteins. Given that Fbs proteins interact with innermost chitobiose in N-glycans, it was proposed that Fbs proteins distinguish native from unfolded glycoproteins by sensing the exposed chitobiose structure (Yoshida et al. 2005). Yamaguchi et al. (2007) suggested that Fbs1 captures malfolded glycoproteins, protecting them from the attack of PNGase, during the chaperoning or ubiquitinating operation in the cytosol (Yamaguchi et al. 2007).

6.3.3 Fbs1 Equivalent Proteins

It has been found that Fbs1 has at least four F-box proteins that show high homology in the substrate-binding domain. Among these proteins, Fbs1/Fgb1/Fbx2/NFB42, Fbs2/Fbg2/Fbx6b, Fbg3/Fbx44, Fbg4/Fbx17, and Fbg5/Fbx27, only Fbs2 and Fbs1 have the ability to interact with N-glycans, but the N-glycan binding activities of other proteins have not been reported. Fbs2 is widely expressed in various tissues in contrast to the limited expression of Fbs1 in neural cells in adult brain (Fbs1 was first named NFB42, meaning neural F Box 42 kDa). As ERAD might be the general feature of

all cells, Fbs2 is thought to be a general ERAD E3 in mammals. Although Fbs1 and Fbs2 recognize high-mannose N-glycans, Fbs1 seems to possess 10^{2-3} higher affinity for oligosaccharides (Yoshida 2003; Yoshida et al. 2002). Thus, Fbs1 is equivalent to neural F-box protein of 42 kDa (NFB42)), Fbx2, (Winston et al. 1999; Cenciarelli et al. 1999), F-box only protein 2 (FBXO2), Fbg1 (Ilyin et al. 2000).

6.3.3.1 NFB42 Mediates Nuclear Export of UL9 Protein After Viral Infection

Neural F-box 42-kDa protein: The neural F-box 42-kDa protein (NFB42) is a component of the SCF(NFB42) E3 ubiquitin ligase that is expressed in all major areas of brain; it is not detected in nonneuronal tissues. Similar to other F-box proteins, NFB42 interacts with Skp1 through its F-box domain (Winston et al. 1999; Cenciarelli et al. 1999). NFB42 is highly enriched in the nervous system, as a binding partner for the herpes simplex virus 1 (HSV-1) UL9 protein. Co-expression of NFB42 and UL9 in human embryonic kidney (293T) cells led to a significant decrease in the level of UL9 protein. The interaction of UL9 protein with NFB42 results in its polyubiquitination and subsequent degradation by the 26S proteasome (Eom and Lehman 2003). The HSV-1 infection promotes the shuttling of NFB42 between the cytosol and the nucleus in both 293T cells and primary hippocampal neurons, permitting NFB42 to bind to the phosphorylated UL9 protein, which is localized in the nucleus. Because the intranuclear localization of the UL9 protein, along with other viral and cellular factors, is an essential step in viral DNA replication, degradation of the UL9 protein in neurons by means of nuclear export through its specific interaction with NFB42 may prevent active replication and promote neuronal latency of HSV-1 (Eom et al. 2004). Although NFB42 is present in the cytosol whereas the UL9 protein is located predominantly in the nucleus, HSV-1 infection promotes nuclear import of NFB42 and NFB42 mediates the nuclear export of the UL9 protein, leading to cytosolic degradation of UL9 protein (Eom et al. 2004)

6.3.3.2 OCP1 and OCP2

Role of selective cochlear degeneration in mice deficient in Fbx2 with specificity for high-mannose glycoproteins has been studied. Originally described as a brain-enriched protein (Yoshida et al. 2002), Fbx2 is also highly expressed in the organ of Corti (the sensory organ of cochlea) and protein, named as organ of Corti protein 1 (OCP1) (Thalmann et al. 1997). OCP1 and OCP2, the most abundant proteins in cochlea, are subunits of an SCF E3 ubiquitin ligase. OCP1 co-localizes exactly with OCP2 in the epithelial gap junction region of the guinea pig organ of Corti (OC), which is rich in OCP1. The full-length OCP1 cDNA—1,180 nt in length—includes a 67 nt 5′ leader sequence, 300 codons (including initiation and termination signals), and a 216 nt 3′ untranslated region. The cDNA encodes a protein having a predicted molecular weight of 33.7 kDa and harbors an F-box motif spanning residues 52–91, consistent with a role for OCP1 and OCP2 in the proteasome-mediated degradation of select OC proteins. OCP1 is equivalent to F-box proteins: Fbs1, Fbx2, or NFB42 - known to bind N-glycosylated proteins and believed to function in the retrieval and recycling of misfolded proteins. The OCP1 displays extensive homology to the rat brain NFB42. But clustered sequence non-identities indicate that the two proteins are transcribed from distinct genes. Located on chromosome 1p35, the inferred translation product exhibits 94% identity with the guinea pig OCP1 coding sequence (Henzl et al. 2001). Although transcribed from a distinct gene, OCP2 is identical to Skp1. The high concentrations of OCP1 and OCP2 in the cochlea suggest that the OCP1-OCP2 heterodimer may serve an additional function independent of its role in a canonical SCF complex. OCP1 and OCP2 associate tightly at room temperature. However, DSC data for the complex suggest that they denature independently, consistent with the highly exothermic enthalpy of complex formation (Tan and Henzl 2009).

Fbs1-deficient mice indicated that the Fbs1-Skp1 dimer is essential to inner ear homeostasis (Nelson et al. 2007). The orthologs of Fbs1 and Skp1 are abundantly present in guinea pig organ of Corti. They are called OCP1 and OCP2 respectively (Henzl et al. 2001). Mice with targeted deletion of Fbs1 develop age-related hearing loss with cochlear degeneration. The inner ear gap junction protein, connexin 26, which interacts with OCP1 (Henzl et al. 2004) increases in $Fbs1^{-/-}$ mouse cochlea. Furthermore, loss of Fbs1 leads to decrease in cochlear Skp1. On the other hand, the Skp1 level remains unchanged and no defects are seen in $Fbs1^{-/-}$ mouse brain (Nelson et al. 2007).

6.3.3.3 F-Box Proteins with a C-Terminal Domain is Homologous to Tobacco Lectin

During the last decade it was shown that plants synthesize minute amounts of carbohydrate-binding proteins upon exposure to stress situations like drought, high salt, hormone treatment, pathogen attack or insect herbivory. In contrast to the ‘classical’ plant lectins, which are typically found in storage vacuoles or in the extracellular compartment this new class of lectins is located in the cytoplasm and the nucleus in animals. Based on these observations the concept was developed that lectin-mediated protein-carbohydrate interactions in the cytoplasm and the nucleus play an important role in the stress physiology of the plant

cell. Hitherto, six families of nucleocytoplasmic lectins have been identified. The putative sugar-binding F-box proteins have been identified in plants. Genome analyses in Arabidopsis and rice revealed the presence of F-box proteins with a C-terminal lectin-related domain homologous with Nictaba, a jasmonate-inducible lectin from tobacco that was shown to interact with the core structure of high-mannose and complex N-glycans. Owing to the high similarity in structure and specificity between Nictaba and the SBD of the mammalian Fbs proteins, a similar role for the plant F-box proteins with a Nictaba domain in nucleocytoplasmic protein degradation in plant cells is suggested (Lannoo and Van Damme 2010; Lannoo et al. 2008).

6.3.4 Ligands for F-Box Proteins

The F-box proteins can be classified into five major families, which can be further organized into multiple subfamilies. Sequence diversity within the subfamilies suggests that many F-box proteins have distinct functions and or substrates. Representatives of all of the major families interact in yeast two-hybrid experiments with members of the *Arabidopsis* Skp family supporting their classification as F-box proteins. Reports show that *Arabidopsis* has exploited the SCF complex and the ubiquitin_26S proteasome pathway as a major route for cellular regulation and that a diverse array of SCF targets is likely present in plants.

6.3.4.1 Binding to High Mannose Oligosaccharides

Fbs1 (equivalent to Fbx2 or NFB42) binds specifically to proteins attached with high mannose oligosaccharides and contributes to elimination of *N*-glycoproteins in cytosol (Yoshida et al. 2002). Fbs2 is another F-box protein that recognizes *N*-glycan and forms an SCF^{Fbs2} ubiquitin ligase complex that targets sugar chains in *N*-glycoproteins for ubiquitylation and plays a role in ERAD pathway (Ilyin et al. 2002; Yoshida et al. 2003). Pull-down analysis revealed that $Man_{3-9}GlcNAc_2$ glycans were required for efficient Fbs2 binding, whereas modifications of mannose residues by other sugars or deletion of inner GlcNAc reduced Fbs2 binding. Fbs2 interacted with *N*-glycans of T-cell receptor α-subunit (TCRα), a typical substrate of ERAD pathway, and the mutant Fbs2ΔF, which lacks the F-box domain essential for forming the SCF complex.

X-ray crystallographic and NMR studies of the SBD of Fbs1 have confirmed that Fbs1 interacts with the innermost chitobiose (GlcNAc-GlcNAc) in N-glycans of glycoproteins by way of a small hydrophobic pocket located at the top of the β sandwich (Mizushima et al. 2004). In general, the internal chitobiose of N-glycans in many native glycoproteins is not accessible by means of macromolecules. Fbs1 interacted with denatured glycoproteins more efficiently than native proteins did, indicating that the innermost position of N-linked oligosaccharides becomes exposed upon protein denaturation and is used as a signal of unfolded glycoproteins to be recognized by Fbs1 (Yoshida et al. 2005)

6.3.4.2 Chitobiose Is More Accessible for SBD

Both Fbs1 and Fbs2 are cytosolic proteins that preferentially bind to free N-glycans with a high-mannose oligosaccharide (Man_{3-9}) attached to a core chitobiose (GlcNAc2) and to high-mannose, N-linked glycoproteins. Fbs1 and Fbs2 do not bind to Man_3, Man_4 or Man_5 structures lacking $GlcNAc_2$, demonstrating the importance of the chitobiose core. In general, the core $GlcNAc_2$ of N-glycans present on correctly folded (native) proteins is not accessible to lectins, since most lectins bind to the non-reducing terminal sugar groups of carbohydrates. However, in unfolded (denatured) N-linked glycoproteins, the chitobiose core of glycan is more accessible for the SBD of Fbs1 and Fbs2. Yoshida et al.(2002, 2003) confirmed that SCF complexes with either Fbs1 or Fbs2 recognize denatured ERAD substrates in the cytosol and act as E3 Ub ligases in the ERAD pathway (Fig. 6.3). This implies that mammalian cells possess a ubiquitination system that uses protein–carbohydrate interactions instead of protein–protein interactions for a specific proteasome mediated degradation of glycoproteins. The crystal structures of sugar-binding domain (SBD) of Fbs1 alone and in complex with chitobiose has been solved. The SBD is composed of a ten-stranded antiparallel β-sandwich. The structure of the SBD-chitobiose complex includes hydrogen bonds between Fbs1 and chitobiose and insertion of the methyl group of chitobiose into a small hydrophobic pocket of Fbs1. Moreover, the amino acid residues adjoining the chitobiose-binding site interact with the outer branches of the carbohydrate moiety. Considering that the innermost chitobiose moieties in N-glycans are usually involved in intramolecular interactions with the polypeptide moieties, it was proposed that Fbs1 interacts with the chitobiose in unfolded N-glycoprotein, pointing the protein moiety toward E2 for ubiquitination (Mizushima et al. 2004).

The substrate recognition domain in Fbs1 has an ellipsoid shape formed by a sandwich of five-stranded, antiparallel β-sheets. One end of the ellipsoid is capped by two alpha-helices and is also the location of the domain N- and C-termini. The sugar binding site is located at the opposite end of the domain and is formed by the loops connecting β-strands 3–4 and 9–10. The binding site is specific for the N-acetylglucosamine (GlcNAc) disaccharide chitobiose. One GlcNAc residue stacks against a tryptophan aromatic

ring, while the other inserts a methyl group into a hydrophobic pocket. Both residues make hydrogen bonds to the protein (Fig. 6.4). Specificity for chitobiose enables Fbs1 to bind to misfolded glycoproteins that have been translocated back into the cytoplasm: a GlcNAc disaccharide is at the core of all N-linked oligosaccharides added to proteins in the ER. In folded proteins, these GlcNAc residues interact with the polypeptide and are shielded, but in unfolded proteins they may become exposed and act as a novel sugar-based degradation signal. In contrast, outer residues in N-linked glycans appear to make only minor interactions with Fbs1.

6.3.5 Evolution of F-Box Proteins

F-box proteins contain a wide range of secondary motifs including zinc fingers, cyclin domains, leucine zippers, ring fingers, tetratricopeptide (TPR) repeats, and proline-rich regions. The diversity of associated protein domains suggests that F-box motifs have been transferred into existing proteins multiple times during eukaryotic evolution. Evolutionary constraints are higher for certain classes of F-box proteins: all of the human FBXW or FBXL proteins have counterparts in *C. elegans* with most also conserved in yeast, but only about half of the human FBXO class of proteins is conserved in nematodes or yeast. An interesting observation is the huge number of F-box proteins in *C. elegans*. The F-box motif is the fourth most common protein domain in *C. elegans*, with their number dwarfing the F-boxes found in other species by a factor often. Over half of the predicted *C. elegans* F-box proteins (135) are found with another motif known as DUF38 (domain of unknown function 38) or FTH (FOG-2 homology). The FTH/DUF38 domain is found mostly in nematodes, with none in humans or yeast. A second domain, PfamB-45, is found in another 56 *C. elegans* F-box proteins. Both of these cases suggest the expansion of single progenitor genes within nematodes (Kipreos and Pagano 2000).

6.3.6 Localization of F-Box Proteins

There have been a limited number of studies analyzing the subcellular localization of F-box proteins, and in all but a couple of cases analysis was performed with over-expressed tagged proteins (Winston et al. 1999). Some F-box proteins were found to be distributed both in the cytoplasm and in the nucleus. The identical localization of wild-type and mutant F-box proteins demonstrates that the presence of the F-box and the F-box-dependent binding to Skp1 does not determine the subcellular localization of these proteins. While the expression of mRNAs encoding some F-box proteins has been found in all tissues tested, others are clearly tissue-specific. Because of the large number of F-box proteins, this information is too complex to be summarized here (Kipreos and Pagano 2000).

6.3.7 Regulation of F-Box Proteins

F-box proteins are regulated by several mechanisms and at different levels: such as synthesis, degradation, and association with SCF components. The three yeast F-box proteins Cdc4, Grr1, and Met30 are intrinsically unstable proteins whose levels do not oscillate during cell cycle. It appears that they are subjected to ubiquitin-proteasome mediated degradation by an autocatalytic mechanism. Whereas the degradation of Cdc4 and Grr1 is dependent on their abilities to bind Skp1 through their F-boxes (Galan and Peter 1999), Met30 seems to be ubiquitinated in a cullin-dependent manner but in an F-box-independent manner (Rouillon et al. 2000).

Mammalian Skp2 is degraded by the ubiquitin-proteasome pathway but its expression is mostly regulated at a transcriptional level (Zhang et al. 1995). The expression of both Skp2 mRNA (Zhang et al. 1995) and Skp2 protein (Carrano et al. 1999) are cell-cycle-regulated, peaking in S phase and declining as cells progress through M phase. In contrast, the expression of other subunits of the SCF-Skp2 ligase complex (Cull, Skp1 and Roc1), as well as its ubiquitin-conjugating enzyme (Ubc3) does not fluctuate through cell cycle. Thus, although the ubiquitination of Skp2 substrates is regulated by their own phosphorylation, which allows their recognition by Skp2, a second level of control is ensured by the cell-cycle oscillations in Skp2 levels. The only characterized post-translational modification of an F-box protein is phosphorylation of Skp2 on Ser76 by the cyclin A-cdk2 complex (Yam et al. 1999), but the significance of this modification is not well known.

Enforced expression of β-catenin induces the expression of the F-box protein β-TrCP (Spiegelman et al. 2000). Although β-catenin can act as a transcriptional regulator, induction of β-TrCP by β-catenin is due to a stabilization of β-TrCP mRNA. As β-catenin is an SCFβ-Trcp substrate, stimulation of β-TrCP expression by β-catenin results in an accelerated degradation of β-catenin itself, suggesting that a negative feedback loop may control the β-catenin pathway. Finally, the association of Grr1 with Skp1 is regulated by glucose levels (Li and Johnston 1997). Grr1 is required to transduce the glucose signal to transcriptional regulatory proteins. When glucose levels are high, the post-translational association of Grr1 with Skp1 is markedly increased; this

effect is dependent on the carboxy-terminal region of Grr1 (Kipreos and Pagano 2000; Frescas and Pagano 2008).

6.4 α-Mannosidases and M-Type Lectins

6.4.1 α-Mannosidases

α-Mannosidases in eukaryotic cells participates in both glycan biosynthetic reactions and glycan catabolism. Two broad families of enzymes have been identified that cleave terminal mannose linkages from Asn-linked oligosaccharides (Moremen 2000), including the Class 1 mannosidases (CAZy GH family 47 (Henrissat and Bairoch 1996)) of the early secretory pathway involved in the processing of N-glycans and quality control and the Class 2 mannosidases (CAZy family GH38 (Henrissat and Bairoch 1996) involved in glycoprotein biosynthesis or catabolism. Within the Class 1 family of α-mannosidases, three subfamilies of enzymes have been identified (Moremen 2000). The ER α1,2-mannosidase I (ERManI) subfamily acts to cleave a single residue from Asn-linked glycans in ER. The Golgi α-mannosidase I (GolgiManI) subfamily has at least three members in mammalian systems (Lal et al. 1998; Tremblay and Herscovics 2000) that help in glycan maturation in the Golgi complex to form the $Man_5GlcNAc_2$ processing intermediate. The third subfamily of GH47 proteins comprises the ER degradation, enhancing α-mannosidase-like proteins (EDEM proteins) (Helenius and Aebi 2004; Hirao et al. 2006; Mast et al. 2005; Mast and Moremen 2006). These proteins have been proposed to accelerate the degradation of misfolded proteins in the lumen of the ER by a lectin function that leads to retrotranslocation to the cytosol and proteasomal degradation. Recent studies have also indicated that ERManI acts as a timer for initiation of glycoprotein degradation via the ubiquitin-proteasome pathway (Hosokawa et al. 2003; Wu et al. 2003). Gong et al. (2005) discussed methods for analysis of the GH47 α-mannosidases, including expression, purification, activity assays, generation of point mutants, and binding studies by surface plasmon resonance. ERManI inhibitor kifunensine and down-regulation of EDEM suppressed the degradation of Y611H mutant channel proteins (Gong et al. 2005). ERAD of the misfolded genetic variant-null Hong Kong (NHK) α1-antitrypsin is enhanced by overexpression of ER processing α1,2-mannosidase (ERManI) in HEK 293 cells, indicating the importance of ERManI in glycoprotein quality control. In addition, EDEM, the enzymatically inactive mannosidase homolog, interacts with misfolded α1-antitrypsin and accelerates its effect showing a combined effect of ER ManI and EDEM on ERAD of misfolded α1-antitrypsin degradation (Hosokawa et al. 2001). It was also shown that misfolded α1-antitrypsin NHK contains labeled $Glc_1Man_9GlcNAc$ and $Man_{5\text{-}9}GlcNAc$ released by endo-β-N-acetylglucosaminidase.

Humans have four α-Mannosidases: ER mannosidase and Golgi mannosidases IA, IB and IC (gene names Man1B1, Man1A1, Man1A2 and Man1C1 respectively). ER mannosidase mediates the first mannose trimming reaction in the processing of N-linked glycans, reducing a $Man_9GlcNAc_2$ oligosaccharide to $Man_8GlcNAc_2$ by removal of a specific terminal mannose residue**.** ER mannosidase is conserved in eukaryotes; the yeast protein is known as Mns1. The trio of *cis*-Golgi mannosidases performs further specific trimming reactions on the $Man_8GlcNAc_2$ oligosaccharide, reducing the glycan to a $Man_5GlcNAc_2$ core that may be elaborated upon by a number of glycosyltransferases in the medial and *trans*-Golgi. The three Golgi mannosidases are conserved in vertebrates only and often have just a single homologue in invertebrate organisms. High-resolution crystal structures of the human and yeast ER α-1,2-mannosidases have been determined, the former complexed with the inhibitors 1-deoxymannojirimycin and kifunensine, both of which bind in its active site in the unusual $^1C_4$ conformation. Data revealed the roles of potential catalytic acid and base residues and the identification of a novel $^3S_1$ sugar conformation for the bound substrate analog. The co-crystal structure, in combination with $^1C_4$ conformation of a previously identified co-complex with the glycone mimic, 1-deoxymannojirimycin, indicated that glycoside bond cleavage proceeds through a least motion conformational twist of a properly predisposed substrate in the –1 subsite. A novel $^3H_4$ conformation is proposed as the exploded transition state (Karaveg et al. 2005).

6.4.2 ER-associated Degradation-enhancing α-Mannosidase-like Proteins (EDEMs)

The M-type lectins are members of glycosylhydrolase family 47 protein structural group. They are closely related to α-mannosidases of ER and *cis*-Golgi and function alongside these proteins in the handling of N-linked glycoproteins. These lectins lack key catalytic residues, as well as a key disulphide bond thought to be essential for enzymatic activity. As a result they bind to high mannose glycans attached to glycoproteins in ER lumen, but have no catalytic function (Hosokawa et al. 2001). Like other intracellular lectin families, the M-type lectin family is modest in size. Mammals have three M-type lectins, EDEM1, EDEM2 and EDEM3 (ER-associated degradation-enhancing α-mannosidase-like proteins), and these are generally conserved in metazoa, although EDEM1 is missing in *Drosophila* for example. Yeast has a single M-type lectin, Mnl1 that is related to EDEM1. Different M-type lectins are found in plants. M-type lectins are type II transmembrane proteins with very short cytoplasmic tails (although in some

cell types the expressed protein may lack transmembrane domain). In common with the ligand/substrate binding domains of glycoside hydrolase family 47 proteins, such as human ER mannosidase, the M-type CRD is a barrel-like structure with both α-helices and β-sheets. Some of the M-type lectins have C-terminal extensions after the M-type CRD that are not related to known protein folds (Fig. 6.3). The ERAD system is upregulated under stress conditions, when a greater proportion of proteins remain unfolded or misfolded, and serves to clear the secretory pathway of 'waste' proteins and thus prevent clogging. EDEM1 was the first M-type lectin established as having a role in recognizing unfolded or misfolded proteins in ERAD (Hosokawa et al. 2001).

More recently, EDEMs 2 and 3 have also been shown to accelerate ERAD, but EDEM3 is unique in retaining mannosidase activity, suggesting it enhances ERAD by a different mechanism to EDEMs 1 and 2. Endogenous EDEM1 exists mainly as a soluble glycoprotein. High-resolution analysis showed that endogenous EDEM1 is sequestered in buds that form along cisternae of the rough ER at regions outside of the transitional ER. They give rise to approximately 150-nm vesicles scattered throughout the cytoplasm that are lacking a recognizable COPII coat. Some of the EDEM1 vesicles also contain Derlin-2 and the misfolded Hong Kong variant of α-1-antitrypsin, a substrate for EDEM1 and ERAD. Experiments demonstrate the existence of a vesicle budding transport pathway out of the rough ER that does not involve the canonical transitional ER exit sites and therefore represents a passageway to remove potentially harmful misfolded luminal glycoproteins from ER (Zuber et al. 2007). EDEM may function as an acceptor of terminally misfolded glycoproteins. To test this hypothesis, Hosokawa et al. (2006) constructed several genetically manipulated cell lines and suggested that EDEM may function as a molecular chaperone. To examine this possibility, researchers found that the accumulation of covalent NHK dimers was selectively prevented by the over-expression of EDEM, and therefore EDEM may maintain the retrotranslocation competence of NHK by inhibiting aggregation so that unstable misfolded proteins can be accommodated by the dislocon for ERAD.

The structure of ER mannosidase with a bound substrate analogue suggests that the ligand binding site in M-type CRDs is a deep cleft at one end of the barrel. This unusually deep binding site for a lectin allows selective interaction with high mannose glycans rather than just terminal residues (Fig. 6.6).

6.4.3 Functions of M-Type Lectins in ERAD

The folding of glycoproteins in ER lumen is assisted by chaperone proteins including the calnexin family lectins (Chap. 2). Proteins which have failed to fold correctly are re-glucosylated to signal that re-binding to calnexin is necessary. Trimming of mannose from a $Man_9GlcNAc_2$ glycan on an unfolded glycoprotein is the signal that the glycoprotein should be exported from ER to cytoplasm for degradation, and concordantly inhibits further participation of the glycoprotein in the calnexin cycle. Despite several dedicated chaperones and folding factors that ensure efficient maturation, protein folding remains error-prone and mutations in the polypeptide sequence may significantly reduce folding-efficiency. Folding-incompetent proteins carrying N-glycans are extracted from futile folding cycles in the calnexin chaperone system upon intervention of EDEM1, EDEM2 and EDEM3, three ER-stress-induced members of the glycosyl hydrolase 47 family (Olivari and Molinari 2007). The plant toxin ricin is transported retrogradely from the cell surface to the ER from where the enzymatically active part is retrotranslocated to the cytosol, presumably by the same mechanism as used by misfolded proteins. The EDEM is responsible for directing aberrant proteins for ERAD (Fig. 6.3). EDEM is involved in ricin retrotranslocation. from the ER to cytosol (Slominska-Wojewodzka et al. 2006).

All glycoproteins - folded or unfolded - bear a $Man_8GlcNAc_2$ glycan following ER mannosidase processing, so ERAD receptors are required to distinguish between folded and unfolded glycoproteins bearing this structure, in order to trigger degradation of the unfolded ones only. If the protein is permanently misfolded, the mannose residue in the middle branch of the oligosaccharide is removed by ERManI. This leads to recognition by EDEM, which probably targets glycoproteins for ERAD. The EDEM proteins are believed to fulfil this role, probably alongwith other proteins such as the P-type lectin OS-9. EDEMs in turn hand over bound glycoproteins to the translocon machinery for retrotranslocation into the cytoplasm, where the glycoproteins are recognized by proteins including F-box lectins and targeted for proteasomal degradation. That the protein generating the $Man_8GlcNAc_2$ degradation signal (ER mannosidase) and the proteins recognizing the signal (EDEMs) are structurally related points to concurrent evolution of both aspects of the ERAD system in a very early eukaryotic ancestor.

EDEM has been shown to interact with calnexin, but not with calreticulin, through its transmembrane region. Both binding of substrates to calnexin and their release from calnexin were required for ERAD to occur. Over-expression of EDEM accelerated ERAD by promoting the release of terminally misfolded proteins from calnexin. Thus, EDEM appeared to function in the ERAD pathway by accepting substrates from calnexin (Oda et al. 2003). Overexpression of ERManI greatly increases the formation of $Man_8GlcNAc$, induced the formation of Glc1Man8GlcNAc and increased trimming to $Man_{5-7}GlcNAc$. A model whereby the misfolded glycoprotein interacts with ERManI and with EDEM, before being recognized by downstream ERAD

Fig. 6.6 Human ER class I α1,2-mannosidase structure showing catalytic domain of human ER mannosidase with bound substrate analogue. The thiodisaccharide substrate analogue is shown in *green* and the Ca^{2+} ion in *dark blue* (PDB: ID: 1X9D; Karaveg et al. 2005).

components, suggests that the carbohydrate recognition determinant triggering ERAD may not be restricted to $Man_8GlcNAc2$ isomer B as suggested earlier (Hosokawa et al. 2003). It was further demonstrated that overexpression of Golgi α1,2-mannosidase IA, IB, and IC also accelerates ERAD of terminally misfolded human α1-antitrypsin variant, and mannose trimming from the N-glycans on NHK in 293 cells (Hosokawa et al. 2007).

EDEM extracts misfolded glycoproteins, but not glycoproteins undergoing productive folding, from the calnexin cycle. EDEM overexpression resulted in faster release of folding-incompetent proteins from the calnexin cycle and earlier onset of degradation, whereas EDEM down-regulation prolonged folding attempts and delayed ERAD. Up-regulation of EDEM during ER stress may promote cell recovery by clearing the calnexin cycle and by accelerating ERAD of terminally misfolded polypeptides (Molinari et al. 2003). Non-maintenance of efficient glycoprotein folding in cells with defective ERAD caused by lack of adaptation of the intralumenal level of EDEM may increase in the ER cargo load. Eriksson et al. (2004) indicated that up-regulation of EDEM to strengthen the ERAD machinery (but not up-regulation of calnexin to reinforce the folding machinery) was instrumental in maintaining folding efficiency and secretory capacity and thus underscore the important role for degradation machinery in maintaining a functional folding environment in ER.

The efficiency of ERAD, which orchestrates the clearance of structurally aberrant proteins under basal conditions, is boosted by the unfolded protein response (UPR) as one of several means to relieve ER stress. However, the underlying mechanism that links the two systems in higher eukaryotes has remained elusive. Results of transient expression, RNAi-mediated knockdown and functional studies demonstrate that the transcriptional elevation of EDEM1 boosts the efficiency of glycoprotein ERAD through the formation of a complex that suppresses the proteolytic downregulation of ER mannosidase I (ERManI). A model is proposed in which ERManI, by functioning as a downstream effector target of EDEM1, represents a checkpoint activation paradigm by which the mammalian UPR coordinates the boosting of ERAD (Termine et al. 2009). EDEM1 is a novel chaperone of rod opsin and its expression promoted the degradation of P23H rod opsin and decreased its aggregation. Thus, EDEM1 can be used to promote correct folding, as well as enhanced degradation, of mutant proteins in ER to combat protein-misfolding disease (Kosmaoglou et al. 2009).

6.4.3.1 EDEM1

EDEM-1 is a crucial regulator of ERAD. Under normal growth conditions, the intralumenal level of EDEM1 must be low to prevent premature interruption of ongoing folding programs. In unstressed cells, EDEM1 is segregated from the bulk ER into LC3-I-coated vesicles and is rapidly degraded. The rapid turnover of EDEM1 is regulated by a novel mechanism that shows similarities but is clearly distinct from macroautophagy (Cali et al. 2009). Cells with defective EDEM1 turnover contain unphysiologically high levels of EDEM1, show enhanced ERAD activity and are characterized by impaired capacity to efficiently complete maturation of model glycopolypeptides. EDEM1 specifically binds nonnative proteins in a glycan-independent manner. Inhibition of mannosidase activity with kifunensine or disruption of the EDEM1 mannosidase-like domain by mutation had no effect on EDEM1 substrate binding but diminished its association with the ER membrane adaptor protein SEL1L. These results support a model whereby EDEM1 binds nonnative proteins and uses its mannosidase-like domain to target aberrant proteins to the ER membrane dislocation and ubiquitination complex containing SEL1L (Cormier et al. 2009).

Endogenous over-expression of EDEM1 not only resulted in inappropriate occurrence throughout the ER but also caused cytotoxic effects. Proteasome inhibitors had no effect on the clearance of endogenous EDEM1 in non-starved cells. However, EDEM1 could be detected in purified autophagosomes. Furthermore, influencing the lysosome-autophagy by vinblastine or pepstatin A/E64d and inhibiting autophagosome formation by 3-methyladenine or siRNA knockdown stabilized EDEM1. It was demonstrated that endogenous EDEM1 in cells not stressed by the expression of a transgenic misfolded protein reaches the cytosol and is degraded by basal autophagy (Le Fourn et al. 2009). Overexpression of EDEM1 produces $Glc_1Man_8GlcNAc_2$ isomer C on terminally misfolded null Hong Kong 1-antitrypsin (NHK) in vivo. It was suggested that EDEM1 activity trims

mannose from the C branch of *N*-glycans in vivo (Hosokawa et al. 2010; Ushioda et al. 2008).

6.4.3.2 EDEM2

In humans there are a total of three EDEM homologs. One of the EDEM homologs from Homo sapiens was termed EDEM2 (C20orf31). Over-expression of EDEM2 accelerates the degradation of misfolded α1-antitrypsin, indicating that the protein is involved in ERAD (Mast et al. 2005). The transcriptional up-regulation of EDEM2 depends on the ER stress-activated transcription factor Xbp1 and selectively accelerates ERAD of terminally misfolded glycoproteins by facilitating their extraction from the calnexin cycle (Olivari et al. 2005).

6.4.3.3 EDEM3

Mouse EDEM3, a soluble homolog of EDEM consists of 931 amino acids and has all the signature motifs of Class I α-mannosidases in its N-terminal domain and a protease-associated motif in its C-terminal region. EDEM3 accelerates glycoprotein ERAD in transfected HEK293 cells, as shown by increased degradation of misfolded α1-antitrypsin variant (null (Hong Kong) and of TCRα. Overexpression of EDEM3 also greatly stimulates mannose trimming not only from misfolded α1-AT null (Hong Kong) but also from total glycoproteins, in contrast to EDEM, which has no apparent α1,2-mannosidase activity. Results suggest that EDEM3 has α1,2-mannosidase activity in vivo, suggesting that the mechanism whereby EDEM3 accelerates glycoprotein ERAD is different from that of EDEM (Hirao et al. 2006). Overexpression of EDEM3 enhances glycoprotein ERAD with a concomitant increase in mannose-trimming activity in vivo.

6.4.3.4 HTM1

In Saccharomyces cerevisiae, Jakob et al. (2001) identified a gene coding for a non-essential protein that is homologous to mannosidase I (HTM1), which is required for degradation of glycoproteins. Deletion of the HTM1 gene does not affect oligosaccharide trimming, yet, deletion of HTM1 did reduce the rate of degradation of the mutant glycoproteins but not of mutant non-glycoprotein. This mannosidase homolog is a lectin that recognizes $Man_8GlcNAc_2$ oligosaccharides that serve as signals in the degradation pathway. Clerc et al. (2009) defined the function of the Htm1 protein as an α1,2-specific exomannosidase that generates the $Man_7GlcNAc_2$ oligosaccharide with a terminal α1,6-linked mannosyl residue on degradation substrates. This oligosaccharide signal is decoded by ER-localized lectin Yos9p that in conjunction with Hrd3p triggers the ubiquitin-proteasome-dependent hydrolysis of these glycoproteins. The Htm1p exomannosidase activity requires processing of the N-glycan by glucosidase I, glucosidase II, and mannosidase I, resulting in a sequential order of specific N-glycan structures that reflect the folding status of the glycoprotein.

6.4.3.5 *S. pombe* ER α-Mannosidase

It has been postulated that creation of $Man_8GlcNAc_2$ isomer B (M_8B) by ER α-mannosidase I constitutes a signal for driving irreparably misfolded glycoproteins to proteasomal degradation. ER α-mannosidase I is present in extremely feeble form in *Schizosaccharomyces pombe*. The enzyme yielded M8B on degradation of $Man_9GlcNAc_2$ and was inhibited by kifunensin. Disruption of the α-mannosidase encoding gene almost totally prevented degradation of a misfolded glycoprotein. The enzyme, behaving as a lectin binding polymannose glycans of varied structures, would belong together with its enzymatically inactive homologue Htm1p/Mnl1p/EDEM, to a transport chain responsible for delivering irreparably misfolded glycoproteins to proteasomes. Kifunensin and 1-deoxymannojirimycin, being mannose homologues, would behave as inhibitors of ER mannosidase or/and Htm1p/Mnl1p/EDEM putative lectin properties (Movsichoff et al. 2005).

6.5 Derlin-1, -2 and -3

Derlin-1, a member of a family of proteins that bears homology to yeast Der1p, is a factor that is required for the human cytomegalovirus US11-mediated dislocation of class I MHC heavy chains from the ER membrane to the cytosol. Derlin-1 acts in concert with the AAA ATPase p97 to remove dislocation substrate proteins from the ER membrane. Mammalian genomes encode two additional, related proteins (Derlin-2 and Derlin-3). The similarity of the mammalian Derlin-2 and Derlin-3 proteins to yeast Der1p suggested that Derlins also play a role in ER protein degradation. Derlin-1 and Derlin-2 are ER-resident proteins that participates in the degradation of proteins from the ER. Furthermore, Derlin-2 forms a robust multiprotein complex with the p97 AAA ATPase as well as the mammalian orthologs of the yeast Hrd1p/Hrd3p ubiquitin-ligase complex (Lilley and Ploegh 2005).

Derlin-2 and -3 showed weak homology to Der1p, a transmembrane protein involved in yeast ERAD. Both Derlin-2 and -3 are up-regulated by unfolded protein response (UPR), and at least Derlin-2 is a target of the IRE1 branch of the response, which is known to up-regulate EDEM and EDEM2, receptor-like molecules for misfolded glycoprotein. Over-expression of Derlin-2 or -3 accelerated degradation of misfolded glycoprotein, whereas their knock-down blocked degradation. Derlin-2 and -3 are associated with EDEM and p97, a cytosolic ATPase responsible for extraction of ERAD substrates. Report indicates that Derlin-2 and -3 provide missing link between EDEM and p97 in the process of degrading misfolded glycoproteins (Lilley et al. 2006; Oda et al. 2006; Dixit et al. 2008).

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F-Box Lectins

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M-Type Lectins

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Part III

L-Type Lectins

L-Type Lectins in ER-Golgi Intermediate Compartment

7

G.S. Gupta

7.1 L-Type Lectins

7.1.1 Lectins from Leguminous Plants

L-type lectins possess a luminal carbohydrate recognition domain (CRD) that binds to high-mannose-type oligosaccharides in a Ca^{2+}-dependent manner. The L-type CRD is named after the lectins found in abundance in the seeds of leguminous plants, such as concanavalin A from jack beans. The history of L-type lectins is as old as discovery of plant lectins from seeds of leguminous plants in nineteenth century. The structural motifs of L-type lectins are now known to be present in a variety of glycan-binding proteins from other eukaryotic organisms. The domain is present in plant, fungal, and animal proteins, but plant and animal L-type lectins have divergent sequences and different molecular properties. While plant lectins are secreted-soluble proteins and found at high level in specialised tissues, animal L-type lectins are (often membrane-bound) luminal proteins that are found at low levels in many different cell types. This observation suggests that animal L-type lectins have different functions. The crystal structures of some of the legume seed lectins show structural similarities among these lectins and to some other lectins, including the galectins and a variety of other lectins. Therefore, the term "L-lectins" has been designated as a classification for all lectins with this legume seed lectin-like structure. The L-type lectin-like domain has an overall globular shape composed of a β-sandwich of two major twisted antiparallel β-sheets. The β-sandwich comprises a major concave β-sheet and a minor convex β-sheet, in a variation of the jelly roll fold (Velloso et al. 2002, 2003; Satoh et al. 2006, 2007).

7.1.2 L-Type Lectins in Animals and Other Species

L-type lectins in animal cells are involved in protein sorting in luminal compartments of animal cells. In humans and other mammals there are four L-type lectins: ERGIC-53, ERGIC-53 like (ERGL), vesicular integral membrane protein-36 (VIP36), and VIP36 like (VIPL). ERGIC-53 is found only in mammals and VIP36 is restricted to vertebrates, but ERGIC-53 and VIPL are also found in invertebrates. A protein similar to ERGIC-53 is present in the slime mold *Dictyostelium dyscoideum*, a very simple eukaryote. L-type lectins have different intracellular distributions and dynamics in the ER-Golgi system of secretory pathway and interact with N-glycans of glycoproteins in a Ca^{2+}-dependent manner, suggesting a role in glycoprotein sorting and trafficking. The VIP36 is an intracellular animal lectin that acts as a putative cargo receptor, which recycles between the Golgi and the ER (Hauri et al. 2002).

Proteins more distantly related to ERGIC-53 and VIP36 are present in yeast and other fungi and in protozoa. Emp46p and Emp47p are L-type lectins from *S. cerevisiae* which cycle between the ER and the Golgi to facilitate the exit of N-linked glycoproteins from the ER. Unlike ERGIC-53, binding of high mannose glycans does not require a Ca^{2+} ion. Emp46p binds a K^+ ion, which is essential for glycoprotein transport, at a different location to that of Ca^{2+} ion in ERGIC-53, and Emp47p does not bind any metal ions. The differences in metal binding are evident in the primary structure of the proteins. Members of the galectin family of lectins may be considered as members of the L-type lectin family and are the subject of a separate chapter. The pentraxins are a superfamily of plasma proteins that are involved in innate immunity in invertebrates and vertebrates. They contain L-type lectin folds and require Ca^{2+} ions for ligand binding (Chap. 8). Other carbohydrate-binding proteins that may fit into this category are discussed in this chapter.

VP4 is a monomeric sialic acid–binding domain with an L-type lectin fold. This domain is required for infectivity of most animal rotaviruses. Sialidases or neuraminidases are a superfamily of N-acylneuraminate-releasing (sialic-acid-releasing) exoglycosidases found mainly in higher eukaryotes

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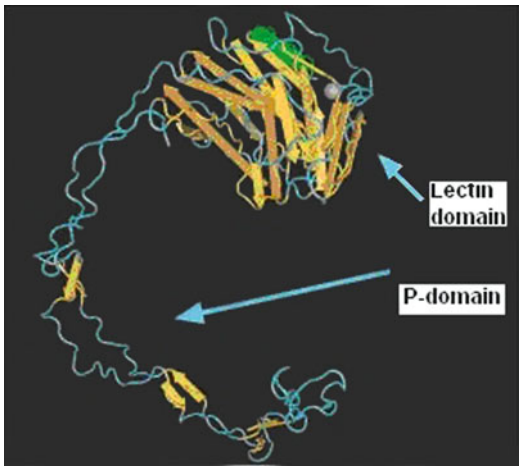

Fig. 7.1 Domain organization of calnaxin (Adapted with permission from Schrag et al. 2001 © Elsevier)

and in some, mostly pathogenic, viruses, bacteria and protozoans and contain L-type lectin domain. Several bacterial toxins such as exotoxin A (ETA) from *Pseudomonas aeruginosa* and Leech intramolecular trans-Sialidase and Vibrio cholerae Sialidase are known to possess CRD as observed in many lectins. Calnexin (Cnx) and calreticulin (Crt) are homologous chaperones that mediate quality control of proteins in the ER (see Chap. 2). Both Crt and Cnx are Ca^{2+}-binding proteins, and their carbohydrate-binding activity is sensitive to changes in Ca^{2+} concentration. Cnx is a type I membrane protein with its carboxy-terminal end in the cytoplasm. The lumenal portion of the protein is divided into three domains: a Ca^{2+}-binding domain (which is adjacent to the transmembrane domain), a proline-rich long hairpin loop called the P domain, and the amino-terminal L-type lectin domain. The Crt has a similar structure, but it is missing the cytoplasmic and transmembrane regions; it is retained in the ER through its KDEL-retrieval signal at the carboxyl terminus (Fig. 7.1)

7.2 ER-Golgi Intermediate Compartment

Protein traffic moving from ER to Golgi complex in mammalian cells passes through the tubulovesicular membrane clusters of the ER-Golgi intermediate compartment (ERGIC), the marker of which is the lectin ERGIC-53. Because the functional borders of the intermediate compartment (IC/ERGIC) are not well defined, the spatial map of the transport machineries operating between the ER and the Golgi apparatus remains incomplete. However, studies showed that the ERGIC consists of interconnected vacuolar and tubular parts with specific roles in pre-Golgi trafficking. The identification of ERGIC-53 has added to the complexity of the exocytic pathway of higher eukaryotic cells. A subcellular fractionation procedure for the isolation of the ERGIC from Vero cells provided a means to study more precisely the compartmentalization of the various enzymic functions along the early secretory pathway. The results suggested that in the secretory pathway of Vero cells O-glycan initiation and sphingomyelin as well as glucosylceramide synthesis mainly occur beyond the ERGIC in the Golgi apparatus (Schweizer et al. 1994). The dynamic nature and functional role of the ERGIC have been debated for quite some time. In the most popular current view, the ERGIC clusters are mobile transport complexes that deliver secretory cargo from ER-exit sites to the Golgi. Recent live-cell imaging data revealing the formation of anterograde carriers from stationary ERGIC-53-positive membranes, however, suggest a stable compartment model in which ER-derived cargo is first shuttled from ER-exit sites to stationary ERGIC clusters in a COPII-dependent step and subsequently to the Golgi in a second vesicular transport step. This model can better accommodate previous morphological and functional data on ER-to-Golgi traffic. Such a stationary ERGIC would be a major site of anterograde and retrograde sorting that is controlled by coat proteins, Rab and Arf GTPases, as well as tethering complexes, SNAREs and cytoskeletal networks. The ERGIC also contributes to the concentration, folding, and quality control of newly synthesized proteins (Appenzeller-Herzog and Hauri 2006). Marie et al. (2009)provided novel insight into the compartmental organization of the secretory pathway and Golgi biogenesis, in addition to a direct functional connection between the intermediate compartment and the endosomal system, which evidently contributes to unconventional transport of the cystic fibrosis transmembrane conductance regulator to the cell surface. The ERGIC defined by ERGIC-53 also participates in the maturation of (or is target for) several viruses such as corona virus, cytomegalovirus, flavivirus, poliovirus, Uukuniemi virus, and vaccinia virus. Further analysis of the function of ERGIC-53, and the use of ERGIC-53 as a marker protein, should provide novel results about the mechanisms controlling traffic in the secretory pathway.

7.3 Lectins of Secretory Pathway

The most prominent cycling lectin is mannose-binding type I membrane protein ERGIC-53 (ERGIC protein of 53 kDa), a marker for ERGIC, which functions as a cargo receptor to facilitate export of an increasing number of glycoproteins with different characteristics from the ER. The ERGIC-53 is a homo-hexameric transmembrane lectin localized to the ERGIC that exhibits mannose-selective properties in vitro. Two ERGIC-53-related proteins, VIP36 (vesicular integral

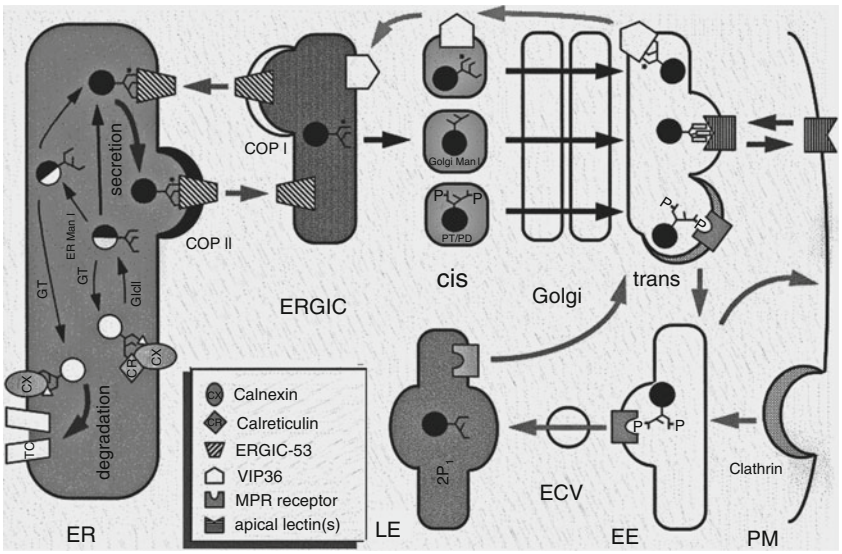

Fig. 7.2 Lectin-mediated glycoprotein transport in the secretory pathway. The secretory pathway is composed of membrane compartments specialized in protein folding, modification, transport, and sorting. Several lines of evidence indicate that glycan moieties are essential for folding, sorting and targeting of glycoproteins through the secretory pathway to various cellular compartments. Numerous transient protein-protein interactions guide the transport-competent proteins through the secretory pathway (Lee et al. 2004). Crystallographic and NMR studies of proteins located in ER, Golgi complex and ERGIC have illuminated their roles in glycoprotein folding and secretion. Calnexin and calreticulin, both ER-resident proteins with lectin domains are crucial for their function as chaperones (Chap. 2). After synthesis and removal of the two outermost glucose residues of their *N*-glycans many glycoproteins bind to calnexin and/or calreticulin which recognize monoglucosylated *N*-glycans. Subsequently the glycoproteins are trimmed by glucosidase II (*GlcII*). Thel incompletely folded protein (marked in *white*) is reglucosylated by ER enzyme UDP-glucose:glycoprotein glucosyltransferase (*GT*) which redirects them to another cycle of quality control. After prolonged time in the ER, ER mannosidase I (*ER Man I*) removes one mannose residue of the middle branch of the *N*-glycan. Incompletely folded, and thus reglucosylated, Man_8 glycoproteins are targeted for calnexin-dependent retrotranslocation to the cytosol and subsequent degradation by the proteasome. By contrast, correctly folded proteins (marked in *black*) are no longer recognized by GT after deglucosylation by GlcII and are transport-competent. They may or may not undergo some additional trimming by mannosidase I and II before leaving the ER. Some of these $Man_{7–9}$ glycan-bearing proteins (*) now bind to the lectin ERGIC-53 which recruits them to COPII buds and thereby facilitates transport to the ERGIC. Dissociation of ERGIC-53 and its glycoprotein ligand occurs in the ERGIC and free ERGIC-53 recycles to the ER via COPI vesicles. In the *cis*-Golgi, glycoproteins are either trimmed to Man_5 prior to reglucosylation by Golgi glycosyltransferases, or tagged with the lysosomal signal Man-6-P by sequential action of phosphotransferase (*PT*) and phosphodiesterase (*PD*). Some glycoproteins escape *cis*-Golgi trimming but may be recognized by VIP36 in the *trans*-Golgi and recycled to the *cis*-Golgi for another trimming attempt. Proteins carrying Man-6-P residues are recognized by MPRs in the *trans*-Golgi and sorted to endosomes via clathrin-coated vesicles. Secreted Man-6-P-bearing glycoproteins can also be internalized from the plasma membrane by the large MPR. *N*-Glycans also serve as signals for Golgi exit and apical targeting in epithelial cells. These processes may also be mediated by lectins that are, however, unknown. *ECV* endosomal carrier vesicles, *EE* early endosome, *LE* late endosome, *TC* translocation channel (Reprinted with permission from Hauri et al. 2000a © Elsevier)

membrane protein 36) and a ERGIC-53-like protein (ERGL) are also found in early secretory pathway. The homologous lectin VIP36 may operate in quality control of glycosylation in the Golgi. In addition to well-understood role of mannose 6-phosphate receptors in lysosomal protein sorting, the VIP36 functions as a sorting receptor by recognizing high-mannose type glycans containing α1—>2Man residues for transport from Golgi to cell surface in polarized epithelial cells (Hauri et al. 2000a, b). The ERGL may act as a regulator of ERGIC-53. Exit from the Golgi of lysosomal hydrolases to endosomes requires mannose 6-phosphate receptors and exit to the apical plasma membrane may also involve traffic lectins. Analysis of the cycling of ERGIC-53 uncovered a complex interplay of trafficking signals and revealed novel cytoplasmic ER-export motifs that interact with COP-II coat proteins. These motifs are common to type I and polytopic membrane proteins including presenilin 1 and presenilin 7. The results support the notion that protein export from the ER is selective (Yamashita et al. 1999; Hauri et al. 2000b, 2002; Yerushalmi et al. 2001) (Fig. 7.2).

7.4 ER-Golgi Intermediate Compartment Marker-53 (ERGIC-53) or LMAN1

7.4.1 ERGIC-53 Is Mannose-Selective Human Homologue of Leguminous Lectins

Secretory proteins are cotranslationally translocated into lumen of ER, where they interact with ER-resident chaperones such as calnexin, and/or calreticulin. Only secretory proteins

that fold correctly are transported through the Golgi apparatus to their final destinations. Several proteins are known to be transported by specific receptors. Such receptors may include membrane proteins ERGIC-53, VIP36, the p24 family, and Erv29p, which cycle between ER and the Golgi apparatus (Nichols et al. 1998; Appenzeller et al. 1999; Muniz et al. 2000; Belden and Barlowe 2001). Protein ERGIC-53, in humans, is encoded by the *LMAN1* gene. The protein encoded by this gene is a type I integral membrane protein localized in the intermediate region between the endoplasmic reticulum and the Golgi, presumably recycling between the two compartments. Also named LMAN1, the protein is a mannose-specific lectin and is a member of L-type lectin family.

ERGIC-53 bears homology to leguminous lectins and binds to mannose (Itin et al. 1996). It is, therefore, proposed to recognize high mannose-type oligosaccharides attached to proteins and to transport these glycoproteins from the ER to Golgi apparatus. The ERGIC-53, a member of a putative new class of animal lectins, is associated with the secretory pathway. It is type I transmembrane lectin, which facilitates the efficient export of a subset of secretory glycoproteins from ER. Indeed, the lack of ERGIC-53 impairs the secretion of procathepsin C and blood coagulation factors V (FV) and VIII (FVIII) glycoproteins (Nichols et al. 1998; Vollenweider et al. 1998). Chemical cross-linking studies revealed that ERGIC-53 interacts with procathepsin Z in a mannose- and calcium-dependent manner (Appenzeller et al. 1999; Appenzeller-Herzog et al. 2005). However, ERGIC-53 and its mutant, which is unable to bind to mannose, both coimmunoprecipitate with FVIII, and treatment with tunicamycin does not reduce the interaction between ERGIC-53 and FVIII (Cunningham et al. 2003), which indicates that protein–protein interactions also contribute to this interaction. It is therefore possible that ERGIC-53 also acts as a molecular chaperone in addition to transporting glycoproteins.

Although ERGIC-53 has selectivity for mannose yet it has a low affinity for glucose and GlcNAc, but not for galactose. Since leguminous family of lectin proteins possesses a highly conserved invariant asparagine essential for carbohydrate binding, the corresponding mutation in ERGIC-53 as well as a mutation affecting a second site in the putative CRD abolished mannose-column binding and co-staining with mannosylated neoglycoprotein. Based on its monosaccharide specificity, domain organization, and recycling properties, it was proposed that ERGIC-53 functions as a sorting receptor for glyco-proteins in the early secretory pathway (Itin et al. 1996). ERGIC-53 is present as reduction-sensitive homo-oligomers, i.e. as a balanced mixture of disulfide-linked hexamers and dimers, with the two cysteine residues located close to the transmembrane domain playing a crucial role in oligomerization. It is present exclusively as a hexameric complex in cells. Beyond its interest as a transport receptor, ERGIC-53 is an attractive probe for studying numerous aspects of protein trafficking in the secretory pathway, including traffic routes, mechanisms of anterograde and retrograde traffic, retention of proteins in the ER, and the function of the ERGIC.

7.4.2 Cells of Monocytic Lineage Express MR60: A Homologue of ERGIC-53

Most mammalian macrophages express mannose receptor (membrane lectin of 175 kDa) allowing endocytosis of their ligands, but cells of monocytic lineage (HL60, U937, monocyte) lack this receptor. However, after permeabilization, promyelocytic, promonocytic cells and monocytes bind D-mannose-terminated neoglycoproteins. The intracellular mannose binding protein from the human promyelocytic cell line HL60 is a 60 kDa protein (MR60). Under similar conditions, mouse macrophages express a 175 kDa mannose receptor but not the 60 kDa receptor (Pimpaneau et al. 1991). Under non-reducing conditions, MR60 migrates as a 120 kDa protein. MR60 does not contain any N-glycan moiety that could be cleaved by N-glycanase. MR60 induces a sugar selective aggregation of beads coated with α-D-mannosyl residues while beads bearing α-D-glucosyl residues are not.

Independently, a promyelocytic protein (MR60) was purified by mannose-column chromatography, and a cDNA was isolated that matched MR60 peptide sequences. This cDNA was identical to that of ERGIC-53 and homologies with the animal lectin family of the galectins were noticed. Not all peptide sequences of MR60, however, were found in ERGIC-53, raising the possibility that another protein associated with ERGIC-53 might possess lectin activity. This lectin is a type I transmembrane protein which includes a luminal N-terminal domain, a transmembrane domain, and a short C-terminal cytosolic domain (Fig. 7.3). The sequence of MR60 is identical (except for one amino acid) to that of ERGIC-53 (Arar et al. 1995; Schindler et al. 1993). The sequence of MR60/ERGIC-53 from human cells and that of the rat homologue p58 revealed 89% identity at the amino acid level (Lahtinen et al. 1996). Homologous proteins of MR60/ERGIC-53 have been characterized in *Caenorrhabditis elegans* and *Xenopus*. MR60 is not present at the cell surface and is structurally and immunochemically distinct from 175 kDa mannose receptor of mature macrophages. MR60/ERGIC-53 shares significant homologies with VIP-36 and with leguminous plant lectins (Fiedler and Simons 1994).

The recombinant protein binds mannosides and is oligomeric, up to the hexameric form. Two truncated proteins

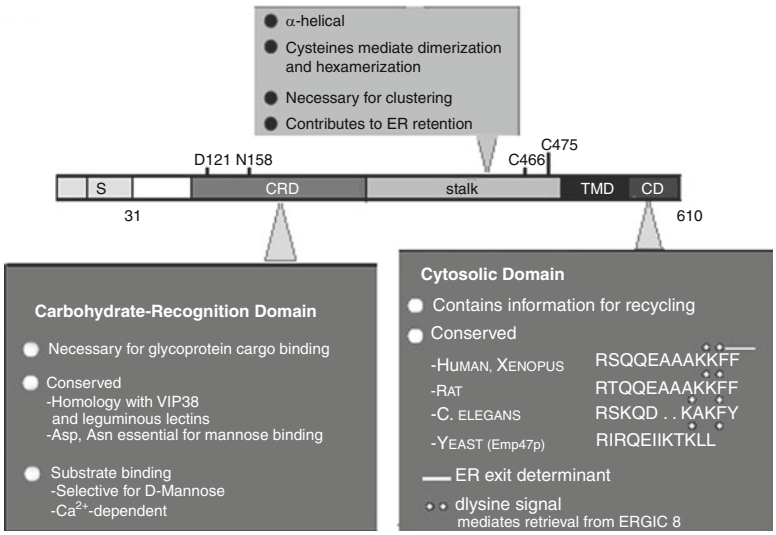

Fig. 7.3 Schematic diagram of ERGIC-53 showing functional domains. *CD* cytosolic domain, *S* signal sequence, *TMD* transmembrane domain. Some functionally important amino acid sequences (Adapted from Hauri et al. 2000b)

showed that the luminal moiety of MR60/ERGIC-53 contains a device, which allows both its oligomeric pattern and its sugar binding capability (Carriere et al. 1999a, b). ERGIC-53 is present as reduction-sensitive homo-oligomers, i.e. as a balanced mixture of disulfide-linked hexamers and dimers, with two cysteine residues located close to transmembrane domain playing a crucial role in oligomerization. It is present exclusively as a hexameric complex in cells. However, the hexamers exist in two forms, one as a disulfide-linked, Triton X-100, perfluoro-octanic acid, and SDS-resistant complex, and the other as a non-covalent, Triton X-100, perfluoro-octanoic acid-resistant, but SDS-sensitive, complex made up of three disulfide-linked dimers which are likely to interact through the coiled-coil domains present in the luminal part of the protein (Neve et al. 2005).

The cDNA sequence of the monkey homologue of ERGIC-53 revealed a sequence of 2,422 nt with 96.2% identity to the human ERGIC-53 cDNA and 87% and 67% identity to the rat and amphibian cDNA, respectively. The translated CV1 ERGIC-53 protein is 96.47% identical to the human ERGIC-53, 87% identical to rat p58 and 66.98% to *Xenopus laevis* protein. ERGIC-53 is expressed as a major transcript of about 5.5 kb in either monkey CV1 or in human CaCo7. A shorter transcript of 7.3 kb was detected in both cell lines and in mRNAs derived from human pancreas and placenta (Sarnataro et al. 1999).

7.4.3 Rat Homologue of ERGIC53/MR60

7.4.3.1 The 58-kDa Protein in Rat

The 58-kDa (p58), the type I homodimeric and hexameric microsomal membrane protein, has been characterized and localized to tubulo-vesicular elements at the ER-Golgi interface and the cis-Golgi cisternae in pancreatic acinar cells in rat (Lahtinen et al. 1992). The rat cDNA encodes a 517-amino acid protein having a putative signal sequence, a transmembrane domain close to the C terminus and a short cytoplasmic tail. The C-terminal tail contains a double-lysine motif (KKFF), known to mediate retrieval of proteins from the Golgi back to the endoplasmic reticulum. The rat p58 sequence is 89% identical with those of ERGIC-53 and MR60 and a strong homology with the frog sequence, indicating a high evolutionary conservation. Over-expression of c-Myc-tagged p58 resulted in accumulation of the protein both in the ER and in an apparently enlarged Golgi complex, as well as its leakage to the plasma membrane. The C-terminal tail of p58 located in the ER and transport intermediates is hidden, but becomes exposed when the protein reaches the Golgi complex (Lahtinen et al. 1996).

Shortly after synthesis, p58 forms dimers and hexamers, after which they are in equilibrium. The mature p58 contains four cysteine residues in the lumenal domain which are capable of forming disulphide bonds. The membrane-proximal half of the lumenal domain consists of four predicted alpha-helical domains, one heavily charged and three amphipathic in nature, all candidates for electrostatic or coiled-coil interactions. Using single-stranded mutagenesis, the cysteine residues were individually changed to alanine and the contribution of each of the alpha-helical domains was probed by internal deletions. The N-terminal cysteine to alanine mutants C198A and C238A and the double mutant, C198/238A oligomerized like the wild-type protein. The two membrane-proximal cysteines were found to be necessary for the oligomerization of p58. Mutants lacking one of the membrane proximal cysteines, either C473A or C482A, were unable to form hexamers, while dimers were formed normally. The C473/482A double

mutant formed only monomers. Deletion of any of the individual alpha-helical domains had no effect on oligomerization. The dimeric and hexameric forms bound equally well to D-mannose. The dimeric and monomeric mutants displayed a cellular distribution similar to the wild-type protein, indicating that the oligomerization status played a minimal role in maintaining the sub-cellular distribution of p58 (Lahtinen et al. 1999).

7.4.3.2 Crystal Structure

The structures of many of L-type lectins have been thoroughly characterized, and many are employed in a wide range of biomedical and analytical procedures. All soluble L-type lectins found to date are multimeric proteins, although all do not have the same quaternary structure. Thus, these lectins are multivalent with more than one glycan-binding site per lectin molecule. The same multivalent principle applies to the membrane bound L-type lectins because the presence of two or more molecules on a membrane surface essentially presents a multivalent situation. The L-type CRD is a β-sandwich structure with a concave sheet of seven β-strands and a convex sheet of five. The ligand binding site is in a negatively-charged cleft of conserved residues. Most L-type CRDs require metal ions for ligand binding: in concanavalin A, a transition metal is bound at one site and Ca^{2+} at the second site. Despite divergent sequences and function, it seems likely that the L-type CRDs have retained similar mechanisms of sugar binding. Certain key residues in four loop regions that contribute to the binding sites in the plant proteins are conserved in all of the animal and plant L-type CRDs. The L-type lectins are distinguished from other lectins primarily on the basis of tertiary structure. In general, either the entire lectin monomer or the CRDs of the more complex lectins are composed of antiparallel β-sheets connected by short loops and β-bends, and they are usually devoid of any α-helical structure. These sheets form a dome-like structure related to the 'jelly-roll fold,' and it is often called a 'lectin fold'. The carbohydrate-binding site is generally localized toward the apex of this dome (Srinivas et al. 2001).

Velloso et al. (2002) determined the crystal structure of CRD of p58, the rat homologue of human ERGIC-53, to 1.46 Å resolution. The CRD of rat p58 was over-expressed in insect cells and *E. coli,* purified and crystallized using Li_2SO_4 as a precipitant. The crystals belong to space group I222, with unit-cell parameters a = 49.6, b = 86.1, c = 128.1 Å, and contain one molecule per asymmetric unit. The fold and ligand binding site are most similar to those of leguminous lectins. The structure also resembles that of the CRD of the ER folding chaperone calnexin and the neurexins, a family of non-lectin proteins expressed on neurons. The CRD comprises one concave and one convex β-sheet packed into a β-sandwich. The ligand binding site resides in a negatively charged cleft formed by conserved residues. A large surface patch of conserved residues with a putative role in protein-protein interactions and oligomerization lies on the opposite side of the ligand binding site (Fig. 7.4a). Knowledge of the structure of p58/ERGIC-53 provides a starting model for understanding receptor-mediated glycoprotein sorting between the ER and the Golgi (Velloso et al. 2002).

7.4.4 Structure-Function Relations

7.4.4.1 The Recycling of ERGIC-53 in the Early Secretory Pathway

COPII proteins are necessary to generate secretory vesicles at ER. Investigation on the targeting of ERGIC-53 by site-directed mutagenesis revealed that its lumenal and transmembrane domains together confer ER retention (Schokman and Orci 1996). In addition the cytoplasmic domain is required for exit from the ER indicating that ERGIC-53 carries an ER-exit determinant. Two phenylalanine residues at the C terminus are essential for ER-exit. Thus, ERGIC-53 contains determinants for ER retention as well as anterograde transport which, in conjunction with a dilysine ER retrieval signal, control the continuous recycling of ERGIC-53 in the early secretory pathway. In vitro binding studies revealed a specific phenylalanine-dependent interaction between an ERGIC-53 cytosolic tail peptide and the COPII coat component Sec23p. Results suggest that the ER-exit of ERGIC-53 is mediated by direct interaction of its cytosolic tail with the Sec23pSec24p complex of COPII and that protein sorting at the level of the ER occurs by a mechanism similar to receptor-mediated endocytosis or Golgi to ER retrograde transport (Kappeler et al. 1997).

Nufer et al. (2003a) suggested that the ER export determinant of ERGIC-53, which cycles in the early secretory pathway, requires a phenylalanine motif at the C-terminus, known to mediate COPII interaction, which is assisted by a glutamine in the cytoplasmic domain. Disulfide bond-stabilized oligomerization is also required. Efficient hexamerization depends on the presence of a polar and two aromatic residues in the transmembrane domain (TMD). ER export is also influenced by TMD length, 21 amino acids being most efficient. Results suggest an ER-export mechanism in which transmembrane and luminal determinants mediate oligomerization required for efficient recruitment of ERGIC-53 into budding vesicles via the C-terminal COPII-binding phenylalanine motif (Nufer et al. 2003a).

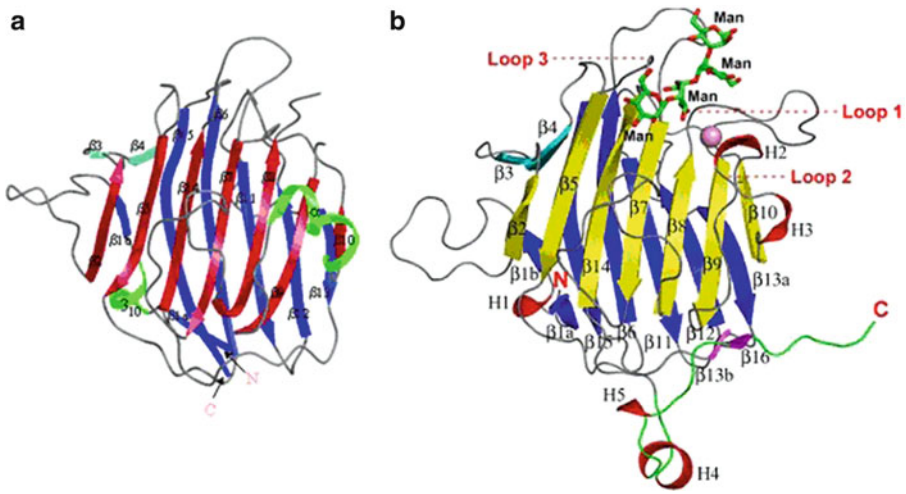

Fig. 7.4 (**a**) **Overall structure of Rat homologue (p58) of ERGIC53/MR60.** Ribbon diagram of p58 monomer shown perpendicular to the β-sheets. Secondary structure elements are labeled. Positions of the N and C termini are indicated. β-Strands belonging to the concave and convex β-sheets are shown in *red* and *dark blue*, respectively. Strands that do not take part in the β-sheets and are variable when compared with leguminous lectin structures are shown in *light blue*. Loops and helices are shown in *gray* and *green*, respectively (PDB ID: 1GV9 (Adapted with permission from Velloso et al. 2003 © Elsevier). (**b**) **Overall structure of exoplasmic/luminaldomain of VIP36.** Ribbon model of VIP36 (Man2-bound form) (PDB ID: 2DUR) is shown. The secondary structures are highlighted (β-strands belonging to concave β-sheets, *yellow*; β-strands belonging to convex β-sheets, *blue*; β-strands belonging to β-hairpin, *cyan*; β-strands belonging to short β-sheet formed between the stalk domain and one of the loops of the CRD, *magenta*; helices, *red*), and the loops of the CRD and stalk domain are colored *gray* and *green*, respectively. The bound Ca^{2+} is shown as a *pink sphere*. The bound oligomannoses are superimposed from the VIP36 complex structures with Man-α-1,2-Man and Man-α-1,2-Man-α-1,3-Man and are shown as a *green stick* model. The reducing-end mannose residue in the Man2-bound form is omitted because its position is almost the same as that of the $Man_3GlcNAc$-bound form. Positions of Loops 1, 2, and 3, which are bound to the oligomannose, are indicated (Adapted by permission from Satoh et al. 2007 © The American Society for Biochemistry and Molecular Biology)

7.4.4.2 Cytoplasmic ER-Retrieval Signal in ERGIC-53

Although ERGIC-53 is not a resident protein of the rough ER its cDNA sequence carries a double lysine ER retention motif at cytoplasmically exposed COOH terminus. Cell surface ERGIC-53 is efficiently endocytosed by a mechanism that is disturbed when the two critical lysines of the ER retention motif are replaced by serine residues. Results suggested a similarity between pre-Golgi retention by the double lysine motif and lysine based endocytosis (Kappeler et al. 1994). Although ERGIC-53 contains a cytoplasmic ER-retrieval signal, KKFF, over-expressed ERGIC-53 is transported to the cell surface and rapidly endocytosed. The ERGIC-53 endocytosis signal KKFF and like the ER-retrieval signal require a C-terminal position. In fact, the minimal consensus sequence determined by substitutional mutagenesis was also related to the ER-retrieval consensus (K-K-X-X). Evidence shows that internalization of VIP36, a protein that cycles between plasma membrane and Golgi, is mediated by a signal at its C-terminus that matches the internalization consensus sequence. The relatedness of the two signals suggests that coatomer-mediated retrieval of proteins may be mechanistically more related to clathrin-dependent sorting than previously anticipated (Itin et al. 1995a). Further dissection of the cytoplasmic domain revealed a COOH-terminal di-lysine ER-retrieval signal, KKFF, and an RSQQE targeting determinant adjacent to the transmembrane domain. Surprisingly, the two COOH-terminal phenylalanine residues influence the targeting. They reduce the ER-retrieval capacity of the di-lysine signal and modulate the RSQQE determinant (Itin et al. 1995b).

KKAA Retrieval Signal in Yeast: Studies on ERGIC-53 KKAA as a signal revealed a mechanism for static retention of mammalian proteins in the ER. This mechanism is conserved in yeast. Making use of a genetic assay, this signal was shown to induce COPI-dependent ER retrieval. ER retention of KKAA-tagged proteins was impaired in yeast mutants affected in COPI subunits. Furthermore, post-ER carbohydrate modifications detected on reporter proteins indicated that KKAA-tagged proteins recycle continuously within early compartments of the secretory pathway. Therefore in yeast, the KKAA signal might only function as a classical dilysine ER retrieval signal (Dogic et al. 2001).

A Single C-Terminal Valine Is Export Signal: The ERGIC-53 carries a C-terminal diphenylalanine motif that

is required for efficient ER export. Replacement of C-terminal diphenylalanine motif by a single C-terminal valine accelerates transport of inefficiently exported reporter constructs and hence operates as an export signal. The valine signal is position dependent. Results suggest that cytoplasmic C-terminal amino-acid motifs, either alone or in conjunction with other transport determinants, accelerate ER export of numerous type I and probably polytopic membrane proteins by mediating interaction with COPII coat components (Nufer et al. 2002).

7.4.4.3 Two Distinct Pathways for Golgi-To-ER Transport

The cytosolic COP-I interacts with cytoplasmic 'retrieval' signals present in membrane proteins that cycle between ER and the Golgi complex, and is required for both anterograde and retrograde transport in the secretory pathway. The role of COP-I in Golgi-to-ER transport of distinct marker proteins has been described. For example, anti-COP-I antibodies inhibit retrieval of ERGIC-53 and of the KDEL receptor from the Golgi to the ER. Transport to the ER of protein toxins, which contain a sequence that is recognized by the KDEL receptor, is also inhibited. Results indicated the existence of at least two distinct pathways for Golgi-to-ER transport, one COP-I dependent and the other COP-I independent. The COP-I-independent pathway is specifically regulated by Rab6 and is used by Golgi glycosylation enzymes and Shiga toxin/Shiga-like toxin-1 (Girod et al. 1999).

7.4.4.4 Site of Transport Arrest of Mitotic Cells

Using ERGIC-53/p58 and plasma membrane protein CD8, Farmaki et al. (1999) established the site of transport arrest between ER and Golgi stack of mitotic animal cells. Recycled ERGIC 53/p58 and newly synthesised CD8 accumulate in ER cisternae but not in COPII-coated export structures or more distal sites. During mitosis the tubulovesicular ER-related export sites were depleted of the COPII component Sec13p, which indicated that COPII budding structures are the target for mitotic inhibition. Findings established that the site of ER-Golgi transport arrest of mitotic cells is COPII budding structures (Farmaki et al. 1999).

7.4.5 Functions of ERGIC-53

7.4.5.1 ERGIC-53 as a Cargo Receptor

Soluble secretory proteins are transported from the ER to ERGIC in vesicles coated with COP-II coat proteins. The sorting of secretory cargo into these vesicles is thought to involve transmembrane cargo-receptor proteins. According to Appenzeller et al. (1999) a cathepsin-Z-related glycoprotein binds to ERGIC-53. Binding that occurs in the ER, is carbohydrate- and calcium- dependent and is affected by untrimmed glucose residues. Binding does not, however, require oligomerization of ERGIC-53, although oligomerization is required for exit of ERGIC-53 from the ER. Dissociation of ERGIC-53 occurs in the ERGIC and is delayed if ERGIC-53 is mislocalized to the ER. These results indicate that ERGIC-53 may function as a receptor facilitating ER-to-ERGIC transport of soluble glycoprotein cargo (Appenzeller et al. 1999).

7.4.5.2 ERGIC-53 in Traffic and in the Secretory Pathway

Functional deficiency in ERGIC-53 leads to a selective defect in secretion of glycoproteins in cultured cells and to hemophilia in humans. Beyond its interest as a transport receptor, ERGIC-53 is an attractive probe for studying numerous aspects of protein trafficking in the secretory pathway (Hauri et al. 2000b). Studies suggest that ERGIC is a dynamic membrane system composed of a constant average number of clusters and that the major recycling pathway of ERGIC-53 bypasses the Golgi apparatus (Klumperman et al. 1998). To investigate if ERGIC-53 is involved in glycoprotein secretion, a mutant form of this protein was generated that is incapable of leaving the ER. If expressed in HeLa cells in a tetracycline-inducible manner, this mutant accumulated in the ER and retained the endogenous ERGIC-53 in this compartment, thus preventing its recycling. It was suggested that recycling of ERGIC-53 is required for efficient intracellular transport of a small subset of glycoproteins, but it does not appear to be essential for the majority of glycoproteins (Vollenweider et al. 1998).

pH-Induced Conversion of ERGIC-53 Triggers Glycoprotein Release: Binding of cargo to ERGIC-53 in the ER requires Ca^{2+}. Cargo release occurs in the ERGIC, but the signals required for the cargo-receptor interaction are largely unknown. Though the efficient binding of ERGIC-53 to immobilized mannose occurs at pH 7.4, the pH of the ER, binding fails at slightly lower pH. pH sensitivity of the lectin was more prominent when Ca^{2+} concentrations were low. A conserved histidine in the center of the CRD was required for lectin activity suggesting it may serve as a molecular pH/Ca^{2+} sensor. Acidification of cells inhibited the association of ERGIC-53 with the known cargo cathepsin Z-related protein (Appenzeller-Herzog et al. 2004).

7.4.5.3 ERGIC-53 as a Marker of ER-Cargo Exit Site

A two-step reconstitution system for the generation of ER cargo exit sites from starting ER-derived low density microsomes (LDMs) has been described. The first step

involves the hydrolysis of Mg^{2+}ATP and Mg^{2+}GTP, leading to the formation of a transitional ER (tER) with the soluble cargo albumin, transferrin, and the ER-to-Golgi recycling membrane enriched proteins α2p24 and p58/ERGIC-53. Upon further incubation (step two) with cytosol and mixed nucleotides, interconnecting smooth ER tubules within tER transform into vesicular tubular clusters (VTCs). The cytosolic domain of α2p24 and cytosolic COPI coatomer affect VTC formation. This was observed from the effect of Abs to the C-terminal tail of α2p24, but not of Abs to the C-terminal tail of calnexin on this reconstitution. Therefore, the p24 family member, α2p24, and its cytosolic coat ligand, COPI coatomer play a role in the de novo formation of VTCs and the generation of ER cargo exit sites (Lavoie et al. 1999).

Stephens and Pepperkok (2002) examined the ER-to-Golgi transport of procollagen, which, when assembled in the lumen of the ER, is thought to be physically too large to fit in classically described 60–80 nm COPI- and COPII-coated transport vesicles. Using ERGIC-53 as a marker, data indicated the existence of an early COPI-dependent, pre-Golgi cargo sorting step in mammalian cells (Stephens and Pepperkok 2002).

Carbohydrate- and Conformation-Dependent Cargo Capture for ER-Exit: The targeting motif in ERGIC-53 is composed of a high-mannose type oligosaccharide intimately associated with a surface-exposed peptide β-hairpin loop. The motif accounts for ERGIC-53-assisted ER-export of the lyososomal enzyme pro-cathepsin Z. The second oligosaccharide chain of pro-cathepsin Z exhibits no binding activity for ERGIC-53, illustrating the selective lectin properties of ERGIC-53. Evidences suggest that the conformation-based motif is only present in fully folded pro-cathepsin Z and that its recognition by ERGIC-53 reflects a quality control mechanism that acts complementary to the primary folding machinery in the ER. A similar oligosaccharide/β-hairpin loop structure is present in cathepsin C, another cargo of ERGIC-53, suggesting the general nature of this ER-exit signal. Perhaps, the molecular mechanism underlying reversible lectin/cargo interaction involves the ERGIC as the earliest low pH site of the secretory pathway. Possibly, this is a report on an ER-exit signal in soluble cargo in conjunction with its decoding by a transport receptor (Appenzeller-Herzog et al. 2004, 2005).

Regeneration of Golgi Stacks at Peripheral ER Exit Sites: After microtubule depolymerization, Golgi membrane components are found to redistribute to a distinct number of peripheral sites that are not randomly distributed, but correspond to sites of protein exit from the ER. Whereas Golgi enzymes redistributed gradually over hours to these peripheral sites, ERGIC-53 redistributed rapidly (within minutes) to these sites after first moving through the ER. It was proposed that a slow but constitutive flux of Golgi resident proteins through the same ER/Golgi cycling pathways as ERGIC-53 underlies Golgi dispersal upon microtubule depolymerization. Both ERGIC-53 and Golgi proteins would accumulate at peripheral ER exit sites due to failure of membranes at these sites to cluster into the centrosomal region. Regeneration of Golgi stacks at these peripheral sites would re-establish secretory flow from the ER into the Golgi complex and result in Golgi dispersal (Cole et al. 1996).

Heat Shock Affects Translation and Recycling Pathway of ERGIC-53: ERGIC-53 accumulates during heat shock response. However, at variance with unfolded protein response, which results in enhanced transcription of ERGIC-53 mRNA, heat shock leads only to enhanced translation of ERGIC-53 mRNA. In addition, the half-life of the protein does not change during heat shock. Therefore, distinct pathways of the cell stress modulate the ERGIC-53 protein level. Heat shock also affects the recycling pathway of ERGIC-53. The protein rapidly redistributes in a more peripheral area of the cell, in a vesicular compartment that has a low sedimentation density in comparison to the compartment that contains majority of ERGIC-53 at 37°C. Moreover, the anterograde transport of two unrelated reporter proteins is not affected. Interestingly, MCFD2, which interacts with ERGIC-53 to form a complex required for the ER-to-Golgi transport of specific proteins, is regulated similar to ERGIC-53 in response to cell stress. These results support the view that ERGIC-53 alone, or in association with MCFD2, plays important role during cellular response to various stress conditions (Spatuzza et al. 2004).

ER stress induces transcription of ERGIC-53. The ERGIC-53 promoter contains a single cis-acting element that mediates induction of gene by thapsigargin and other ER stress-causing agents. This ER stress response element is highly conserved in mammalian ERGIC-53 genes. The ER stress response element contains a 5′-end CCAAT sequence that constitutively binds NFY/CBF and, 9 nt away, a 3′-end region (5′-CCCTGTTGGCCATC-3′) that is equally important for ER stress-mediated induction of the gene. This sequence is the binding site for endogenous YY1 at the 5′-CCCTGTTGG-3′ part and for undefined factors at the CCATC 3′-end. ATF6 alpha-YY1, but not XBP1, interacted with the ERGIC-53 regulatory region and activated ERGIC-53 ER stress response element-dependent transcription (Renna et al. 2007).

7.4.5.4 ERGIC-53 (LMAN1) and MCFD2 Form a Cargo Receptor Complex

Interaction of ERGIC-53 and MCFD2 with Factor VIII: The ERGIC is the site of segregation of secretory proteins for anterograde transport, via packaging into COPII-coated transport vesicles (Schekman and Orci 1996). Correctly folded proteins destined for secretion are packaged in the ER into COPII-coated vesicles, which subsequently fuse to form the ERGIC. Multiple coagulation factor deficiency 2 (MCFD2) is a soluble EF-hand-containing protein that is retained in ER through its interaction with ERGIC-53. Exit of soluble secretory proteins from ER can occur by receptor-mediated export as exemplified by blood coagulation factors V and VIII. Their efficient secretion requires ERGIC-53 and its soluble luminal interaction partner MCFD2, which form a cargo receptor complex in early secretory pathway. ERGIC-53 also interacts with two lysosomal glycoproteins cathepsin Z and cathepsin C. In absence of ERGIC-53, MCFD2 was secreted, whereas knocking down MCFD2 had no effect on the localization of ERGIC-53. Endogenous LMAN1 and MCFD2 are present primarily in complex with each other with a 1:1 stoichiometry, although MCFD2 is not required for oligomerization of ERGIC-53. Coimmunoprecipitation of ERGIC-53 and FVIII from transfected HeLa and COS-1 cells and results of the crystal structure of CRD of ERGIC-53 demonstrated an interaction between ERGIC-53 and FVIII in vivo, mediated via high mannose-containing asparagine-linked oligosaccharides, which were densely situated within B domain of FVIII, as well as protein-protein interactions (Cunningham et al. 2003). Perhaps ERGIC-53 and MCFD2 form a cargo receptor complex and that the primary sorting signals residing in the B domain direct the binding of factor VIII to ERGIC-53 1-MCFD2 through calcium-dependent protein-protein interactions. MCFD2 may function to specifically recruit factor V and factor VIII to sites of transport vesicle budding within the ER lumen (Zhang et al. 2003, 2005). These findings suggest that MCFD2-ERGIC-53 complex forms a specific cargo receptor for the ER-to-Golgi transport of selected proteins. However, MCFD2 is dispensable for the binding of cathepsin Z and cathepsin C to ERGIC-53, since ERGIC-53 can bind cargo glycoproteins in an MCFD2-independent fashion and that MCFD2 is a recruitment factor for blood coagulation factors V and VIII (Nyfeler et al. 2006).

7.4.5.5 ERGIC-53 in Quality Control

Activating Transcription Factors 6, a Regulator of Mammalian UPR: Newly synthesized secretory and transmembrane proteins are folded and assembled in ER where an efficient quality control system operates so that only correctly folded molecules are allowed to move along the secretory pathway. The productive folding process in ER has been thought to be supported by the unfolded protein response (UPR). The accumulation of unfolded proteins in the ER triggers a signaling response. In yeast the UPR affects several hundred genes that encode ER chaperones and proteins operating at later stages of secretion. In mammalian cells the UPR appears to be more limited to chaperones of the ER and genes assumed to be important after cell recovery from ER stress that are not important for secretion.

The mRNA of ERGIC-53 and its related protein VIP36 is induced by inducers of ER stress, tunicamycin and thapsigargin. The rate of synthesis of the ERGIC-53 protein is also induced by these agents. The response was due to the UPR since it was also triggered by castanospermine, a specific inducer of UPR, and inhibited by genistein. Thapsigargin-induced upregulation of ERGIC-53 could be fully accounted for by the activating transcription factors 6 (ATF6) pathway of UPR. It has been suggested that in mammalian cells the UPR also affects traffic from and beyond the ER (Nyfeler et al. 2003). However, a dilemma has emerged; activation of ATF6, the key regulator of mammalian UPR, requires intracellular transport from the ER to the Golgi apparatus. This suggests that unfolded proteins might be leaked from the ER together with ATF6 in response to ER stress, exhibiting proteotoxicity in the secretory pathway. It has been found that ATF6 and correctly folded proteins are transported to the Golgi apparatus via the same route and by the same mechanism under conditions of ER stress, whereas unfolded proteins are retained in the ER. Thus, the activation of UPR is compatible with the quality control in the ER and the ER possesses a remarkable ability to select proteins to be transported in mammalian cells in marked contrast to yeast cells, which actively utilize intracellular traffic to deal with unfolded proteins accumulated in the ER (Nadanaka et al. 2004).

ERGIC-53 in the Formation of Russell Bodies: Owing to the impossibility of reaching the Golgi for secretion or the cytosol for degradation, mutant Ig-micro chains that lack the first constant domain (micro-δCH1) accumulate as detergent-insoluble aggregates in dilated ER cisternae, called Russell bodies. The pathological role(s) of similar structures in ER storage diseases remains obscure. In cells containing smooth Russell bodies, ERGIC-53 co-localizes with micro-δCH1 aggregates in a Ca^{2+}-dependent fashion. Studies suggest that interaction with light chains or ERGIC-53 seed micro-δCH1condensation in different stations of the early secretory pathway (Mattioli et al. 2006).

7.4.6 Mutations in ERGIC-53 *LMAN1* Gene and Deficiency of Coagulation Factors V and VIII lead to bleeding disorder

Mutations in the ERGIC-53 gene are associated with a coagulation defect. Using positional cloning, the gene was

identified as the disease gene leading to combined factor V-factor (FV) and VIII (FVIII) deficiency, a rare, autosomal recessive disorder in which both coagulation factors V and VIII are diminished. ERGIC-53 was mapped to a YAC and BAC contig containing the critical region for the combined factors V and VIII deficiency gene. DNA sequence analysis identified two different mutations, accounting for all affected individuals in nine families studied. Findings indicated that ERGIC-53 may function as a molecular chaperone for the transport from ER to Golgi of a specific subset of secreted proteins, including coagulation factors V and VIII (Nichols et al. 1998). The crystal structure of CRD of ERGIC-53 complements the biochemical and functional characterization of the protein, confirming that a lectin domain is essential for this protein in sorting and transfer of glycoproteins from the ER to the Golgi complex. The lectin domains of calnexin and ERGIC-53 are structurally similar, although there is little primary sequence similarity. By contrast, sequence similarity between ERGIC-53 and VIP36, a Golgi-resident protein, leaves little doubt that a similar lectin domain is central to the transport and/or sorting functions of VIP36. The theme emerging from these studies is that carbohydrate recognition and modification are central to mediation of glycoprotein folding and secretion (Schrag et al. 2003).

Studies suggest that mutations in genes *LMAN1* and *MCFD2* are responsible for FVFVIII action. The binding of ERGIC-53 to sugar is enhanced by its interaction with MCFD2, and defects in this interaction in FV/FVIII patients may be the cause for reduced secretion of factors V and VIII (Kawasaki et al. 2008). Though clinically indistinguishable, MCFD2 mutations generally exhibit lower levels of FV and FVIII than LMAN1 mutations. The LMAN1 is a mannose-specific lectin which cycles between ER and ER-Golgi intermediate compartment. MCFD2 is an EF-hand domain protein that forms a calcium-dependent heteromeric complex with LMAN1 in cells. Missense mutations in the EF-hand domains of MCFD2 abolish the interaction with LMAN1. The LMAN1-MCFD2 complex may serve as a cargo receptor for the ER-to-Golgi transport of FV and FVIII, and perhaps a number of other glycoproteins. The B domain of FVIII may be important in mediating its interaction with the LMAN1-MCFD2 complex (Zhang 2009). Loss of functional ERGIC-53 leads to a selective defect in secretion of glycoproteins in cultured cells. Studies on the effect of defective ER to Golgi cycling by ERGIC-53 on the secretion of factors V and VIII showed that efficient trafficking of factors V and VIII requires a functional ERGIC-53 cycling pathway and that this trafficking is dependent on post-translational modification of a specific cluster of asparagine (N)-linked oligosaccharides to a fully glucose-trimmed, mannose9 structure (Moussalli et al. 1999).

7.4.6.1 DNA Polymorphism in ERGIC-53/*LMAN1* and *MCFD2* Genes

Mutations in a candidate gene of, *ERGIC-53*/*LMAN1*, were found to be associated with the coagulation defect in human population. Single-strand conformation and sequence analysis of the *ERGIC-53* gene in families of different ethnic origins identified 13 distinct mutations accounting for 52 of 70 mutant alleles. These were 3 splice site mutations, 6 insertions and deletions resulting in translational frame shifts, 3 nonsense codons, and elimination of the translation initiation codon. These mutations predict the synthesis of either a truncated protein product or no protein at all. This study revealed that FVFVIII shows extensive allelic heterogeneity and all ERGIC-53 mutations resulting in FV/FVIII are "null." Approximately 26% of the mutations have not been identified, suggesting that lesions in regulatory elements or severe abnormalities within the introns may be responsible for the disease in these individuals. In two such families, ERGIC-53 protein was detectable at normal levels in patients' lymphocytes, raising the further possibility of defects at other genetic loci (Neerman-Arbez et al. 1999; Nyfeler et al. 2005).

Nineteen additional families were analyzed by direct sequence analysis of the entire coding region and the intron/exon junctions. Seven novel mutations were identified in ten families, with one additional family found to harbor one of the two previously described mutations. All of the identified mutations were predicted to result in complete absence of functional ERGIC-53 protein. In 8 of 19 families, no mutation was identified. Thus a significant subset of combined factors V and VIII deficiency is due to mutation in one or more additional genes (Nichols et al. 1999). Two mutations in ERGIC-53 gene have been observed in Jews: a guanine (G) insertion in exon 1 among Middle Eastern Jewish families, and a thymidine (T) to cytosine (C) transition in intron 9 at a donor splice site among Tunisian families. All affected Tunisian families belonged to an ancient Jewish community. Screening this community for the intron 9T—>C transition, among 233 apparently unrelated individuals five heterozygotes were detected, predicting an allele frequency of 0.0107, while among 259 North African Jews none was found to carry the mutation. The G insertion in exon 1 was found in one of 245 Iraqi Jews, predicting an allele frequency of 0.0022, but in none of 180 Iranian Jews examined. In view of the relatively low frequency of the mutations in the respective populations it seems reasonable to advocate carrier detection and prenatal diagnosis only in affected families (Segal et al. 2004).

Three Indian families with FV/FVIII were analyzed for the presence of mutations in their LMAN1 and MCFD2 genes. One of the three families showed the presence of a G to A substitution in exon 2 of the MCFD2 gene, whereas

another family showed a nonsense mutation, i.e., G to T substitution, in exon 2 of the LMAN1 gene, the latter being a novel mutation not previously reported. The third family did not show mutations in either of the two genes, suggesting that a significant subset of FV/FVIII cases may be due to additional genes resulting in a similar phenotype (Mohanty et al. 2005). Immunoprecipitation and Western blot analysis detected a low level of LMAN1-MCFD2 complex in lymphoblasts derived from patients with missense mutations in LMAN1 (C475R) or MCFD2 (I136T), suggesting that complete loss of the complex may not be required for clinically significant reduction in FV and FVIII (Zhang et al. 2006).

7.4.6.2 *LMAN1* Expression in MSI-H Tumorigenesis

Roeckel et al. (2009) analyzed mutation frequencies of genes of glycosylation machinery in microsatellite unstable (MSI-H) tumors, focusing on frameshift mutations in coding MNRs (cMNRs). Among 28 candidate genes, LMAN1/ERGIC53 showed high mutation frequency in MSI-H colorectal cancer cell lines (52%; 12 of 23), carcinomas (45%; 72 of 161), and adenomas (40%; 8 of 20). Analysis of LMAN1-mutated carcinomas and adenomas revealed regional loss of LMAN1 expression due to biallelic LMAN1 cMNR frameshift mutations. In LMAN1-deficient colorectal cancer cell lines, secretion of α-1-antitrypsin (A1AT), an inhibitor of angiogenesis and tumor growth, was significantly impaired but could be restored upon LMAN1 re-expression. Results suggest that LMAN1 mutational inactivation is a frequent and early event potentially contributing to MSI-H tumorigenesis.

7.5 Vesicular Integral Membrane Protein (VIP36) OR LMAN2

7.5.1 The Protein

The vesicular integral membrane lectin (VIP36), also called -Lectin, mannose binding 2 (LMAN2) belongs to a family of lectins, conserved from yeast to mammals, trafficking in secretory pathway and closely related to lectin ERGIC-53 that acts as a cargo receptor, facilitating ER to Golgi transport of certain glycoproteins. VIP36 was originally identified as a component of apical post-Golgi vesicles in virally infected, polarized Madin-Darby canine kidney (MDCK) cells (Fiedler et al. 1994). VIP36 was shown to localize not only to the early secretory pathway but also to the plasma membrane of MDCK and Vero cells. The VIP36 recognizes high-mannose type glycans containing α1—>2 Man residues and α-amino substituted asparagine. The binding of VIP36 to high-mannose type glycans was independent of Ca^{2+} at optimum pH 6.0 at 37°C. The association constant between Man7-9GlcNAc2 in porcine thyroglobulin and immobilized VIP36 was $7.1 \times 10^8\ M^{-1}$. This shows that VIP36 functions as an intracellular lectin recognizing glycoproteins which possess high-mannose type glycans, $(Man\alpha1—>2)_{2–4}Man_5GlcNAc_2$ (Hara-Kuge et al. 1999).

Although VIP36 interacts with glycoproteins carrying high mannose-type oligosaccharides, further analysis using the frontal affinity chromatography (FAC) and the sugar-binding properties of a rCRD of VIP36 (VIP36-CRD) have shown that glucosylation and trimming of D1 mannosyl branch (Fig. 7.5) interfere with the binding of VIP36-CRD. VIP36-CRD exhibits an optimal pH value of ~6.5. Examining the specificity and optimal pH of sugar -VIP36 interaction and its subcellular localization, along with organellar pH, it was suggested that VIP36 binds glycoproteins that retain the intact D1 mannosyl branch in cis-Golgi network and recycles to ER where, due to higher pH, it releases its cargos, thereby contributing to quality control of glycoproteins.

In the plasma membrane, VIP36 exhibited an apical-predominant distribution, the apical/basolateral ratio being approximately 7. Localization of over-expressed VIP36 to plasma membrane, endosomes, and Golgi structures, together with evidence for lectin activity lead to the hypothesis that it functions to segregate apical cargo into distinct vesicles within the *trans*-Golgi network by binding specific *N*-glycans. Results indicated that VIP36 was involved in the transport and sorting of glycoproteins carrying high mannose-type glycan(s) (Fiedler and Simons 1994, 1995, 1996; Hara-Kuge et al. 2002). Punctate cytoplasmic structures co-localize with coatomer and ERGIC-53, labeling ER-Golgi intermediate membrane structures. Cycling of VIP36 is suggested by colocalisation with anterograde cargo trapped in pre-Golgi structures and modification of its N-linked carbohydrate by glycosylation enzymes of medial Golgi cisternae. Furthermore, after brefeldin A treatment VIP36 is segregated from resident Golgi proteins and codistributes with ER-Golgi recycling proteins (Fullekrug et al. 1997, 1999).

VIP36 shares significant homology with leguminous lectins as well as with ERGIC-53. Its ability to recognize high-mannose type glycans (Hara-Kuge et al. 1999; Kamiya et al. 2005, 2008) and its broad localization from ER to *cis*-Golgi (Fullekrug et al. 1999; Shimada et al. 2003a, 2003b) indicates that VIP36 also functions as a cargo receptor that facilitates the transport of various glycoproteins. Proteins that interact with VIP36 during quality control of secretory proteins have been identified. An 80 kDa immunoglobulin-binding protein (BiP), a major protein of Hsp70 chaperone family, binds VIP36. The interaction between VIP36 and BiP is not due to chaperone-substrate complex. The interaction depends on divalent cations but not on ATP. These observations suggest a new role for VIP36 in the quality

control of secretory proteins (Nawa et al. 2007; Kamiya et al. 2008). It was speculated that VIP36 binds to sugar residues of glycosphingolipids and/or glycosylphosphatidyl-inositol anchors and might provide a link between the extracellular/luminal face of glycolipid rafts and the cytoplasmic protein segregation machinery (Fiedler et al. 1994).

VIP36 is highly expressed in salivary glands, especially the parotid gland, which secretes α-amylase in large quantities. Endogenous VIP36 is localized in trans-Golgi network, on immature granules, and on mature secretory granules in acinar cells and co-localized with amylase. VIP36 is involved in the post-Golgi secretory pathway, suggesting that VIP36 plays a role in trafficking and sorting of secretory and/or membrane proteins during granule formation. EM demonstrated that VIP36 was primarily localized to secretory vesicles in glandula parotis of the rat, where α-amylase also resided. It was suggested that VIP36 is involved in the secretion of α-amylase in the rat parotid gland (Hara-Kuge et al. 2004; Shimada et al. 2003a). In GH3 cells endogenous VIP36 is localized mainly in 70–100-nm-diameter uncoated transport vesicles between the exit site on ER and the neighboring cis-Golgi cisterna. The thyrotrophin-releasing hormone (TRH) stimulation and treatment with actin filament-perturbing agents, cytochalasin D or B or latrunculin-B, caused marked aggregation of the VIP36-positive vesicles and the appearance of a VIP36-positive clustering structure located near cis-Golgi cisterna. The size of this structure, which comprised conspicuous clusters of VIP36, depended on the TRH concentration. Furthermore, VIP36 colocalized with filamentous actin in the paranuclear Golgi area and its vicinity. It suggests that actin filaments are involved in glycoprotein transport between the ER and cis-Golgi cisterna by using the lectin VIP36 (Shimada et al. 2003b).

7.5.2 VIP36-SP-FP as Cargo Receptor

To investigate the trafficking of transmembrane lectin VIP36 and its relation to cargo-containing transport carriers (TCs), Dahm et al. (2001) analyzed a C-terminal fluorescent-protein (FP) fusion, VIP36-SP-FP. At moderate levels of expression, VIP36-SP-FP is localized to the ER, Golgi apparatus, and intermediate transport structures, and colocalized with epitope-tagged VIP36. VIP36-SP-FP recycles in the early secretory pathway, exhibiting trafficking representative of a class of transmembrane cargo receptors, including the closely related lectin ERGIC-53. The VIP36-SP-FP trafficking structures comprised tubules and globular elements, which translocated in a salutatory manner. Simultaneous visualization of anterograde secretory cargo and VIP36-SP-FP indicated that the globular structures were pre-Golgi carriers, and that VIP36-SP-FP segregated from cargo within the Golgi and was not included in post-Golgi TCs (Dahm et al. 2001).

7.5.3 Structure for Recognition of High Mannose Type Glycoproteins by VIP36

It has been shown that ERGIC-53 interacts with glycoproteins carrying high mannose type glycan by endo-β-*N* acetylglucosaminidase H treatment (Appenzeller 1999; Moussalli 1999) and binds glycoproteins in a Ca^{2+} and pH-dependent manner (Appenzeller-Herzog et al. 2004, 2005). VIP36 has high affinity for high mannose type glycans containing Man-α-1,2-Man residues in Man7–9(GlcNAc)2-Asn peptides (Hara-Kuge et al. 1999). Kamiya et al. (2005) reported carbohydrate binding properties of VIP36 by frontal affinity chromatography and suggested Ca^{2+} dependence of carbohydrate binding and the specificity for D1 arm, Man-α-1,2-Man-α- 1,2-Man residues, of high mannose type glycans (corresponding to Man(D1)-Man (C)-Man(4) (Fig. 7.5).

The exoplasmic/luminal domain of VIP36 as well as the luminal domain of ERGIC-53 and Emp46/47p share homology with L (leguminous)-type lectins and are thus identified as CRDs. It has been shown that ERGIC-53 interacts with glycoproteins carrying high mannose type glycan by endo-β-*N*-acetylgluco- saminidase H treatment (Appenzeller et al. 1999; Moussalli, et al. 1999; Appenzeller-Herzog et al. 2005) and binds glycoproteins in a Ca^{2+}- and pH-dependent manner (Appenzeller-Herzog et al. 2004). It has been found that VIP36 has high affinity for high mannose type glycans containing Man-α-1,2- Man residues in Man7–9(GlcNAc)2-Asn peptides (Hara-Kuge et al. 1999). Kamiya et al. (2005) reported the carbohydrate binding properties of VIP36 by frontal affinity chromatography. This study suggested Ca^{2+} dependence of carbohydrate binding and the specificity for D1 arm, Man-α-1,2-Man-α-1,2-Man residues, of high mannose type glycans (corresponding to Man(D1)-Man(C)-Man (4); (Fig. 7.5). In addition, using a VIP36 binds glycoproteins carrying high mannose type glycans (Kawasaki et al. 2007). These observations suggested that VIP36 is involved in the transport of glycoproteins via high mannose type glycans.

Crystal structures of the CRD of rat ERGIC-53 in the absence and presence of Ca^{2+} have been determined, confirming its structural similarity to the L-type lectins (Velloso et al. 2002, 2003). In these reports, it was shown that the putative ligand-binding site of ERGIC-53 is similar to the mannose-binding site of the L-type lectins. The crystal structures of the CRD of Ca^{2+}-independent K^{+}-bound Emp46p and the metalfree form of Emp47p have also been reported (Satoh et al. 2006). Satoh et al. (2007) determined structures of transport lectin in complex with high mannose type glycans. and determined crystal structures of the exoplasmic/luminal domain of VIP36 alone and in complex

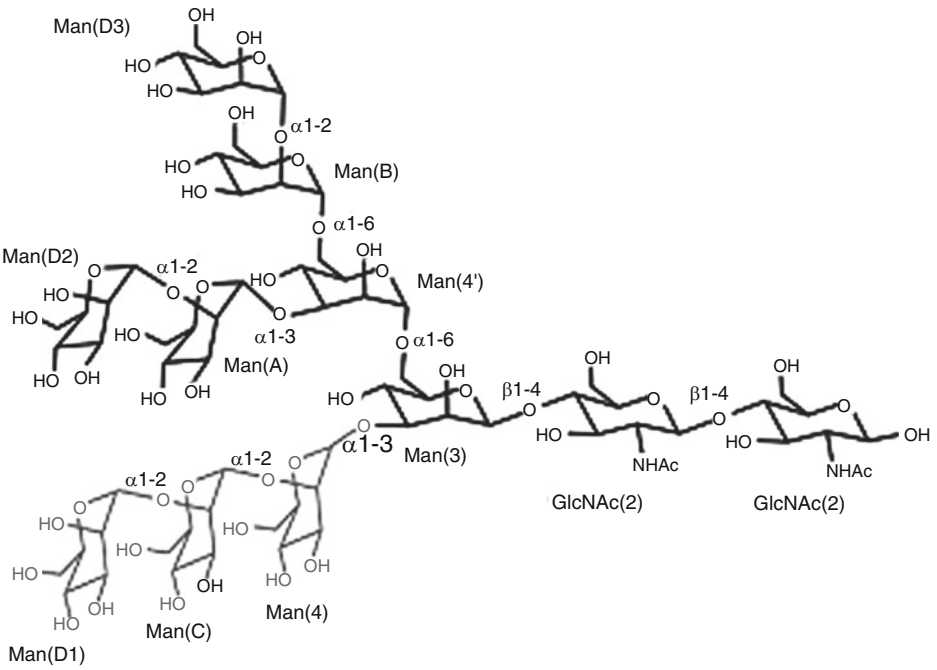

Fig. 7.5 Chemical structures of $Man_9(GlcNAc)_7$. The individual carbohydrate residues of $Man_9(GlcNAc)_2$ are labeled. The D1 arm of $Man_9(GlcNAc)_2$ is colored in *light grey*

with Ca^{2+} and mannose, Man-α-1,2-Man (termed Man2, which corresponds to Man(D1)-Man(C), Man(C)-Man(4), Man(D2)-Man(A), or Man(D3)-Man(B) of Man9(GlcNAc)2; and Man-α-1,2- Man-α-1,3-Man-β-1,4-GlcNAc (termed Man3GlcNAc, which corresponds to Man(C)-Man(4)-Man(3)-GlcNAc(2) (Fig. 7.5).

Satoh et al. (2007) reported the crystal structure of VIP36 exoplasmic/luminal domain comprising a CRD and a stalk domain and in complexed form with Ca^{2+} and mannosyl ligands. The CRD is composed of a 17-stranded antiparallel β-sandwich and binds one Ca^{2+} adjoining the carbohydrate-binding site. The structure reveals that a coordinated Ca^{2+} ion orients the side chains of Asp131, Asn166, and His190 for carbohydrate binding. This result explains the Ca^{2+}-dependent carbohydrate binding of this protein. The Man-α-1,2-Man-α-1,2-Man, which corresponds to the D1 arm of high mannose type glycan, is recognized by eight residues through extensive hydrogen bonds. The complex structures reveal the structural basis for high mannose type glycoprotein recognition by VIP36 in a Ca^{2+}-dependent and D1 arm-specific manner (Fig. 7.4b).

7.5.4 Emp47p of *S. cerevisiae:* A Homologue to VIP36 and ERGIC-53

Whereas mannose 6-phosphate receptor functions as a cargo receptor for lysosomal proteins in the *trans*-Golgi network, ERGIC-53 (Hauri et al. 2000a, b; Zhang et al. 2003) and its yeast orthologs Emp46/47p (Sato and Nakano 2003; Schroder et al. 1995) are transport lectins for glycoproteins that are transported out of ER. The *S. cerevisiae EMP47* gene encodes a type-I transmembrane protein with sequence homology to ERGIC-53 and VIP36. The 12-amino acid COOH-terminal cytoplasmic tail of Emp47p ends in the sequence KTKLL, which agrees with the consensus for di-lysine-based signals in ER. Despite the presence of this motif, Emp47p is known as a Golgi protein at steady-state. The di-lysine motif of Emp47p was functional when transplanted onto Ste2p, a plasma membrane protein, conferring ER localization. Emp47p cycles between the Golgi apparatus and the ER and requires a di-lysine motif for its α-COP-independent, steady state localization in Golgi (Schroder et al. 1995).

7.6 VIP36-Like (VIPL) L-Type Lectin

The profiles of human L-type lectin-like membrane proteins ERGIC-53, ERGL, and VIP36 and optimal alignment of entire CRD of these proteins revealed numerous orthologous and homologous L-type lectin-like proteins in animals, protozoans, and yeast, as well as the sequence of a novel family member related to VIP36, named VIPL for VIP36-like. VIPL has 43% similarity to ERGIC-53 and 68% similarity to VIP36. Its orthologues are broadly distributed in

many eukaryotes from human to fission yeast, whereas VIP36 is restricted to higher organisms. This phylogenetic finding suggests that VIP36 evolved from VIPL by gene duplication. VIPL is also predicted to have type I transmembrane topology with a putative CRD homologous to L-type lectins (Neve et al. 2003; Nufer et al. 2003b). Although the structural similarity between VIP36 and VIPL is very high, their characteristics are quite different. VIP36 and VIPL have the same cytoplasmic ER exit motif (KRFY) on their C-termini, but VIPL has an additional arginine at the fifth position from the C-terminus, creating the ER localization motif RKR. As a result, VIPL is a resident of ER (Neve et al. 2003; Nufer et al. 2003b), unlike ERGIC-53 and VIP36, which cycles in the secretory pathway. The sugar-binding activity of VIPL is also thought to be different from those of ERGIC-53 and VIP36; for example, HA-tagged VIPL does not bind to immobilized Man or BSA conjugated to Glc, Man, or GlcNAc as ERGIC-53 does (Nufer et al. 2003b). Moreover, VIPL does not compete with ERGIC-53 for binding to immobilized Man (Nufer et al. 2003b). Based on these observations, VIPL is thought to be an L-type lectin without sugar-binding activity. On the other hand, another group reported that knockdown of VIPL mRNA using short interfering RNA in HeLa cells significantly slowed down the secretion of two glycoproteins (35 and 250 kDa) into the medium (Neve et al. 2003), suggesting that VIPL does function in the secretion of glycoproteins, possibly by serving as an ER export receptor, similarly to ERGIC-53 (c/r Yamaguchi et al. 2007).

Although VIPL is structurally similar to VIP36, VIPL was thought not to be a lectin, because its sugar-binding activity had not been detected in several experiments. Yamaguchi et al. (2007) examined the sugar-binding activity and specificity of recombinant soluble VIPL (sVIPL) using cells with modified cell surface carbohydrates as ligands. Competition experiments with several high-mannose-type *N*-glycans indicated that VIPL recognizes the Man1–2Man1–2Man sequence and that the glucosylation of the outer mannose residue of this portion decreased the binding. Although the biochemical characteristics of VIPL are similar to those of VIP36, the sugar-binding activity of VIPL was stronger at neutral pH, corresponding to the pH in the lumen of the ER, than under acidic conditions. Unlike VIP36 and ERGIC-53 that are predominantly associated with ER membranes and cycle in the early secretory pathway, VIPL is a non-cycling resident protein of the ER. The results suggest that VIPL may function as a regulator of ERGIC-53 (Nufer et al. 2003a).

VIPL has been conserved through evolution from zebra fish to man. The 7.4-kb VIPL mRNA was widely expressed to varying levels in different tissues. The 32-kDa VIPL protein was detected in various cell lines. VIPL localized primarily to the ER and partly to the Golgi complex. Like VIP36, the cytoplasmic tail of VIPL terminates in the sequence KRFY, a motif characteristic for proteins recycling between the ER and ERGIC/cis-Golgi. Mutating the retrograde transport signal KR to AA resulted in transport of VIPL to the cell surface. Knockdown of VIPL mRNA using siRNA slowed down the secretion of two glycoproteins (M_r 35 and 250 kDa) to the medium, suggesting that VIPL may also function as an ER export receptor (Neve et al. 2003).

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Pentraxins: The L-Type Lectins and the C-Reactive Protein as a Cardiovascular Risk

8

G.S. Gupta

8.1 Pentraxins and Related Proteins

The pentraxins (PTX) are a superfamily of plasma proteins that are involved in innate immunity in invertebrates and vertebrates. They contain L-type lectin folds and require Ca^{2+} ions for ligand binding. Three of the principal members of the pentraxin family are serum proteins: namely, C-reactive protein (CRP), serum amyloid P component protein (SAP), and female protein (FP). PTX3 (or TSG-14) protein is a cytokine-induced protein that is homologous to CRPs and SAPs, but its exact function has not yet been determined. Beckmann et al. (1998) identified the superfamily of protein modules comprising the pentraxin families. Beckmann et al. (1998) predicted a jellyroll fold for all members of this superfamily. Pentraxins are made up of five noncovalently bound identical subunits that are arranged in a annular pentameric disc in shape. Proteins with this type of configuration are known as *pentraxins*. Based on the primary structure of the subunit, the pentraxins are divided into two groups: short pentraxins and long pentraxins. C-reactive protein (CRP), the first innate immunity receptor and serum amyloid P-component (SAP) are the two short pentraxins. Soluble pentraxins act as pattern recognition receptors with a dual role: protection against extracellular microbes and autoimmunity. The prototype protein of the long pentraxin group is pentraxin 3 (PTX3). The "long pentraxins" are an emerging family of genes that have conserved in their carboxy-terminal halves a pentraxin domain homologous to the prototypical acute phase protein pentraxins (CRP and SAP component) and acquired novel amino-terminal domains. Long pentraxins, including the prototype PTX3, are expressed in a variety of tissues and cells and in particular by innate immunity cells and most notably by dendritic cells and macrophages, in response to Toll-like receptor (TLR) engagement and in response to proinflammatory signals. PTX3 interacts with several ligands, including growth factors, extracellular matrix components and selected pathogens, playing a role in complement activation and facilitating pathogen recognition by phagocytes. Some long pentraxins are expressed in the brain and some are involved in neuronal plasticity and degeneration. PTX3 acts as a functional ancestor of antibodies, recognizing microbes, activating complement, and facilitating pathogen recognition by phagocytes, hence playing a non-redundant role in resistance against selected pathogens. In addition, PTX3 is essential in female fertility because it acts as a nodal point for the assembly of the cumulus oophorus hyaluronan-rich extracellular matrix. Thus, the prototypic long pentraxin PTX3 is a multifunctional soluble pattern recognition receptor at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Unlike the classical pentraxins, the PTX3 is expressed after exposure to the inflammatory cytokines to IL-1β and TNF-α, but not to IL-6, in various cell types. PTX3 has been shown to be produced in response to microbial infections, and highly elevated levels have been reported in patients with sepsis (al-Ramadi et al. 2004; Bottazzi et al. 2006; Garlanda et al. 2005; Mantovani et al. 2006).

8.2 Short Pentraxins

C-reactive protein (CRP) and serum amyloid P component (SAP) are short pentraxins which have been conserved through evolution. In humans both have pentameric structures and both play complex roles in the immune response, CRP being the classical acute-phase (AP) reactant produced in response to tissue damage and inflammation. Serum amyloid A (SAA) and the pentraxins CRP and SAP are major acute-phase proteins: their serum levels can rise by 1,000-fold, indicating that they play a critical role in defense and/or the restoration of homeostasis. The name "pentraxin" relates to the radial symmetry of five monomers forming a ring approximately 95 Å across and 35 Å deep (Fig 8.1–8.3). SAP exhibits multispecific calcium-dependent binding to oligosaccharides with terminal N-acetyl-galactosamine, mannose and glucuronic acid.

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Pentraxins have been conserved in evolution and share sequence homology, similar subunit assembly and the capacity for calcium-dependent ligand binding. The sequence homology and gene organization indicate that CRP and SAP arose from a gene duplication of an ancestral pentraxin gene. SAP is an integral component of all amyloid deposits. Inflammation induces dramatic changes in the biosynthetic profile of the liver, leading to increased serum concentrations of positive acute-phase proteins and decreased concentrations of negative acute-phase proteins. Hepatocytes are the single major site of pentraxin clearance and catabolism in vivo. This is consistent with the observation that SAP that has localized to amyloid deposits persists there and is not degraded (Hutchinson et al. 1994).

8.3 C-Reactive Protein

8.3.1 General

C-reactive protein was originally discovered in 1930 as a substance in the serum of patients with acute inflammation that reacted with the C polysaccharide of *pneumococcus* (Tillett and Francis 1930). It was named as C-reactive protein for its ability to precipitate the 'C' polysaccharide extracted from the pneumococcal cell wall (Black et al. 2004). Initially it was thought that CRP might be a pathogenic secretion, as it was elevated in people with a variety of illnesses, including cancer (Pepys and Hirschfield 2003). However, with the discovery of hepatic synthesis and secretion of CRP closed that debate. Nuclear localization of CRP with a high nuclear to cytoplasmic ratio is consistent with its active nuclear transport. Nuclear localization of SAP, a related protein, with a homologous nuclear localization signal was also identified. Because CRP has been known to inhibit RNA transcription and enhance chromatin degradation it was proposed that CRP may play a unique role in injured cells to alter processing of damaged nuclei (Du Clos 1996).

CRP is a glycoprotein and may have sugars—sialic acid, glucose, galactose and mannose—attached to it. In different disease states, one or two amino-acids get lopped off CRP. It retains its activity, but these losses open it up to glycosylation. Different diseases, which raise CRP add sugars to it in different patterns. The patterns of CRP are different across diseases, but similar among patients that had the same disease. Patients with lupus, leukemia, tuberculosis, leishmaniasis, Cushing's syndrome, and bone cancer have been reported in 2003. Although CRP increased the rate at which a particular parasite could invade blood cells, Tanusree et al. (2003) showed that different CRPs had very different potencies in this regard. It was speculated that subtyping CRP may give us more insight into heart attack mechanisms. The study offered circumstantial evidence that proves that the differing glycation is part of CRPs mode-of-action. CRPs, purified from several samples in different pathological conditions demonstrated that human CRP is glycosylated differently in some pathological conditions (Das et al. 2003). In rat serum, CRP is complexed with lipoprotein and interacts with apolipoprotein E. In contrast, human CRP showed no evidence of an interaction with rat serum or with the affinity-purified proteins. This selectivity coincided with the ability of these pentraxins to bind to O-phosphorylethanolamine (O-PE) with high affinity. The sedimentation properties of serum lipoproteins suggested an interaction with rat CRP (Schwalbe et al. 1995).

8.3.2 CRP Protein

CRP is a 224-residue protein with a monomer molar mass of 25,106 Da. Native CRP is somewhat different, as it has 10 subunits making two pentameric discs, with an overall molecular mass of 251,060 Da. Nucleotide sequencing of the coding regions of both cDNA and genomic DNA of CRP revealed an additional 19 amino acid peptide. The CRP gene contains a single 278 base pair intron within the codon specifying the third residue of mature CRP. The intron contains a repetitive sequence (GT)15 G(GT)3 which is similar to structures capable of adopting the Z-DNA form. A comparison of CRP coding and amino acid sequences with those of serum amyloid P component revealed striking overall homology which was not uniform: a region of limited conservation is bounded by two highly conserved regions (Woo et al. 1985).

The *CRP* gene is located on chromosome 1 (1q21-q23) and contains only one intron, which separates the region encoding the signal peptide from that encoding the mature protein. Induction of CRP in hepatocytes is principally regulated at the transcriptional level by cytokine IL-6, an effect which can be enhanced by IL-1β (Kushner et al. 1995). Both IL-6 and IL-1β control expression of many acute phase protein genes through activation of the transcription factors STAT3, C/EBP proteins, and Rel proteins (NF-kB). The unique regulation of each acute phase gene is due to cytokine-induced specific interactions of these and other transcription factors on their promoters. Thus, while C/EBP family members C/EBPβ and C/EBPδ are essential for induction of CRP, the NF-κB is essential for serum amyloid A genes. Interactions among these factors that result in enhanced stable DNA binding of C/EBP proteins result in maximum induction of the gene (Agrawal et al. 2003; Black et al. 2004).

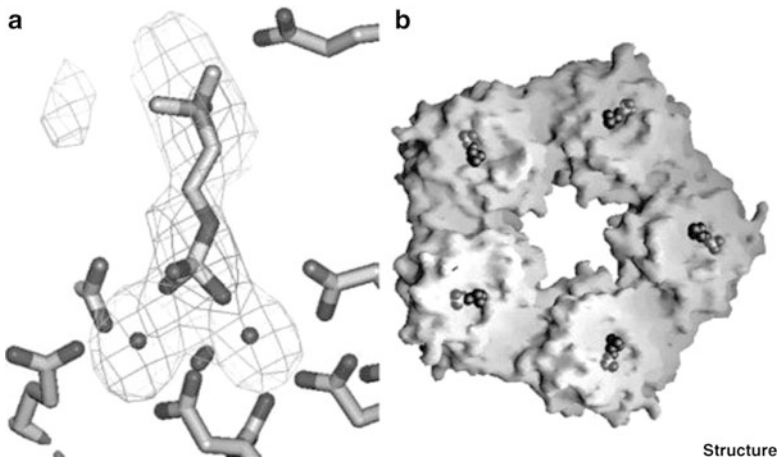

Fig. 8.1 Crystal structure of C-reactive protein complexed with phosphocholine. Ribbon diagram of the x-ray crystal structure of CRP-phosphocholine complex (PDB ID: 1B09). (**a**) Difference Fourier map at 2.5 Å resolution, contoured at 2σ, shows the positions of the two calcium ions (*spheres*) and a molecule of phosphocholine. (**b**) GRASP representation of CRP illustrating the positions of the five bound molecules of phosphocholine (*gray* and *black*) (Adapted by permission from Thompson et al. 1999 © Elsevier)

8.3.3 Structure of CRP

The CRP exists as a pentamer in the presence or absence of Ca^{2+}. Pentamers of CRP were shown to form mixed decamers in Ca^{2+}-free buffer; however, in the presence of Ca^{2+}, this interaction was not observed. Furthermore, no exchange of monomeric subunits was observed between the SAP and CRP oligomers, suggesting a remarkable stability of the individual pentameric complexes (Aquilina and Robinson 2003). The CRP structure contains a remarkable crystal contact, where the calcium binding loop including Glu^{147} from one protomer (Type II) coordinates into the calcium site of a protomer (Type I) in a symmetry related pentamer, revealing the mode of binding of the principal ligand PC and providing information concerning conformational changes associated with calcium binding. The Glu^{147}-Phe^{146} dipeptide from this loosely associated 140–150 loop mimics phosphate-choline (PC) binding in the accepting Type I protomer. The movement of the loop also results in the loss of calcium in the donating Type II protomer where large concerted movements of the structure, involving 43–48, 67–72 and 85–91 are seen. A striking structural cleft on the pentameric face opposite to the PC binding site, suggests an important functional role, perhaps in complement activation. There are significant conformational differences from SAP, both at the tertiary and molecular levels. In the region of the two-residue insertion with respect to SAP, human CRP shows large concerted movements of three loops due to both the insertion and sequence differences. These movements are reflected in the assembly of five protomers to form the pentamer, with a 15° rotation of each protomer, about the conformational axis, with respect to SAP. The calcium coordination differs from that in SAP due to the substitution $Asp^{147}Glu$ (Shrive et al. 1996).

CRP consists of five identical, noncovalently associated ~23-kDa protomers arranged symmetrically around a central pore. Each protomer has been found to be folded into two antiparallel β-sheets with a flattened jellyroll topology similar to lectins such as concanavalin A (Shrive et al. 1996; Thompson et al. 1999). Each protomer has a recognition face with a phosphocholine binding site consisting of two coordinated calcium ions adjacent to a hydrophobic pocket. The co-crystal structure of CRP with phosphocholine (Fig. 8.1) suggests that Phe^{66} and Glu^{81} are the two key residues mediating the binding of phosphocholine to CRP (Thompson et al. 1999). Phe^{66} provides hydrophobic interactions with the methyl groups of phosphocholine whereas Glu^{81} is found on opposite end of the pocket where it interacts with positively charged choline nitrogen. The importance of both residues has been confirmed by mutagenesis studies (Agrawal et al. 2002; Black et al. 2003, 2004).

The opposite face of the pentamer is the effector face, where complement C1q binds and Fcγ receptors are presumed to bind. A cleft extends from the center of the protomer to the central pore of the pentamer, and several residues along the boundaries of this cleft have been shown to be critical for the binding of CRP to C1q, including Asp^{112} and Tyr^{175} (Agrawal and Volanakis 1994; Agrawal et al. 2001). Gaboriaud et al. (2003) proposed a model for C1q binding to CRP. The model suggests that top of the predominantly positively charged C1q head interacts with the

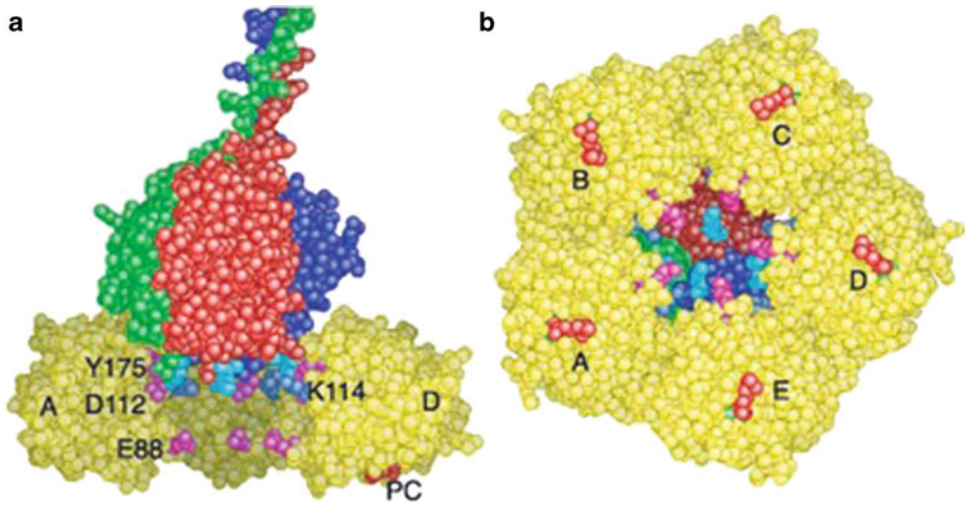

Fig. 8.2 Model of the interaction of CRP with C1q from Gaboriaud et al. (2003). (**a**) Side view. Subunits B and C of CRP have been omitted for clarity. (**b**) Perpendicular bottom view. Modules A, B, and C of the C1q subunit are shown in *blue*, *green*, and *red*, respectively. The lysines at the *top* of the C1q head (Ala^{173}, Ala^{200}, Ala^{201}, Cys^{170}) and Tyr^{B175} are in *light blue*. A–E designate the CRP protomers as described by Shrive et al. (1996). The phosphocholine (PC) ligand is in *red*, and the nearby Ca^{2+} ion is in *green*. Color coding for CRP mutations is as follows. Mutations impairing complement activation (Glu^{88}, Asp^{112}, Tyr^{175}) are *magenta*, and mutations enhancing complement activation (Lys^{114}) are *blue* (Adapted by permission from Gaboriaud et al. (2003) © The American Society for Biochemistry and Molecular Biology)

predominantly negatively charged central pore of the CRP pentamer; the globular head of C1q spans the central pore of CRP and interacts with two of the five protomers of the pentamer (Fig. 8.2). The steric requirements for CRP interaction with C1q imply that optimal C1q binding is accompanied by conformational changes in CRP structure (Gaboriaud et al. 2003). These conformational changes appear to differ depending on the ligand to which CRP is bound (c/r Black et al. 2004).

8.3.4 Functions of CRP

8.3.4.1 Innate Immunity

CRP is a member of the class of acute-phase reactants as its levels rise dramatically during inflammatory processes occurring in the body. This increment is due to a rise in the plasma concentration of IL-6, which is produced predominantly by macrophages (Pepys and Hirschfield 2003) as well as adipocytes (Lau et al. 2005). Its rapid synthesis after infection suggests it contributes to host defense. Unlike the cytokines, IL-6, IL-1β, and TNF-α that elicit CRP production from the liver (Mortensen 2001), CRP has a plasma half-life of 19 h and is quite stable in vitro (Aziz et al. 2003). Synthesis of the CRP is stimulated in response to many pathogens including gram-positive (Mold et al. 1981) and gram-negative pathogens, fungi, and malarial parasites (Volanakis 2001; Szalai 2002). It is thought to bind to phosphocholine, thus initiating recognition and phagocytosis of damaged cells (Pepys and Hirschfield 2003).

Volanakis and Kaplan (1971) identified the specific ligand for CRP in the pneumococcal C polysaccharide as phosphocholine, part of the techoic acid of the pneumococcal cell wall. CRP binding to phosphocholine is thought to assist in complement binding to foreign and damaged cells and to enhance phagocytosis by macrophages, which express a receptor for CRP (Tharia et al. 2002). C-reactive protein not only opsonizes a bacterium, but can also activate the complement cascade by binding to C1q, the first component of the classical pathway of complement activation (Gaboriaud et al. 2003). By binding to specific ligands of the pathogen's cell wall, CRP provides a means of defense against the invading pathogen (Mold et al. 1999). Unlike the activation of complement by immunoglobulin, complement activation initiated by CRP is limited to C1–C4 by the complement-control protein, factor H (Du Clos 2002; Giannakis et al. 2001, 2003). Therefore, CRP promotes phagocytosis of particles without generating a strong inflammatory response (Du Clos 2002). As a result, the strong inflammatory responses typically associated with C5a and the C5–C9 are limited.

In addition to interacting with various ligands and activating the classical complement pathway, CRP can stimulate phagocytosis, and bind to Ig receptors (FcγR). There is evidence that CRP can interact with the Ig receptors FcγRI and FcγRII as well, eliciting a response from phagocytic cells. The ability to recognize pathogens with subsequent recruitment and activation of complement, as well as effects

on phagocytic cells, constitute important components of the first line of host defense. Although phosphocholine was the first defined ligand for CRP, a number of other ligands have since been identified. Phosphocholine is found in a number of bacterial species and is a constituent of sphingomyelin and phosphatidylcholine in eukaryotic membranes. However, the head groups of these phospholipids are inaccessible to CRP in normal cells, so that CRP can bind to these molecules only in damaged and apoptotic cells (Gershov et al. 2000; Chang et al. 2002).

8.3.4.2 Clearance of Apoptotic Cells

Besides phosphocholine, CRP can bind to a variety of other ligands, including phosphoethanolamine, chromatin, histones, fibronectin, small nuclear ribonucleoproteins, laminin, and polycations (Black et al. 2003; Szalai et al. 1999). Due to interaction of CRP with phosphorylcholine in calcium-dependent fashion human CRP is classified as a phosphocholine (PC)-binding protein, whereas SAP is identified as a polysaccharide-binding protein (Christner and Mortensen 1994a). Mouse SAP showed binding interactions and specificity similar to human SAP. Female protein (FP) from hamster and rat CRP showed a hybrid specificity and bound to both phosphoryl- ethanolamine and phosphorylcholine. All of the proteins that bound phosphorylethanolamine also associated with human C4b-binding protein (C4BP) suggesting more functions for pentraxins (Schwalbe et al. 1992). Gershov et al. (2000) assessed binding of apoptotic lymphocytes with CRP and the effect of binding on innate immunity. As expected, CRP bound to apoptotic cells and augmented the classical pathway of complement activation but protected the cells from assembly of the terminal complement components. Furthermore, CRP enhanced opsonization and phagocytosis of apoptotic cells by macrophages associated with the expression of the antiinflammatory cytokine TGF-β. The antiinflammatory effects of CRP required C1q and factor H and were not effective once cells had become necrotic. Thus, CRP and the classical complement components act in concert to promote noninflammatory clearance of apoptotic cells and may help to explain how deficiencies of the classical pathway and certain pentraxins lead to impaired handling of apoptotic cells and increased necrosis with the likelihood of immune response to self (Gershov et al. 2000).

8.3.4.3 Pro- and Anti-inflammatory Activity

CRP has pleiotropic effects and shows both "pro-inflammatory" and "anti-inflammatory" activities. CRP has been shown to induce the expression of IL-1 receptor antagonist (Tilg et al. 1993) and increase release of the anti-inflammatory cytokine IL-10 (Mold et al. 2002a, b; Szalai et al. 2002b) while repressing synthesis of interferon-γ (Szalai et al. 2002b). Expression of IL-10 is induced by CRP's binding to Fcγ receptors on macrophages (Ogden and Elkon 2005). However, many other functions that can be regarded as pro-inflammatory are recognized. For example, CRP activates complement and enhances phagocytosis. CRP up-regulates the expression of adhesion molecules in endothelial cells, inhibits endothelial nitric-oxide synthase expression in aortic endothelial cells (Venugopal et al. 2002), stimulates IL-8 release from several cell types, increases plasminogen activator inhibitor-1 expression and activity, and increases the release of IL-1, IL-6, IL-18, and tumor necrosis factor-α (Ballou and Lozanski 1992). The CRP also exhibits a distinct anti-inflammatory activity indicated by its protective effects against endotoxic shock, allergic encephalitis, inflammatory alveolitis, nephrotoxic nephritis, and systemic lupus erythematosus (SLE) (Black et al. 2004; Rodriguez et al. 2005; Szalai et al. 2000a). This activity is believed to be mediated, at least in part, by the immunosuppressive cytokine IL-10. In addition, CRP appears to play a very important role in preventing autoimmunity (Du Clos and Mold 2004; Russell et al. 2004) by targeting apoptotic and necrotic cells removal (Du Clos 1996; Black et al. 2004); this suggests for at least this autoimmune disease, a failure of the normal CRP clearance mechanisms.

Increased levels of CRP are associated with endothelial dysfunction. The glycocalyx decorates the luminal surface and affords critical protection of the endothelium. C-reactive protein dose-dependently increased HA release in vitro and in vivo. There was a significant positive correlation between HA release and monocyte–endothelial cell adhesion, plasminogen activator inhibitor-1, and ICAM-1 release and a negative correlation with endothelial nitric oxide synthase activity (Devaraj et al. 2009b). Although some of these in vitro properties are consistent with the net in vivo effects of CRP observed in mice and described below, it is likely that the function of CRP is context-dependent and that it can either enhance or dampen inflammatory responses depending on the circumstance.

CRP Receptor: Functional effects of CRP on phagocytic cells have been recognized for many years. Recently the receptors for CRP have been identified as the already known receptors for IgG, FcγRI and FcγRII. Two general classes of FcγRs include stimulatory receptors and inhibitory receptors. The stimulatory receptors are characterized by a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) sequence. The inhibitory receptor is characterized by the presence of an immunoreceptor tyrosine-based inhibition motif (ITIM) sequence. Biological responses triggered by ITAM-containing FcγRs include phagocytosis, respiratory bursts, and secretion of cytokines. ITIM-containing FcγRs, when found co-aggregated with ITAM-containing FcγRs, negatively regulate ITAM-mediated activity. In both humans

and mice, CRP binds to ITAM- and ITIM-containing receptors, which include FcγRI and FcγRII. Phagocytosis of CRP-opsonized particles and apoptotic cells has been shown to proceed through FcγRI in the mouse (Mold et al. 2001, 2002). CRP has also been shown to induce signaling through human FcγRIIa, an ITAM-containing receptor, in granulocytes (Chi et al. 2002). The enhancement of phagocytosis by CRP is likely due to its interactions with FcγRs. However, some investigators have voiced doubts about CRP binding to FcγRs (Hundt et al. 2001).

In Vivo Effects: In contrast to humans, plasma levels of mouse CRP rarely exceed 2 μg/ml following inflammatory stimuli. The murine CRP response represents an evolutionary oddity, a natural knockdown that has been exploited in a variety of studies utilizing exogenous or transgenic CRP to study the effects of CRP in vivo. The ability of CRP to protect mice against bacterial infection by various species has been well established. These species include *S. pneumoniae* (Szalai et al. 1995) and *Haemophilus influenza* (Weiser et al. 1998; Lysenko et al. 2000), which have phosphocholine-rich surfaces, and *Salmonella enterica* serovar Typhimurium, which has no known surface phosphocholine, although its cell membrane is known to be rich in phosphoethanolamine (Szalai et al. 2000). Protection appears to be mediated through CRP binding to phosphocholine or phosphoethanolamine, followed by activation of classical complement pathway. CRP protection of mice infected with *S. pneumoniae* has been shown to require an intact complement system (Mold et al. 2002b) but does not require interaction with FcγRs (Mold et al. 2002a; Szalai et al. 2002b). CRP has been also shown to play a protective role in a variety of inflammatory conditions, including protecting mice from lethal challenge with bacterial LPS and various mediators of inflammation (Xia and Samols 1997). In addition, CRP has been found to delay the onset and development of experimental allergic encephalomyelitis, an aseptic animal model of multiple sclerosis (Szalai et al. 2002a). In a murine model of chemotactic factor-induced alveolitis, CRP has also been shown to inhibit the influx of neutrophils and protein into the lungs (Heuertz et al. 1994; Ahmed et al. 1996). Taken together, these experiments suggest that the net effect of CRP in mice is anti-inflammatory.

It is of interest that CRP may exert an ameliorative effect upon murine models of SLE in which CRP levels are often unexpectedly low (ter Borg et al. 1990). Two reports (Szalai et al. 2003; Du Clos 1996) and mouse models implicate polymorphism in human CRP gene resulting in a lower basal level of CRP with an increased risk of developing SLE (Russell et al. 2004). These findings suggest the possibility that decreased amounts of CRP may contribute to the pathogenesis of SLE. Since CRP is important in clearance of the cellular debris of necrotic and apoptotic cells by binding to damaged cell membranes and nuclear material, decreased clearance of such material might enhance development of autoantibodies to them.

8.4 CRP: A Marker for Cardiovascular Risk

8.4.1 CRP: A Marker for Inflammation and Infection

C-reactive protein is well known to rheumatologists. Levels of CRP in the blood serve as a reliable marker of disease activity in rheumatoid arthritis and various vasculitides. In systemic lupus erythematosus (SLE), the expression of CRP does not appear to correlate with disease activity, and levels generally remain low despite active disease. CRP is also known to infectious disease specialists; it serves as a marker of infection and participates in host defense (Du Clos 2003). Cardiologists have become intensely interested in CRP, and it has been widely examined as a risk factor for cardiovascular disease. These properties suggest that CRP is a sensitive marker for acute and chronic inflammation of diverse causes and various infection (Du Clos 2003). However, since many things can cause elevated CRP, this is not a very specific prognostic indicator (Lloyd-Jones et al. 2006). A high-sensitivity CRP test measures low levels of CRP and a level above 2.4 mg/l has been associated with a doubled risk of a coronary event compared to levels below 1 mg/l (Pepys and Hirschfield 2003). C-reactive protein is not normally found in the blood of healthy people. It appears after an injury, infection, or inflammation and disappears when the injury heals or the infection or inflammation disappears. CRP rises up to 50,000-fold in acute inflammation, such as infection. It rises above normal limits within 6 h, and peaks at 48 h. Serum amyloid A is a related acute-phase marker that responds rapidly in similar circumstances. Viral infections tend to give a lower CRP level than bacterial infection. CRP is used mainly as a marker of inflammation. Apart from liver failure, there are few known factors that interfere with CRP production (Pepys and Hirschfield 2003). Consumption of red meat is associated with increased colon cancer risk. This association might be due to the heme content of red meat. In rat, PCR confirmed the strong heme-induced down-regulation of mucosal pentraxin gene (*Mptx*) (Van Der Meer-Van Kraaij et al. 2003). The role of inflammation in cancer is now well known. Blood samples of persons with colon cancer have an average CRP concentration of 2.69 mg/l. Persons without colon cancer average 1.97 mg/l (Erlinger et al. 2004). These findings concur with previous studies that indicate that anti-

inflammatory drugs could lower colon cancer risk (Baron et al. 2003).

8.4.2 CRP: A Marker for Cardiovascular Risk

Patients with prolonged elevated levels of CRP are at an increased risk for heart disease, stroke, hypertension (high blood pressure), diabetes, and metabolic syndrome (insulin resistance, a precursor of type 2 diabetes). The amount of CRP produced by the body varies from person to person, and this difference is affected by lifestyle. Higher CRP levels tend to be found in individuals who smoke, have high blood pressure, are overweight and do not exercise, whereas lean, athletic individuals tend to have lower CRP levels. The research shows that too much inflammation can sometimes have adverse effects on the blood vessels which transport oxygen and nutrients throughout the body. Atherosclerosis, which involves the formation of fatty deposits or plaques in the inner walls of the arteries, is considered in many ways an inflammatory disorder of the blood vessels, similar to arthritis, which is considered an inflammatory disorder of the bones and joints. Inflammation affects the atherosclerotic phase of heart disease and can cause plaques to rupture, which produces a clot and interfere with blood flow, causing a heart attack or stroke. CRP has been localized to monocytes and tissue macrophages, which are present in the necrotic core of lesions prone to plaque rupture. Leukocyte-derived myeloperoxidase (MPO), primarily hosted in human polymorphonuclear cells (PMNs), has also been shown to be present in human atherosclerotic lesions. CRP stimulates MPO release both in vitro and in vivo, providing further cogent data for the proinflammatory effect of CRP. These results might further support the role of CRP in patients of acute coronary syndrome (Singh et al. 2009).

Inflammation is pivotal in all phases of atherosclerosis. Among the numerous inflammatory biomarkers, the largest amount of published data supports a role for CRP as a robust and independent risk marker in the prediction of primary and secondary adverse cardiovascular events. In addition to being a risk marker, there is much evidence indicating that CRP may indeed participate in atherogenesis. This correlation applies even to apparently healthy men and women who have normal cholesterol levels. Recent research suggests that patients with elevated basal levels of CRP are at an increased risk of diabetes, hypertension and cardiovascular disease (Dehghan et al. 2007; Pradhan et al. 2001). CRP level can be used by physicians as part of the assessment of a patient's risk for heart disease since it can be easily measured in blood. Studies provided evidence that the CRP level in human blood is an important and highly accurate predictor of future heart disease. CRP is an indicator of inflammation in the walls of arteries. Studies show that reducing the inflammation by lowering CRP levels with a class of drugs known as statins significantly lowers the rate of heart attacks and coronary-artery disease in people with acute heart disease. In fact, studies indicated that CRP levels may be as important—if not more important—in predicting and preventing heart disease as cholesterol levels are. Devaraj et al. (2009b) suggested that CRP is clearly a risk marker for cardiovascular disease and is recommended for use in primary prevention. In addition, CRP appears also to contribute to atherogenesis. A study of over 700 nurses showed that those in the highest quartile of trans fat consumption had blood levels of CRP that were 73% higher than those in the lowest quartile (Lopez-Garcia et al. 2005). Although CRP may only be a moderate risk factor for cardiovascular disease (Danesh et al. 2004), Reykjavik Study found to have some problems for this type of analysis related to the characteristics of the population, and an extremely long follow-up time, which may have attenuated the association between CRP and future outcomes (Verma et al. 2003). Others have shown that CRP can exacerbate ischemic necrosis in a complement-dependent fashion and that CRP inhibition can be a safe and effective therapy for myocardial and cerebral infarcts; so far, this has been demonstrated only in animal models (Pepys et al. 2006).

The JUPITER trial was conducted to determine if patients with elevated high-sensitivity CRP levels but without hyperlipidemia might benefit from statin therapy. Statins were selected because they have been proven to reduce levels of CRP. The trial found that patients taking rosuvastatin with elevated high-sensitivity CRP levels experienced a decrease in the incidence of major cardiovascular events (Armani and Becker 2005; Ridker et al. 2008). The trial specifically found, "the number of patients who would need to be treated with rosuvastatin for 2 years to prevent the occurrence of one primary end point is 95, and the number needed to treat for 4 years is 8." In other words, after 4 years of treatment, out of every 31 patients, one cardiovascular event would be prevented.

To clarify whether CRP is a bystander or active participant in atherogenesis, a study compared people with various genetic CRP variants. Those with a high CRP due to genetic variation had no increased risk of cardiovascular disease compared to those with a normal or low CRP (Zacho et al. 2008). However, the clinical utility of CRP measurement in cardiovascular risk prediction is still not well defined (Yeh 2005). Furthermore, there is an intense debate on whether CRP is merely a marker of inflammation or a direct participant. The finding by Dr Janos Filep provides additional insights into the current CRP debate (Khreiss et al. 2005).

Evidence in support of the possibility that CRP itself plays a role in the pathogenesis of atherosclerosis has been elucidated (Devaraj et al. 2009; Jialal et al. 2004). Examples

include the finding that CRP binds the phosphocholine of oxidized LDL (Chang et al. 2002), up-regulates the expression of adhesion molecules in endothelial cells, increases LDL uptake into macrophages (Zwaka et al. 2001), inhibits endothelial nitric-oxide synthase expression in aortic endothelial cells (Venugopal et al. 2002), and increases plasminogen activator inhibitor-1 expression and activity. A recent study utilizing a mouse strain expressing transgenic CRP and deficient in apolipoprotein E reported a modest acceleration in aortic atherosclerosis in male animals expressing high levels of CRP (Paul et al. 2004). Another report demonstrated increased arterial occlusion in transgenic mice expressing CRP in a model of vascular injury (Danenberg et al. 2003). With the recognition that inflammation plays a role in cardiovascular disease (CVD) and precedes myocardial infarction, numerous reports have emerged with plausible explanations for an association between and CVD (Pepys and Hirschfield 2003) and for the characterization of high sensitivity serum C-reactive protein (hs-CRP) as an independent predictor of future cardiovascular events (Verma 2004). Despite these suggestive findings, a role for CRP in the pathogenesis of atherosclerosis is far from established.

CRP is strongly associated with obesity, and weight loss has been shown to decrease CRP in nine of ten studies in which it has been evaluated (Dietrich and Jialal 2005). Race and gender also strongly influence serum hs-CRP concentration (Khera et al. 2005). In Dallas County, characterized as a typical multiethnic U.S. urban population, the median hs-CRP level is 30% higher in blacks than in whites, and almost twice as high in women as men (Khera et al. 2005). A new study demonstrated that cardiorespiratory fitness level, hormone replacement therapy use, and high-density lipoprotein cholesterol accounted for the gender difference in hs-CRP (Huffman et al. 2006). It is also increasingly evident that genetic factors, including apoE genotype (Marz et al. 2004) and polymorphisms in the hs-CRP gene (Russell et al. 2004; Szalai et al. 2002a; Suk et al. 2005), regulate basal hs-CRP concentrations. The substantial variability in hs-CRP concentrations in people of different ethnic origins, led Anand et al. to conclude that uniform hs-CRP cut-points were not appropriate for defining vascular risk across diverse populations (Anand et al. 2004).

8.4.2.1 Lowering of CRP by Drugs

Persons with moderate or high levels of CRP can often reduce the levels with lifestyle changes, including quitting smoking, engaging in regular exercise, taking in healthy nutrition, taking a multivitamin daily, replacing saturated fats such as butter with monounsaturated fats (particularly olive oil), increasing intake of Omega-3 fatty acids, losing weight if overweight, and increasing fiber intake. Statins, usually used to reduce high levels of low density lipoproteins, can also reduce CRP levels. These drugs include: *lovastatin* (Mevacor), simvastatin (Zocor), rosuvastatin (Crestor), pravastatin (Pravacol) and *atorvastatin* (Lipitor). Other drugs that lower CRP levels include the anti-cholesterol drug *ezetimibe* (Zetia) and the diabetes medication *rosiglitazone* (Avandia). Not all physicians are convinced the two studies published in 2005 are accurate, noting that both studies were funded by pharmaceutical companies (Pfizer and Bristol-Meyer Squibb) that make statin drugs used to reduce CRP levels. Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction (Stokes et al. 2009).

8.4.3 Role of Modified/Monomeric CRP

Denatured and aggregated forms of CRP (neo-CRP or modified CRP) are pro-inflammatory in a number of experimental systems, although the existence of this material in vivo has not been unequivocally established (Shields 1993). Khreiss et al. (2005) found that at local sites of deposition, small amounts of modified CRP may be generated with a set of properties distinct from those of the native protein. It has recently been reported that modified CRP increased the release of the inflammatory mediators monocyte chemoattractant protein-1 and IL-8 and up-regulated the expression of ICAM-1 in endothelial cells. In this model, modified CRP was shown to be a much more potent inducer than native CRP.

The capacity of human CRP to activate/regulate complement may be an important characteristic that links CRP and inflammation with atherosclerosis. Emerging advances suggest that in addition to classical pentameric CRP, a conformationally distinct isoform of CRP, termed modified or monomeric CRP (mCRP), may also play an active role in atherosclerosis. Monomeric CRP activates endothelial cells via interaction with lipid raft microdomains (Ji et al. 2009). The capacity of mCRP to interact with and activate the complement cascade was studied by Ji et al. (2006). mCRP binds avidly to C1q, and this binding occurred primarily through collagen-like region of C1q. Fluid phase mCRP inhibited the activation of complement cascade via engaging C1q from binding with other complement activators. In contrast, when immobilized or bound to oxidized or enzymatically modified LDL, mCRP could activate classical complement pathway. Low-level generation of sC5b-9 indicated that the activation largely bypassed the terminal sequence of complement, which appears to involve recruitment of Factor H. Therefore, mCRP can both inhibit and activate the classical complement pathway by binding C1q, depending on whether it is in fluid phase or surface-bound state (Ji et al. 2006, 2007, 2009). The CRP isoforms differ in their effects on thrombus growth (Molins et al. 2008).

Eisenhardt et al. (2009) suggested dissociation of pentameric to monomeric CRP on activated platelets. Since platlet CRP (pCRP) is found neither in healthy nor in diseased vessels, Eisenhardt et al. (2009) suggested that dissociation of pCRP to mCRP involves activated platelets, which play a central role in cardiovascular events. Activated platelets mediate the dissociation of pCRP to mCRP via lysophosphatidylcholine, which is present on activated but not resting platelets. The dissociation of pCRP to mCRP can also be mediated by apoptotic monocytic THP-1 and Jurkat T cells. This mechanism provides a potential link between circulating pCRP and localized platelet-mediated inflammatory and proatherogenic effects (Filep 2009).

Emerging evidence indicates that calcium-dependent binding of pCRP to membranes, including liposomes and cell membranes, results into a rapid but partial structural change, producing molecules that express CRP subunit antigenicity but with retained native pentameric conformation. This hybrid molecule is herein termed $mCRP_m$. The formation of $mCRP_m$ was associated with significantly enhanced complement fixation. The $mCRP_m$ can further detach from membrane to form the well-recognized mCRP isoform converted in solution ($mCRP_s$) and exert potent stimulatory effects on endothelial cells. The membrane-induced pCRP dissociation not only provides a physiologically relevant scenario for mCRP formation but may represent an important mechanism for regulating CRP function (Ji et al. 2009).

8.5 Extra-Hepatic Sources of CRP

The CRP is traditionally thought to be produced by the liver in response to inflammatory cytokines. However, extrahepatic synthesis of CRP has also been reported in neurons, atherosclerotic plaques, monocytes, and lymphocytes (Jialal et al. 2004). Extra-hepatic sources of CRP production point to a more systemic generation of CRP in our body (Yeh 2005). The mechanisms regulating synthesis at these sites are unknown, and it is unlikely that they substantially influence plasma levels of CRP. Studies have shown that both epithelial cells of the respiratory tract and renal epithelium can produce CRP under certain circumstances (Jabs et al. 2003). Moreover, neuronal cells also synthesise acute phase reactants involved in the pathogenesis of neurodegenerative diseases (Yasojima et al. 2000). These new sources provided only tenuous link to atherosclerosis. CRP has been shown to colocalize with the terminal complement complex in atherosclerotic plaques (Torzewski et al. 2000; Yasojima et al. 2001). Human coronary artery smooth muscle cells, but not human umbilical vein endothelial cells, can synthesize CRP after stimulation by inflammatory cytokines. This locally produced CRP may directly participate in the pathogenesis of atherosclerosis. Moreover, human adipocytes can produce CRP after stimulation by inflammatory cytokines and by a specific adipokine, resistin (Calabro et al. 2005).

8.6 Serum Amyloid P Component

In humans serum Amyloid P component (SAP) is a constitutive serum protein that is synthesized by hepatocytes. The SAP is the identical serum form of amyloid P component, a 25 kDa pentameric protein first identified as the pentagonal constituent of in vivo pathological deposits called amyloid (Cathcart et al. 1967). Amyloid P component makes up 14% of the dry mass of amyloid deposits and is thought to be an important contributor to the pathogenesis of a related group of diseases called the Amyloidoses (Botto et al. 1997). These conditions are characterised by the ordered aggregation of normal globular proteins and peptides into insoluble fibres which disrupt tissue architecture and are associated with cell death. Amyloid P component is thought to decorate and stabilise aggregates by preventing proteolytic cleavage and hence inhibiting fibril removal via the normal protein scavenging mechanisms (Tennent et al. 1995).

The SAP, is a component of all amyloid plaques, is also a normal component of a number of basement membranes including the glomerular basement membrane. The association and distribution of SAP within the glomerular basement membrane are altered or completely disrupted in a number of nephritides (e.g. Alport's Syndrome, type II membranoproliferative glomerulonephritis, and membranous glomerulo- nephritis). SAP binds to human laminin and merosin as well as mouse and rat laminins. The K_D of this interaction is 2.74×10^{-7} M at SAP/laminin molar ratio of 1:7.1. The binding of SAP to laminin is inhibited by both SAP and its analog, CRP, as well as phosphatidylethanolamine. Binding of SAP to extracellular matrix components such as type IV collagen, proteoglycans, and fibronectin in concert with above observation suggests that SAP determines the properties of those basement membranes with which it is associated (Zahedi 1997).

8.6.1 Genes Encoding SAP

8.6.1.1 The Human Gene

Both SAP and CRP are evolutionary conserved in all vertebrates and also found in distant invertebrates such as the horseshoe crab (Limulus polyphemus) (Pepys et al. 1997). The serum amyloid P component gene (*APCS*) is human gene for SAP (Floyd-Smith et al. 1986; Ohnishi et al. 1986). It is encoded by a single copy gene on chromosome 1. C-DNA clones corresponding to the human SAP mRNA have been analyzed. The nt sequences of the cDNA and the corresponding regions of the genomic SAP DNA

which were identical, revealed that after coding for a signal peptide of 19 amino acids and the first two amino acids of the mature SAP protein, there is one small intron of 115-bp, followed by a nt sequence coding for the remaining 202 amino acid residues. The SAP gene has an ATATAAA sequence 29-bp upstream from the cap site, but there is no CAAT box-like sequence. A possible polyadenylation signal sequence, ATTAAA, was found to be located 28-bp upstream from the polyadenylation site. A comparison of the genomic SAP DNA sequence with that of human CRP revealed a striking overall homology which was not uniform: several highly conserved regions were bounded by non-homologous regions. This comparison supports for the hypothesis that SAP and CRP are products of a gene duplication event. The genes encoding CRP and SAP are located on the proximal long arm of human chromosome 1, more precisely between bands q12 and q23 by to human metaphase chromosomes (Floyd-Smith et al. 1986; Ohnishi et al. 1986).

8.6.1.2 Mouse SAP Gene

The mouse SAP genomic clone contains the entire SAP gene and specifies a primary transcript of 1,065 nt residues. This comprises a first exon of 206 nt residues containing the mRNA 5′-untranslated region and sequence encoding the pre-SAP leader peptide and the first two amino acid residues of mature SAP separated by a single 110-base intron from a 749-nt-residue second exon containing sequence encoding the bulk of the mature SAP and specifying the mRNA 3′-untranslated region. The overall organization is similar to that of the human SAP gene, and the coding region and intron sequences are highly conserved. The 5′-region of the mouse SAP gene contains modified CAAT and TATA promoter elements preceded by a putative hepatocyte-nuclear-factor-1-recognition site; these structures are in a region that is highly homologous to the corresponding region of the human SAP gene (Whitehead and Rits 1989).

8.6.1.3 Guinea Pig SAP and CRP

CRP and SAP have been isolated from bovine and guinea pig sera (Rubio et al. 1993). In guinea pig, neither SAP nor the CRP is the major acute phase reactant. Both genes have organizations typical of the pentraxin genes in other species. However, some differences were observed in the regions that potentially determine the capacity of the pentraxin gene to be induced during acute inflammation. Nucleotide substitutions in coding regions have occurred at similar rates in the two pentraxin genes. Nonsynonymous substitution rates indicated that SAP and CRP are subject to similar, relatively low levels of constraint. An estimate of the phylogenetic relationship among the pentraxin genes suggested that SAP and CRP arose as the result of a gene duplication event that occurred very early in mammalian evolution, but subsequent to the divergence of the reptilian ancestors of the mammalian and avian lineages. This raised doubts about the identity of proteins from fish, which have been characterized as CRP and SAP (Rubio et al. 1993).

8.6.2 Characterization of SAP

Fourier transform infrared spectroscopy provided estimations of about 50% β-sheet, 12% α-helix, 24% β-turn, and 14% unordered structure for CRP and about 54% β-sheet, 12% α-helix, 25% β-turn, and 9% unordered structure for SAP. With both proteins significant calcium-dependent changes were observed in conformation-sensitive amide I regions assigned to each type of structure. The CRP spectrum was also affected by Mg^{2+}, but the changes differed from those induced by Ca^{2+}. The SAP spectrum was not affected by Mg. Phosphorylcholine (PC) in the presence of Ca^{2+} also affected the spectrum of CRP but not the spectrum of SAP. This study provided a comparison of the secondary structures of human CRP and SAP and hamster female protein (Dong et al. 1992). It indicated that three pentraxins have similar secondary structure compositions and calcium-dependent conformational changes, but differ significantly in their responses to phosphorylcholine and Mg^{2+} (Dong et al. 1994).

8.6.2.1 Structure

SAP is characterised by calcium dependent ligand binding and distinctive flattened β-jellyroll structure similar to that of the legume lectins (Emsley et al. 1994). Human SAP has 51% sequence homology with CRP and is a more distant relative to the "long" pentraxins such as PTX3 (a cytokine modulated molecule) and several neuronal pentraxins. The oligomeric state of human SAP in the absence and presence of known ligands has been investigated using ionization MS. At pH 8.0, in the absence of Ca^{2+}, SAP was shown to consist of pentameric and decameric forms. In the presence of physiological levels of Ca^{2+}, SAP exists primarily as a pentamer, reflecting its in vivo state. dAMP was shown not only to promote decamerization, but also to lead to decamer stacking involving up to 30 monomers. A mechanism for this finding was proposed by Aquilina and Robinson (2003). Furthermore, no exchange of monomeric subunits was observed between the SAP and CRP oligomers, suggesting a remarkable stability of the individual pentameric complexes (Aquilina and Robinson 2003). The three-dimensional structure of pentameric human SAP component at high resolution reveals that the tertiary fold is remarkably similar to that of the legume lectins (Fig. 8.3). Carboxylate and phosphate compounds bind directly to two Ca^{2+}; interactions with a carboxyethylidene ring are mediated by Asn^{59} and Gin^{148} ligands of the Ca^{2+}. X-ray results indicate the probable modes of binding of the biologically important ligands,

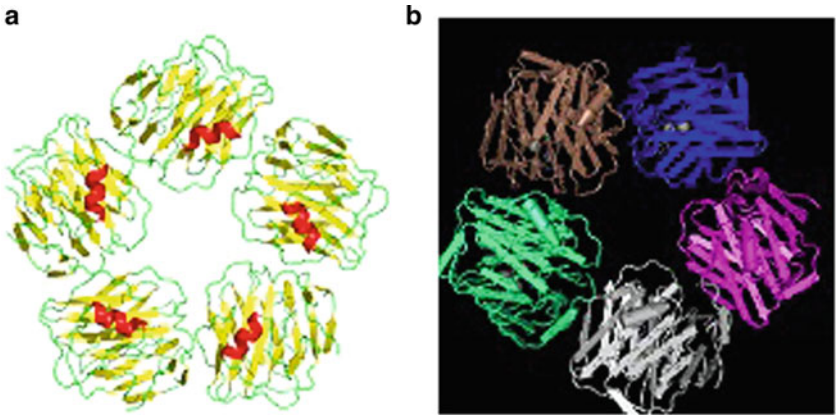

Fig. 8.3 (**a**) Cartoon model of SAP showing helices in *red*, sheets in *yellow* and coils in *green* (**b**) Crystal structure of a decameric complex of human serum Amyloid P component with bound dAMP (PDB ID: 1LGN; Hohenester et al. 1997)

DNA and amyloid fibrils (Emsley et al. 1994). Hohenester et al. (1997) revealed the crystal structure of the SAP-dAMP complex as a decamer in which all interactions between SAP pentamers were mediated by the ligand. The two calcium ions of SAP were bridged by the dAMP phosphate group and five hydrogen bonds are formed between the protein and the ligand, including specific interactions made by the adenine base. The SAP-dAMP decamer is stabilized mainly by base-stacking of adjacent ligand molecules and possibly by electrostatic interactions involving the dAMP phosphate groups; decamerization buries 1,000 A2 (2.6%) of the pentamer solvent-accessible surface.

8.6.3 Interactions of SAP and CRP

8.6.3.1 CRP and SAP Interact with Nuclear Antigens

In the past several years it has been demonstrated that both of these pentraxins interact with nuclear antigens including chromatin and small nuclear ribonucleoproteins (snRNPs). Both CRP and SAP have nuclear transport signals which facilitate their entry into the nuclei of intact cells. Furthermore, these pentraxins have been shown to affect the clearance of nuclear antigens in vivo. It is now believed that one of the major functions of the pentraxins could be to interact with the nuclear antigens released from apoptotic or necrotic cells. This interaction could mitigate against deposition of these antigens in tissue and autoimmune reactivity (Du Clos 1996).

C-reactive protein (CRP) binds to several nuclear Ag, including chromatin, histones, and small nuclear ribonucleoproteins. Binding to sites of tissue inflammation and the nuclei of inflammatory cells has been demonstrated in vivo. A significant similarity has been noticed between CRP and nucleoplasmin, a molecule with nuclear localization activity. Binding of CRP and SAP to chromatin may be involved in the solubilization and clearance of nuclear material. CRP binding to chromatin is mediated by histones. These interactions demonstrated under relatively physiological conditions, with native pentraxins unseparated from serum and with nuclear constituents in situ, are likely to be of functional importance in vivo (Pepys et al. 1994; Shephard et al. 1991).

The CRP and SAP specifically bind to each other only when the CRP is in an immobilized form bound to one of its ligands or to an antibody. CRP did not bind to immobilized SAP. The binding of SAP to immobilized CRP was Ca^{2+}-dependent with sufficient affinity in presence of serum or purified serum proteins. SAP bound preferentially to a synthetic peptide corresponding to the Ca^{2+}-binding region of CRP (Swanson et al. 1992; Christner and Mortensen 1994b).

In man CRP and SAP activate complement through the classical pathway and participate in opsonization of particulate antigens and bacteria. The CRP is an activator of the classical complement pathway. Cleavage of chromatin is C-dependent in the presence of CRP and serum. Oligomers of SAP have been found to bind C1q and consume total C and C4, indicating that SAP can activate complement as well. SAP differs from CRP in being able to bind to DNA. SAP binds to histones H1 and H2A as well as to chromatin. In contrast to CRP, SAP binding to chromatin did not require H1. SAP partially inhibited CRP binding to chromatin and to H1. However, neither pentraxin inhibited binding of the other to H2A. Binding of either CRP or SAP to H2A activated complement in SAP-depleted serum leading to the deposition of C4 and C3. C activation required C1q and produced C4d indicating that it occurred through the classical pathway. These findings demonstrated that both pentraxins can activate the classical C pathway after ligand binding (Hicks et al. 1992).

8.6.3.2 Regulation of Pentraxin Function by Lactic Acid

Carboxylated compounds, especially lactic acid, were capable of dissociating pentraxins from several macromolecular binding sites. Lactate dissociated the hSAP-membrane complex and prevented hSAP self-association. The only interaction that was not dissociated by 10 mM lactate was the hSAP-heparin complex. This suggested that the carboxyl group plus a hydrogen-bonding site on the hydrocarbon chain was important, but a charged amino group was not a contributor to function when the anion was provided by a carboxyl group. Other pentraxins also interacted with lactic acid, but with lower affinities. Importantly, lactic acid was capable of dissociating rat CRP from lipoproteins in rat serum. Human CRP bound very weakly to lactate, so that lactate probably is not a significant regulator of this pentraxin (Evans and Nelsestuen 1995).

8.6.4 Functions of SAP

8.6.4.1 Control of Chromatin Degradation

SAP shows specific calcium-dependent binding to DNA and chromatin in physiological conditions. The avid binding of SAP displaces H1-type histones and thereby solubilizes native long chromatin, which is otherwise profoundly insoluble at the physiological ionic strength of extracellular fluids. Furthermore, SAP binds in vivo both to apoptotic cells, the surface blebs of which bear chromatin fragments, and to nuclear debris released by necrosis. SAP may therefore participate in handling of chromatin exposed by cell death. Mice with targeted deletion of the SAP gene spontaneously develop antinuclear autoimmunity and severe glomerulonephritis, a phenotype resembling human SLE, a serious autoimmune disease. The $SAP^{-/-}$ mice also have enhanced anti-DNA responses to immunization with extrinsic chromatin; and the degradation of long chromatin is retarded in the presence of SAP both in vitro and in vivo. Therefore, SAP has an important physiological role, inhibiting the formation of pathogenic autoantibodies against chromatin and DNA, probably by binding to chromatin and regulating its degradation (Bickerstaff et al. 1997). However, the SAP-deficient mice displayed no obvious phenotypic abnormalities. Though, Soma et al. (2001) reaffirmed that the SAP-deficient mice had high titers of anti-nuclear antibody but did not develop severe glomerulonephritis. On the other hand, as reported that SAP bound to gram-negative bacteria via LPS prevented LPS-mediated activation of a classical complement pathway. Thus, contrary to documented data, SAP-deficient mice do not develop serious autoimmune disease. It was suggested that SAP has a critical role in LPS toxicity (Soma et al. 2001).

8.6.4.2 Amyloid Deposition is Delayed in Mice with Targeted Deletion of SAP Gene

The tissue amyloid deposits that characterize systemic amyloidosis, Alzheimer's disease and the transmissible spongiform encephalopathies always contain SAP bound to the amyloid fibrils. It was proposed that this normal plasma protein may contribute to amyloidogenesis by stabilizing the deposits. Systemic amyloidosis in a transgenic mouse model for an autosomal dominant disease, familial amyloidotic polyneuropathy (FAP) suggested that SAP is not important for the initiation and progression of amyloid deposition (Tashiro et al. 1991). On the contrary, the induction of reactive amyloidosis is retarded in mice with targeted deletion of the SAP gene (Botto et al. 1997). A precise role for SAP in amyloid deposition in vivo is not known. Maeda (2003) indicated that lack of SAP in AA amyloid deposits does not enhance regression of the deposits in vivo and that the dissociation of bound SAP from AA amyloid deposits would not accelerate regression of the deposits in vivo.

8.6.4.3 SAP Forms a Stable Complex with Human C5b6

In serum, SAP binds tightly to C5b6, which is formed by activating C7-depleted human serum with zymosan. The C5b6-SAP complex did not dissociate in the presence of EDTA, which distinguishes SAP-C5b6 binding from SAP's usual Ca^{2+}-dependent binding to other molecules. Purified SAP was able to bind to preformed C5b6, which was isolated from purified components. Functionally, the C5b6-SAP could bind C7, and the resulting C5b67-SAP complex had only moderately lower specific hemolytic activity than that of C5b67. In addition, hemolytically inactive C5b67-SAP, like hemolytically inactive C5b67, was chemotactically active for neutrophils, while isolated SAP had no effect on cell mobility. It is likely that the addition of SAP to terminal complement complexes may affect the fate of these complexes (Barbashov et al. 1997). C4b-binding protein (C4bp) is the main regulatory protein of complement system and regulates C3 convertase activity in classical way of complement activation. The major regulatory function of C4bp is related to its interaction with activated form of C4b. C4bp may also interact with SAP that inhibits complement-regulatory functions of C4bp.

8.6.4.4 Uptake of Apoptotic Cells Through FcγRI and/or FcγRIII

SAP and CRP are opsonins that react with nuclear autoantigens targeted in systemic autoimmunity. CRP and SAP bind to apoptotic and necrotic cells, which are potential sources of these autoantigens. Although SAP binds to DNA and chromatin and affects clearance of these autoantigens, FcγRI and FcγRIII are receptors for SAP in the mouse.

CRP as an opsonin binds to FcγR. The use of FcγR by the pentraxins links innate and adaptive immunity and may have important consequences for processing, presentation, and clearance of the self-Ags to which these proteins bind (Mold et al. 2001). In human neutrophils (PMN) and the Jurkat T-cell line SAP treatment of apoptotic human PMN increased ingestion by autologous macrophages. Mold et al. (2002) suggested that pentraxins promote uptake of apoptotic cells through FcγRI and/or FcγRIII. Ingestion through these receptors is expected to alter the pattern of cytokine production and antigen presentation in response to apoptotic cells.

8.6.4.5 Binding of Pentraxins and IgM to Newly Exposed Epitopes on Late Apoptotic Cells

A random distribution of phospholipids among the inner and outer leaflet of the cell membrane occurs during apoptosis and is known as membrane flip-flop. Flip-flopped cells have binding sites for various plasma proteins, such as IgM and the pentraxins CRP and SAP. Except for SAP which also bound to early apoptotic cells, pentraxins and IgM preferentially bound to late apoptotic cells. It revealed that CRP, SAP, and part of the IgM molecule bind to phospholipid head groups exposed on apoptotic cells. This shared specificity as well as their shared capability to activate complement suggest that IgM and the pentraxins CRP and SAP exert similar functions in the removal of apoptotic cells (Ciurana and Hack 2006).

8.6.4.6 Pentraxins as Receptors for Microbial Pathogens

Attempt to identify LPS-binding proteins from the hemolymph of the horseshoe crab led to the isolation and identification of CRP as the predominant LPS-recognition protein during *Pseudomonas* infection. Investigation of CRP response to *Pseudomonas aeruginosa* unveiled a robust innate immune system in the horseshoe crab, which displays rapid suppression at a dosage of 10^6 CFU of bacteria in the first hour of infection and effected complete clearance of the pathogen by 3 days. Results provide the importance of CRP as a conserved molecule for pathogen recognition (Ng et al. 2004). However, pentraxins do not participate significantly in normal human serum promastigote C3 opsonization. It was indicated that successful *Leishmania* infection in man must immediately follow promastigote transmission (Dominguez et al. 2002).

SAP can bind to influenza A virus and inhibit agglutination of erythrocytes mediated by the virus subtypes H1N1, H2N2 and H3N2. SAP also inhibits the production of haemagglutinin (HA) and the cytopathogenic effect of influenza A virus in MDCK cells. Of several monosaccharides tested only D-mannose interfered with SAP's inhibition of both HA and infectivity. The glycosaminoglycans heparan sulfate and heparin, which bind SAP, reduced SAPs binding to the virus. The results indicate that the inhibition by SAP is due to steric effects when SAP binds to terminal mannose on oligosaccharides of the HA trimer (Andersen et al. 1997).

8.6.5 SAP in Human Diseases

8.6.5.1 Amyloid P Component from Patient of Amyloidosis

Amyloid P component (AP) makes up 14% of the dry mass of amyloid deposits and is thought to be an important contributor to the pathogenesis of a related group of diseases called the Amyloidoses (Botto et al. 1997). Amyloid P component extracted from spleen of a patient with primary idiopathic amyloidosis is a glycoprotein composed of a pair of noncovalently bound pentameric discs with a subunit size of 23–25 kDa. The precursor of AP is the SAP, which has an identical amino acid sequence to that of amyloidosis patient. It shared 52% homology with the amended sequence of human CAP, and 68% homology with the Syrian hamster female protein, whose response is modulated by sex steroids (Prelli et al. 1985).

The mRNAs and proteins of both CRP and AP are concentrated in pyramidal neurons and are upregulated in affected areas of AD brain. Results suggested a more direct role for serum AP and CRP in the pathogenesis of AD. Controlling pentraxin production at the tissue level may be important in reducing inflammatory damage in AD (Duong et al. 1998; Yasojima et al. 2000). Formerly thought to be made primarily if not solely in liver, recent work has shown that AP and CRP are made not only in the brain but in other tissues such as heart and arteries. Their synthesis is markedly upregulated in affected brain regions in AD. Since they are known to activate the complement cascade in an antibody-independent fashion and chronic activation can cause destruction of host tissue, these pentraxins may be important initiators of an autodestructive process. As such, they may be prime targets for therapeutic intervention (McGeer et al. 2001; McGeer and McGeer 2004).

AP is thought to decorate and stabilise aggregates by preventing proteolytic cleavage and hence inhibiting fibril removal via the normal protein scavenging mechanisms (Tennent et al. 1995). This association is utilised in the routine clinical diagnostic technique of SAP scintigraphy whereby radio-labelled protein is injected into patients to locate areas of amyloid deposition (Hawkins and Pepys 1995). The SAP-amyloid association has also been identified as a possible drug target for anti-amyloid therapy, with the development first stage clinical trials of a compound called CPHPC (R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxohexanoyl] pyrrolidine-2-carboxylic acid), a small

molecule able to strip AP from deposits by reducing levels of circulating SAP (Pepys et al. 2002).

8.6.5.2 SAP in Development of Atherosclerosis

SAP is present in human atherosclerotic lesions. SAP from the intima was indistinguishable from plasma or purified SAP with respect to immunological character and molecular weight. Studies suggest a role for SAP in atherogenesis and encourage efforts to determine more precisely the physiological contributions of the pentraxin family to the development of atherosclerosis (Li et al. 1995). It seems that SAP and CRP may represent different facets of inflammation. The association of SAP with CVD in older adults further supports the role of innate immunity in atherosclerosis (Jenny et al. 2007).

8.6.6 SAP from *Limulus Polyphemus*

The structure of SAP from horseshoe crab *Limulus polyphemus* was determined by molecular replacement. Contrary to popular opinion, both CRP and SAP are present in *Limulus* haemolymph. The two independent protomers in the asymmetric unit are related by a ncs two fold bisecting the tetragonal two fold axes, producing a physiological molecule of 16 protomers. In contrast to the known mammalian pentraxin structures, all of which are pentameric, *Limulus* SAP consists of 16 protomers, each with the pentraxin fold, arranged in a novel doubly stacked cyclic octameric structure. The calcium site and the pentraxin helix, which are situated on opposite faces of the homo-oligomeric pentamer in the mammalian pentraxins, are situated on the external and internal edges of the octameric ring in *Limulus* SAP. Interprotomer interactions throughout the molecular assembly are non-covalent and pairs of protomers, one from each octameric ring, form continuous, twisted beta-sheet structures across the hexadecamer. The protomers of the doubly-stacked octameric *Limulus* SAP retain the cyclic oligomeric nature of the pentraxins but aggregate in a distinctly different manner when compared to the known mammalian pentraxins, displaying a 75° rotational shift with respect to the cyclic axis. Alignment of the *Limulus* and human SAP structures with that of the three known polymorphic *Limulus* CRP sequences suggests that the majority of the 10% sequence difference between them is localised in the vicinity of the pentraxin ligand-binding site, giving rise to differing, but overlapping ligand binding specificities (Shrive et al. 1999).

8.7 Female Protein (FP) in Syrian Hamster

8.7.1 Similarity of Female Protein to CRP and APC

Female protein (FP) is a pentraxin of Syrian hamster which is a homologue of CRP and amyloid P component (AP). The pentraxin in the Syrian hamster is unique because it is preferentially expressed in the female at high constitutive levels and accordingly called female protein (FP) or FP (SAP) due to its close homology with human SAP. The high levels of FP in female serum (100-fold greater than male serum) suggested its role in hamster pregnancy, one of the shortest of any eutherian mammal. Serum FP concentration in pregnant Syrian hamsters shows marked decrease (>80%) at term with the nadir at parturition with subsequent increase. A similar down-regulation of FP was found in the normal female Syrian hamster after injury (acute phase response), so in both cases the assumed beneficial effects were achieved with less, rather than more pentraxin, a paradoxical pentraxin response. The fall in serum FP concentration could represent a response to protect the fetus from the high and potentially toxic level of FP normally found in the female that is harmful because of its association with amyloidosis. An FP that is 97.5% identical to Syrian hamster FP is found in the Turkish hamster (Mesocricetus brandti), although serum levels in females are much lower, and amyloid is very rare (Coe et al. 1999).

The serum of Armenian hamster (*Cricetulus migratorius*) contains a protein homologous to Syrian (golden) hamster FP. Whereas serum concentration of FP in Syrian hamsters (SFP) is many fold greater (200–300-fold) in females vs. males, Armenian hamster FP (AFP) is only moderately elevated (approximately three fold) in female vs. males and only for the fall-winter months of the year. In the Armenian hamster testosterone administration to females or castration of males has no effect on AFP serum levels, whereas in Syrian hamster these treatments change SFP serum concentration to that characteristic of the opposite sex. Some sex steroid control of hepatic AFP synthesis is evident, however, as serum levels decrease after exogenous estrogen treatment. In contrast to Syrian hamster FP, normal levels of AFP are dependent on an intact hypophysial-pituitary axis and also are influenced by the season of the year. As an acute-phase protein, AFP responds in a typical fashion, with increasing serum levels detected in both sexes in contrast to the divergent sex-limited response in Syrian hamsters. Although AFP and SFP are similar structurally, morphologically, and antigenetically and share common binding specificities, the regulation of FP synthesis in Armenian hamster is very different from that found in Syrian hamster (Coe and Ross 1990).

Functionally, FP has been shown to be similar to CRP, although FP has more homology at the amino terminus with AP. As an amyloid component, FP appears to be functionally similar to human AP. However, FP synthesis is under sex steroid control and the unique sex-limited expression of this pentraxin was associated with an equally novel propensity for deposition of amyloid in female hamsters under normal or experimental conditions. Thus, a high serum level of FP, as found in normal females or diethylstilbestrol-treated males, was associated with enhanced amyloidosis (Coe and Ross 1985). Female protein (FP) was found to be similar to CRP and SAP in the following ways: (a) hamster FP complexes with phosphorylcholine (PC) in a Ca^{2+} -dependent; (b) electron microscopy of FP indicated a pentameric structure similar in size and appearance to other pentraxins; (c) the parent molecule of FP (150 kDa mol wt) was composed of five noncovalantly assembled subunits of 30 kDa mol wt; and (d) the amino acid analysis and terminal NH2 sequence of FP clearly showed homology with SAP-CRP. Although FP evolved from an ancestral gene common to SAP and CRP, and shares functional, morphological and structural properties with these acute-phase proteins, the biological homology of FP appears quite diverse as this protein is a prominent serum constituent (1–2 mg/ml) of normal female hamsters and under hormonal control (testosterone suppression) in males (Coe et al. 1981). FP is unusual in that it is apparently the only pentraxin produced in hamsters, it is under hormonal control and it shares binding characteristics with both CRP and SAP. The response to inflammation is divergent; FP levels decrease in females and increase in males during an acute phase response (Dong et al. 1992).

Like CRP and SAP, FP shares the ability to bind to chromatin and histones. Similar to CRP, FP bound to histones H1 and H2A, and chromatin. FP shared with SAP the ability to bind to DNA. However, FP binding was inhibited by PC for all ligands, whereas SAP binding was not. FP and SAP also failed to compete with each other for binding to DNA. By cross-inhibition FP bound much less well to PC than CRP, but was a very effective inhibitor of CRP binding to H2A. These studies demonstrate that chromatin and histone binding are conserved among pentraxins (Saunero-Nava et al. 1992). In rats FP was closely related to hamster FP with respect to hormonal regulation and APP nature as well.

8.7.2 Structure of Female Protein

Syrian hamster female protein (SFP), a serum oligomer composed of five identical subunits, was reassociated in vitro from monomer subunits. The reconstituted pentamer was genuine by morphologic, antigenic, and structural criteria. Another female protein (FP), a homologue from Armenian hamsters (AFP), also reassociated into a pentamer after dissociation with 5 M guanidine hydrochloride. These two FP's hybridized when a mixture of them was dissociated and then reassociated. The in vitro dissociation-reassociation of female protein described herein may reflect an in vivo dissociation-reassociation which is functionally important and a common metabolic feature within this family of proteins (Coe and Ross 1987).

Hamster FP is composed of glycosylated subunits of 25,655 MW containing a single intrachain disulphide bridge. In the presence of EDTA the subunits are non-covalently associated as pentamers of mass approximately 128,000 MW, and in the presence of calcium they aggregate further, probably to form decamers. This pentamer-decamer transition at physiological ionic strength has not been described in other pentraxins. It also resembles human CRP in binding only weakly to agarose, to human AA amyloid fibrils in vitro, and to mouse AA amyloid deposits in vivo. It thus differs markedly from human and mouse SAP but it is nevertheless deposited in hamster AA amyloid in vivo and clearly is the hamster counterpart of SAP in other species. These results illustrate the subtle diversity among members of the otherwise conserved pentraxin family of vertebrate plasma proteins (Tennent et al. 1993).

FT infrared spectroscopy indicated that FP is composed of 50% β-sheet, 11% α-helix, 29% β-turn, and 10% random structures. Two putative calcium-binding sites were proposed for FP (residues 93–109 and 150–168) as well as other members of the pentraxin family on the basis of the theoretical secondary structure predictions and the similarity in sequence between the pentraxins and EF-hand calcium-binding proteins. The changes in protein conformation detected upon binding of calcium and PC provide a mechanism for the effects of these ligands on physiologically important properties of the protein, e.g., activation of complement and association with amyloids (Dong et al. 1992).

8.7.3 Gene Structure and Expression of FP

Hamster FP is a unique pentraxin because pretranslational expression of this gene is modulated by mediators of inflammation and sex steroids. The FP gene encodes a 211 amino acid residue mature polypeptide as well as a 22-residue signal peptide. The intron/exon organization is similar to that of other pentraxins, but additional transcripts are generated from alternate polyadenylation sites in the 3′ region. Circulating levels of FP and the corresponding hepatic transcript levels are augmented by estrogen, while testosterone, dexamethasone, and progesterone cause a decrease in these levels. In addition the cytokines interleukin-1, -6, and tumor necrosis factor mediate a decrease in hepatic FP transcript levels in female hamsters

but did not cause a significant elevation of FP mRNA in livers of male hamsters. The differences in expression of the FP gene between male and female hamsters and between unstimulated male hamsters and male hamsters stimulated with an injection of lipopolysaccharide are due, at least in part, to alterations in transcription (Rudnick and Dowton 1993a, b).

8.8 Long Pentraxins

8.8.1 Long Pentraxins 1, -2, -3

The earliest described pentraxins, CRP and SAP, are cytokine-inducible acute phase proteins implicated in innate immunity whose concentrations in the blood increase dramatically upon infection or trauma. The highly conserved family of pentraxins was thought to consist solely of approximately 25 kDa proteins. Later, several distinct larger proteins were identified in which only the C-terminal halves showed characteristic features of the pentraxin family. One of these described "long" pentraxins (TSG-14/PTX3) is inducible by TNF-α or IL-1 and is produced during the acute phase response. Other newly identified long pentraxins are constitutively expressed proteins associated with sperm-egg fusion (apexin/p50), may function at the neuronal synapse (neuronal pentraxin I, NPI), or may serve yet other, unknown functions (NPII and XL-PXN1). Evidence obtained by molecular modeling and by direct physicochemical analysis suggests that TSG-14 protein retains some characteristic structural features of the pentraxins, including the formation of pentameric complexes (Goodman et al. 1996).

8.9 Neuronal Pentraxins (Pentraxin-1 and -2)

Murine or rat neuronal pentraxin 1 (NP1) and human neuronal pentraxin 2 (NPTX2) are expressed in CNS. NP1, NP2, and neuronal pentraxin receptor (NPR) are members of a new family of proteins identified through interaction with a presynaptic snake venom toxin taipoxin. Neuronal pentraxins represent a novel neuronal uptake pathway that may function during synapse formation and remodeling. NP1 and NP2 are secreted, exist as higher order multimers (probably pentamers), and interact with taipoxin and taipoxin-associated calcium-binding protein 49 (TCBP49). NPR is expressed on the cell membrane and does not bind taipoxin or TCBP49 by itself, but it can form heteropentamers with NP1 and NP2 that can be released from cell membranes. Heteromultimerization of pentraxins and release of a pentraxin complex by proteolysis directly effect the localization and function of neuronal pentraxins in neuronal uptake or synapse formation and remodeling (Kirkpatrick et al. 2000).

8.9.1 Functions of Pentraxin 1 and -2

8.9.1.1 Expression of Narp in Orexin Neurons

Evidences suggest that orexin (also known as hypocretin) neurons play a central role in the pathophysiology of narcolepsy, though targeted deletion of orexin does not fully mimic the functional deficits induced by selective ablation of these neurons. Reti et al. (2002) demonstrated that orexin neurons displayed robust expression of neuronal activity-regulated pentraxin (Narp), a secreted neuronal pentraxin, implicated in regulating clustering of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors. Furthermore, it was found that hypothalamic melanin-concentrating hormone (MCH) neurons, which form a peptidergic pathway thought to oppose the effects of the orexin system, express another neuronal pentraxin, NP1. Thus, these pathways utilize neuronal pentraxins, in addition to neuropeptides, as synaptic signaling molecules (Reti et al. 2002).

Glycogen Synthase Kinase 3 Mediates NP 1 Expression and Cell Death: Expression of NP1 is part of the apoptotic cell death program activated in mature cerebellar granule neurons when potassium concentrations drop below depolarizing levels. NP1 is involved in both synaptogenesis and synaptic remodeling. Both activation of the phosphatidylinositol 3-kinase/Akt (PI-3-K/AKT) pathway by insulin-like growth factor I and blockage of the stress activated JNK offer transitory neuroprotection from the cell death evoked by nondepolarizing concentrations of potassium. Impairing the activity of glycogen synthase kinase 3 (GSK3) completely blocks NP1 over-expression induced by potassium depletion and provides transient protection against cell death. Results showed that both the JNK and GSK3 signaling pathways are the main routes by which potassium deprivation activates apoptotic cell death, and that NP1 overexpression is regulated by GSK3 activity independently of the PI-3-K/AKT or JNK pathway (Enguita et al. 2005).

Requirement of NP1 in Hypoxic Neuronal Injury: Neonatal hypoxic-ischemic brain injury is a major cause of neurological disability and mortality. Neuronal pentraxin 1 (NP1), a member of subfamily of "long pentraxins," plays a role in the HI injury cascade. After hypoxia-ischemia (HI) there was an elevated neuronal expression of NP1 in the ipsilateral cerebral cortex and in the hippocampal CA1 and CA3 regions. Results suggest that NP1 induction mediates hypoxic-ischemic injury probably by interacting with and modulating AMPA glutamate receptor subunit (GluR1) and

potentially other excitatory glutamate receptors (Hossain et al. 2004).

Neuronal Pentraxins Mediate Synaptic Refinement in Developing Visual System

Neuronal pentraxins (NPs) have been hypothesized to be involved in activity-dependent synaptic plasticity. NP1/2 knock-out mice exhibited defects in the segregation of eye-specific retinal ganglion cell (RGC) projections to the dorsal lateral geniculate nucleus, a process that involves activity-dependent synapse formation and elimination. Retinas from mice lacking NP1 and NP2 had cholinergically driven waves of activity that occurred at a frequency similar to that of wild-type mice, but several other parameters of retinal activity were altered. Studies indicate that NPs are necessary for early synaptic refinements in the mammalian retina and dorsal lateral geniculate nucleus (Bjartmar et al. 2006).

8.9.1.2 Neuronal Pentraxin Receptor (NPR) in Small Cell Lung Cancer

A global gene analysis showed that the neuronal pentraxin receptor (NPR) is highly and relatively specifically expressed in Small cell lung cancer (SCLC), consistent with the neuroendocrine features of this cancer. Normally, NPR is exclusively expressed in neurons, where it associates with the homologous proteins NP1 and NP2 in complexes capable of binding the snake venom neurotoxin taipoxin. The receptor in SCLC is surface associated. Microarray signals for NP1 and NP2mRNA was detected in a subset of SCLC-cell lines Furthermore, NP1 protein was detected in a few SCLC-cell lines. A number of SCLC-cell lines showed marked sensitivity to taipoxin (IC50: 3–130 nM) (Poulsen et al. 2005).

8.10 Pentraxin 3 (PTX3)

8.10.1 Characterization of PTX3

The long pentraxin 3 (PTX3) is a member of the pentraxin superfamily, a family of proteins highly conserved during evolution and characterized by a multimeric, usually pentameric structure (Bottazzi et al. 2006; Garlanda et al. 2005). PTX3 shares similarities with the classical, short pentraxins but differs for the presence of an unrelated long N-terminal domain coupled to the carboxy-terminal pentraxin domain, and differ, with respect to short pentraxins, in their gene organization, chromosomal localization, cellular source, and in their stimuli-inducing and ligand-recognition ability. PTX3 consists of a C terminal

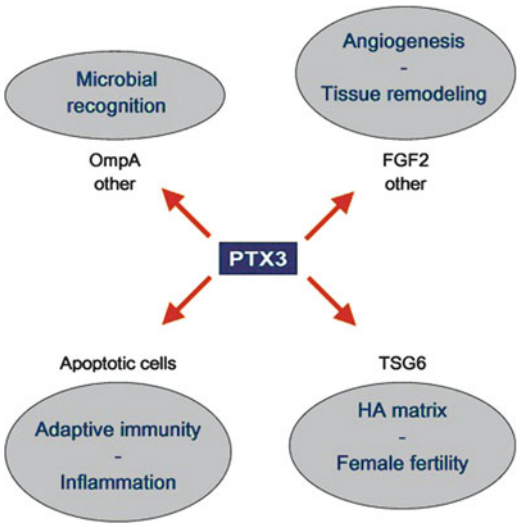

Fig. 8.4 PTX3: a soluble, multifunctional protein. Through interaction with its numerous ligands (fibroblast growth factor 2 (FGF2)) (Bottazzi et al. 2006), the ECM protein TNF-α-induced protein 6 (TSG-6) (Salustri et al. 2004), and the outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA), PTX3 plays complex functions in vivo, recognizing microbes, tuning inflammation, and editing self-nonself discrimination. In addition, PTX3 participates in tissue remodeling and plays an essential role in female fertility by acting as a nodal point in the ECM architecture

203-amino acid pentraxin-like domain coupled with an N-terminal 178-amino acid unrelated portion. PTX3 is induced by primary proinflammatory signals in various cell types, most prominently macrophages and endothelial cells. PTX3 is produced by a variety of cells and tissues, most notably dendritic cells and macrophages, upon Toll-like receptor (TLR) engagement and inflammatory cytokines. PTX3 recognizes microbial moieties, opsonizes fungi and selected bacteria, activates the classic pathway of complement cascade, and participates in the formation of ECM (Fig. 8.4) and apoptotic cell clearance. In addition, PTX3 is involved and female fertility. Unlike short pentraxins, PTX3 primary sequence and regulation are highly conserved in man and mouse. Thus, gene targeting identified PTX3 as multifunctional soluble pattern recognition receptors acting as a nonredundant component of the humoral arm of innate immunity and involved in tuning inflammation, matrix deposition, and female fertility (Deban et al. 2010).

8.10.1.1 Multimer Formation by PTX3

The structure and ligand binding properties of human PTX3 were compared with the CRP and SAP component. Sequencing of CHO cell-expressed PTX3 revealed that the mature secreted protein starts at residue 18 (Glu). Lectin binding

and treatment with N-glycosidase F showed that PTX3 is N-glycosylated, sugars accounting for 5 kDa of the monomer mass (45 kDa). The protein consists predominantly of β-sheets with a minor α-helical component. While in gel filtration the PTX3 is eluted with a molecular mass of congruent with 900 kDa, gel electrophoresis revealed that PTX3 forms multimers predominantly of 440 kDa apparent molecular mass, corresponding to decamers, and that disulfide bonds are required for multimer formation. PTX3 does not have coordinated Ca^{2+}.

8.10.1.2 LG/LNS Domains: Multiple Functions

The three-dimensional structures of LG/LNS domains from neurexin, the laminin α2 chain and sex hormone-binding globulin reveal a close structural relationship to the carbohydrate-binding pentraxins and other lectins. However, these LG/LNS domains appear to have a preferential ligand-interaction site distinct from the carbohydrate-binding sites found in lectins, and this interaction site accommodates not only sugars but also steroids and proteins. In fact, the LG/LNS domain interaction site has features reminiscent of the antigen-combining sites in immunoglobulins. The LG/LNS domain presents an interesting case in which the fold has remained conserved but the functional sites have evolved; consequently, making predictions of structure-function relationships on the basis of the lectin fold alone is difficult (Rudenko et al. 2001).

8.10.1.3 Human PTX3 Gene

A genomic fragment of 1,371 nt from human PTX3 gene was characterized as a promoter on TNFα and IL-1β exposure in transfected 8,387 human fibroblasts. The minimal promoter contains one NF-kB element which is necessary for induction and able to bind p50 homodimers and p65 heterodimers but not c-Rel. Mutants in this site lose the ability to bind NF-kB proteins and to respond to TNFα and IL-1β in functional assays. Sp1- and AP-1 binding sites lying in proximity to the NF-kB site do not seem to play a major role for cytokine responsiveness. The cotransfection experiments with expression vectors validate that the natural promoter contains a functional NF-kB site (Basile et al. 1997).

The mouse *ptx3* gene is organized into three exons on chromosome 3: the first (43 aa) and second exon (175 aa) code for the signal peptide and for a protein portion with no high similarity to known sequences the third (203 aa) for a domain related to classical pentraxins, which contains the "pentraxin family signature." Analysis of the N terminal portion predicts a predominantly α-helical structure, while the pentraxin domain of ptx3 is accommodated comfortably in the tertiary structure fold of SAP (Introna et al. 1996).

8.10.2 Cellular Sources of PTX3

Cells producing PTX3 include DC, mononuclear phagocytes, fibroblasts, endothelial cells, smooth muscle cells, adipocytes, synovial cells, and chondrocytes (Abderrahim-Ferkoune et al. 2003; Bottazzi et al. 2006; Doni et al. 2003; Jeannin et al. 2005). PTX3 is also produced by cells of epithelial origin, such as renal and alveolar epithelial cells (c/r Bottazzi et al. 2006). DCs are major producers of PTX3 in vitro. While production is restricted to DCs of myelomonocytic lineage, plasmacytoid DC do not produce PTX3 (Doni et al. 2003; Baruah et al. 2006a, b). In brain, PTX3 expresses in glial cells, in the white matter (corpus callosum, fimbria) and meningeal pia mater as well as in dentate gyrus hilus and granule cells (Polentarutti et al. 2000). Scleroderma fibroblasts, unlike normal fibroblasts, constitutively expressed high levels of PTX3 in the absence of deliberate stimulation. The constitutive expression of PTX3 in SSc fibroblasts was not modified by anti-TNF-α antibodies or IL-1 receptor antagonist. In contrast, IFN-γ and TGF-β inhibited the constitutive but not the stimulated expression of PTX3 in SSc fibroblasts (Luchetti et al. 2004). IL-1 and TNF-α strongly stimulate the expression of PTX3 by human proximal renal tubular epithelial cells (PTECs). In addition, activation of PTECs with IL-17 and CD40L, respectively, but not with IL-6 or IL-4, resulted in strongly increased production of PTX3, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibited IL-1-induced PTX3 production. Results suggest that local expression of PTX3 may play a role in the innate immune response and inflammatory reactions in the kidney (Nauta et al. 2005).

Human monocyte-derived DC produced copious amounts of PTX3 in response to microbial ligands engaging different members of the Toll-like receptor (TLR) family (TLR1 through TLR6), whereas engagement of the mannose receptor had no substantial effect. DCs were better producers of PTX3 than monocytes and macrophages. In contrast, plasmacytoid DC exposed to influenza virus or to CpG oligodeoxynucleotides engaging TLR9, did not produce PTX3. PTX3-expressing DCs were present in inflammatory lymph nodes from HIV-infected patients. Thus, DCs of myelomonocytic origin are a major source of PTX3, which facilitates pathogen recognition and subsequent activation of innate and adaptive immunity (Doni et al. 2003).

8.10.3 Ligands

Unlike the classical pentraxins CRP and SAP, PTX3 did not bind phosphoethanolamine, phosphocholine, or high pyruvate agarose. PTX3 in solution, bound to immobilized C1q, but not C1s. Binding of PTX3 to C1q is specific with a K_D

7.4×10^{-8} M. The Chinese hamster ovary cell-expressed pentraxin domain bound C1q when multimerized. Thus, on the basis of computer modeling, the long pentraxin PTX3 forms multimers, which differ from those formed by classical pentraxins in terms of protomer composition and requirement for disulfide bonds, and does not recognize CRP/SAP ligands (Bottazzi et al. 1997; Nauta et al. 2003). In addition, PTX3 binds with high affinity to a number of different ligands, the fibroblast growth factor 2 (FGF2) (Bottazzi et al. 2006), the ECM protein TNF-α-induced protein 6 (TSG-6) (Salustri et al. 2004), and the outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA) (Jeannin et al. 2005). Moreover, a specific interaction with apoptotic cells has been described by Rovere et al. (2000). Interaction of PTX3 with its ligands is fundamental for some of the physiological functions attributed to PTX3 (Fig. 8.4).

8.10.4 Regulation of PTX3

Regulation: PTX3 is produced and released by a variety of cell types upon exposure to primary inflammatory signals, such as TNF-α and IL-1ß, TLR ligands, and microbial moieties, such as LPS, lipoarabinomannans, and outer membrane proteins. Normal and transformed fibroblasts, undifferentiated and differentiated myoblasts, normal endothelial cells, and mononuclear phagocytes express *mptx3* mRNA and release the protein in vitro on exposure to IL-1β and TNFα. The *mptx3* was induced by bacterial LPS in vivo in a variety of organs and, strongly in the vascular endothelium of skeletal muscle and heart. Thus, *mptx3* shows a distinct pattern of in vivo expression indicative of a significant role in cardiovascular and inflammatory pathology. Human peripheral blood mononuclear cells exposed to LPS or IL-1β expressed significant levels of PTX3 mRNA. TNF-α was a less-effective inducer of PTX3, whereas IL-6, monocyte chemotactic protein-1, MC-SF, GM-CSF, and IFN-γ were inactive. Thus, PTX3, unlike the classical pentraxins CRP and SAP, is expressed and released by cells of the monocyte-macrophage lineage exposed to inflammatory signals (Bottazzi et al. 2006; Garlanda et al. 2005; Jeannin et al. 2005). IFN-γ, which generally has a synergistic effect with LPS (Ehrt et al. 2001), inhibits LPS induction of PTX3 in DC as well as in monocytes and endothelial cells (Doni et al. 2006; Polentarutti et al. 2000). Conversely, IL-10 is a modest and inconsistent inducer of PTX3 in DC and monocytes (Bottazzi et al. 2006) but significantly amplifies the response to LPS, TLR ligands, and IL-1ß (Doni et al. 2006). PTX3 superinduction, in response to IL-10, is likely to play a role in matrix deposition, tissue repair, and remodeling. Moreover, data suggest that besides stimulation of the humoral arm of adaptive immunity, IL-10 also stimulates the humoral arm of innate immunity (Moore et al. 2001). Cell-specific regulation of *ptx3* by glucocorticoid hormones in hematopoietic and nonhematopoietic cells has been reported by Doni et al. (2008).

Mycobacterial lipoarabinomannan (LAM) induced expression of PTX3 mRNA in human peripheral blood mononuclear cells. The non-mannose-capped version of lipoarabinomannan (AraLAM) was considerably more potent than the mannose-capped version ManLAM or the simpler version phosphatidylinositol mannoside. Whole mycobacteria (Mycobacterium bovis BCG) strongly induced PTX3 expression. LAM-induced PTX3 expression was associated with the production of immunoreactive PTX3. IL-10 and IL-13 did not inhibit the induction of PTX3 by LAM. In contrast, IFNγ inhibited LAM-induced PTX3 expression. Thus, in addition to IL-1, TNF, and LPS, mycobacterial cell wall components also induce expression and production of long pentraxin PTX3 (Vouret-Craviari et al. 1997).

Inducible Expression of PTX3 in CNS: PTX3 is expressed in brain and PTX3 mRNA is induced in the mouse brain by LPS. In contrast NP1 is constitutively expressed in the murine CNS and is not modulated by LPS administration. IL-1β was also a potent inducer of PTX3 expression in the CNS, whereas TNF-α was substantially less effective and IL-6 induced a barely detectable signal. Central administration of LPS and IL-1 induced PTX3 also in the periphery (heart), whereas the reverse did not occur. Expression of PTX3 was also observed in the brain of mice infected with *Candida albicans (C. albicans)* or *Cryptococcus neoformans*. In situ hybridization revealed that i.c.v. injection of LPS induced a strong PTX3 expression in presumptive glial cells, in the white matter (corpus callosum, fimbria) and meningeal pia mater as well as in dentate gyrus hilus and granule cells (Polentarutti et al. 2000).

Elevation of Pentraxins in Haemodialysis Patients: Elevation of CRP in patients with renal failure and its association with cardiovascular disease is well described. PTX3 levels are markedly elevated in haemodialysis (HD) patients. The increase in PTX3 production in whole blood after HD indicates that the HD procedure itself contributes to elevated PTX3 levels in HD patients. The association between PTX3 and cardiovascular morbidity suggests a possible connection of PTX3 with atherosclerosis and cardiovascular disease in HD patients (Boehme et al. 2007).

8.10.5 Functions of PTX3

8.10.5.1 Innate Immunity

Studies so far suggest that PTX3 plays complex, nonredundant functions in vivo, ranging from innate resistance against selected pathogens to the assembly of a hyaluronic acid (HA)-rich extracellular matrix (ECM) and female fertility (Bottazzi et al. 2006; Garlanda et al. 2005). Two main features that characterize *ptx3*-deficient mice are that they are sterile and more susceptible to infections to selected pathogens (Garlanda et al. 2002; Salustri et al. 2004; Varani et al. 2002). The *ptx3*-deficient mice are more susceptible to infection with selected fungal and bacterial microorganisms, such as *A. fumigatus, P. aeruginosa*, and *S. typhymurium*, while they are resistant to infection with *Listeria monocytogens* and *S. aureus* (Garlanda et al. 2005). Susceptibility of *ptx3*-null mice was associated with defective recognition of conidia by alveolar macrophages and DCs, as well as inappropriate induction of an adaptive type 2 response (Garlanda et al. 2002). Binding of PTX3 has been demonstrated for conidia of *A. fumigatus* as well as for *P. aeruginosa* and *S. typhymurium*, while PTX3 does not recognize *L. monocytogens*. Moreover, macrophages from *ptx3*-transgenic mice have an improved phagocytic activity toward zymosan and *Paracoccidioides brasiliensis* (Diniz et al. 2004). These findings provide evidences for a role of PTX3 as an opsonin and imply the existence of a receptor for this molecule.

Pathogen recognition is a common feature among the members of the pentraxin family. PTX3 does not bind LPS as well as lipoteichoic acid, N-acetylmuramyl-L-alanyl-D-isoglutamine, exotoxin A, and enterotoxin A and B, but it binds with high affinity the recombinant KpOmpA, which binds and activates macrophages and DC in a TLR2-dependent way and activates genetic induction of PTX3. In turn, PTX3 binds KpOmpA and plays a crucial role in the amplification of the inflammatory response to this microbial protein, as demonstrated in *ptx3*-deficient mice (Jeannin et al. 2000, 2005) (Fig. 8.4).

Pentraxin 3 in Inflammatory and Immune Responses: Clinical significance of different levels of pentaxins, their role in the induction or protection from autoimmunity, and the presence of specific autoantibodies against them in the different autoimmune diseases have been reviewed (Kravitz et al. 2005; Kravitz and Shoenfeld 2006; Kunes 2005) (Fig. 8.4). In humans, PTX3 blood levels are barely detectable in normal conditions and increase rapidly during a range of inflammatory and infectious conditions. PTX3 levels are elevated in critically ill patients, reflecting the severity of disease (Muller et al. 2001). In patients with acute myocardial infarction (AMI), PTX3 is a prognostic marker of death (Latini et al. 2004). CRP levels increase in AMI, possibly as a consequence of a systemic response to myocardial injury. Emerging studies on rapidity of increase of PTX3 in blood compared with CRP in humans, together with a lack of correlation between CRP and PTX3 reflect a role for this pentraxin in the pathogenesis of damage (Napoleone et al. 2004). Patients with active vasculitis, dengue virus infections or active pulmonary tuberculosis have significantly higher plasma levels of PTX3 than patients with quiescent disease (Fazzini et al. 2001; Mairuhu et al. 2005; Azzurri et al. 2005). Increased levels of blood PTX3 have been observed in patients with pulmonary infection and acute lung injury (He et al. 2007) and in systemic sclerosis (Iwata et al. 2009).

Role of PTX3 in inflammatory conditions was evaluated in *Ptx3*-transgenic and -deficient mice. *Ptx3* over-expression increases resistance to LPS toxicity and *ptx3*-transgenic mice show an exacerbated, inflammatory response and reduced survival rate following intestinal ischemia reperfusion injury (Dias et al. 2001). Moreover, *ptx3*-deficient mice had more widespread and severe IL-1-induced neuronal damage. In these mice, PTX3 confers resistance to neurodegeneration, and rescuing neurons from otherwise irreversible damage (Ravizza et al. 2001). The PTX3 is crucial for tissue inflammation after intestinal ischemia and reperfusion in mice (Souza et al. 2009).

Expression of PTX3 in RA

The expression of PTX3 was studied in dissociated rheumatoid arthritis (RA) and osteoarthritis (OA) type B synoviocytes, cultured in the presence and in the absence of inflammatory cytokines. OA synoviocytes were induced to express high levels of PTX3 mRNA by TNF-α, but not by other cytokines including IL-1β and IL-6. RA synoviocytes, unlike OA synoviocytes, constitutively expressed high levels of PTX3 in the absence of deliberate stimulation. In contrast, IFNγ and TGF-β inhibited PTX3 constitutive expression in RA synoviocytes. The joint fluid from RA patients contained higher levels of immunoreactive PTX3 than controls and the synovial tissue contained endothelial cells and synoviocytes positive for PTX3. Thus, PTX3 may play a role in inflammatory circuits of RA (Luchetti et al. 2000).

8.10.5.2 PTX3 Bound Apoptotic Cells Are Regulated by APCs

The PTX3 is produced in tissues under the proinflammatory signals, such as LPS, IL-1β, and TNF-α, which also promote maturation of DCs. Cell death commonly occurs during inflammatory reactions. It was shown that PTX3 specifically binds to dying cells in dose dependent and saturable manner. Recognition was restricted to extranuclear membrane domains and to a chronological window after UV irradiation or after CD95 cross-linking-induced or spontaneous cell

death in vitro. PTX3 bound to necrotic cells to a lesser extent. Studies suggest that PTX3 sequesters cell remnants from antigen-presenting cells, possibly contributing to preventing the onset of autoimmune reactions in inflamed tissues (Rovere et al. 2000).

PTX3 Limits C1q-Mediated Complement Activation and Phagocytosis of Apoptotic Cells by DCs: The PTX3 and C1q are innate opsonins involved in the disposal of dying cells by phagocytes. C1q increases the phagocytosis of apoptotic cells by DC and the release of interleukin-12 in the presence of TLR4 ligands and apoptotic cells; PTX3 inhibits both events. Results suggest that the coordinated induction by primary, proinflammatory signals of C1q and PTX3 and their reciprocal regulation during inflammation influences the clearance of apoptotic cells by APCs and possibly plays a role in immune homeostasis (Baruah et al. 2006a). PTX3 levels have been shown to parallel disease activity in small-vessel vasculitis, which is often characterized by leukocytoclasia, a persistence of leukocyte remnants in the vessel wall. Therefore, PTX3 can play a role in the development of leukocytoclasia by affecting the clearance of apoptotic PMNs, thereby inducing their accumulation in the vessel wall (van Rossum et al. 2004).

PTX3 is Recruited at the Synapse Between Dying and Dendritic Cells: The PTX3 is specifically recruited at both sides of the phagocytic synapse between DCs and dying cells and remains stably bound to the apoptotic membranes. Apoptotic cells per se influence the production of PTX3 by maturing DCs. When both microbial stimuli and dying cells are present, PTX3 behaves as a flexible adaptor of DC function, regulating the maturation program and the secretion of soluble factors. Moreover a key event associated with autoimmunity (i.e., the cross-presentation of epitopes expressed by apoptotic cells to T cells) abates in the presence of PTX3, as evaluated using self, viral, and tumor-associated model antigens (vinculin, NS3, and MelanA/MART1). In contrast, PTX3 did not influence the presentation of exogenous soluble antigens, an event required for immunity against extracellular pathogens. These data suggest that PTX3 acts as a third-party agent between microbial stimuli and dying cells, contributing to limit tissue damage under inflammatory conditions and the activation of autoreactive T cells (Baruah et al. 2006b).

8.10.5.3 Role in Female Fertility

Ptx3 deficiency results in a severe defect in female fertility (Garlanda et al. 2002; Salustri et al. 2004; Varani et al. 2002). Infertility of *ptx3*-deficient mice is due to an abnormal cumulus oophorus characterized by an unstable ECM in which cumulus cells are dispersed uniformly instead of radiating out from a central oocyte (Salustri et al. 2004). However, oocytes from *ptx3*-deficient mice can be fertilized in vitro, suggesting that oocyte develops normally in the absence of PTX3 and that lack of in vivo is a result of the defective cumulus expansion. Cumulus cells express *ptx3* mRNA, and no or barely detectable expression was observed in peripheral granulosa cells (Salustri et al. 2004; Zhang et al. 2005). PTX3 has been identified as a potential marker of oocyte competence expressed in bovine cumulus cells which were matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro (Assidi et al. 2008). Human cumulus cells also express *PTX3* (Zhang et al. 2005), and PTX3 is present in human cumulus matrix, suggesting some role for PTX3 in human female fertility. Results show a higher abundance of *PTX3* mRNA in cumulus cells from fertilized oocytes compared with cumulus cells from unfertilized oocytes, indicating in *PTX3* a possible marker for oocyte quality (Zhang et al. 2005), although this is not the case for PTX3 protein (Paffoni et al. 2006). In addition, pre-eclampsia is associated to elevated levels of PTX3 (Cetini et al. 2006). Thus, it is important to assess the potential of PTX3 in human fertility and pregnancy.

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Part IV

Animal Galectins

Overview of Animal Galectins: Proto-Type Subfamily 9

Anita Gupta and G.S. Gupta

9.1 Galectins

Lectins recognize and bind carbohydrates covalently linked to proteins and lipids on the cell surface and within the extracellular matrix, and they mediate many cellular functions ranging from cell adhesion to pathogen recognition. Phylogenetically conserved family of Galectins was defined in 1994 as a shared consensus of amino-acid-sequences of about 130 amino acids and the CRD responsible for ß-galactoside binding (Barondes et al. 1994a, b). The galectin (Gal) CRDs bind small β-galactosides/poly-N-acetyllactosamine-enriched glycoconjugates. But the overall binding affinity for more complex glycoconjugates varies substantially. To date, 15 members of the mammalian galectin family have been identified. Some, such as galectin-1, are isolated as dimers and have a single CRD in each monomer, whereas others, such as galectin-4, are isolated as monomers and have two CRDs in a single polypeptide chain. While CRDs of all galectins share an affinity for minimum saccharide ligand N-acetyllactosamine—a common disaccharide found on many cellular glycoproteins—individual galectins can also recognize different modifications to this minimum saccharide ligand and so demonstrate the fine specificity of certain galectins for tissue- or developmentally-specific ligands (Ahmad et al. 2004a). Location studies of galectins have established that these proteins can segregate into multiple cell compartments in function of the status of the cells in question (Danguy et al. 2002; Liu and Rabinovich 2005). Although galectins as a whole do not have the signal sequence required for protein secretion through usual secretory pathway, some galectins are secreted and are found in the extracellular space. While the intracellular activity of galectin-1 is mainly independent on its lectin activity, its extracellular activity is mainly dependent on it. The functions and distribution of Gal-1 and Gal-3 are well characterized.

9.2 Galectin Sub-Families

Galectins are localized to the cytoplasm, nucleus, and the extracellular environment (Liu et al. 2002). Secretion of galectins occurs via a nonclassical secretion pathway that requires association of galectins with glycosylated counter-receptors (Seelenmeyer et al. 2005). Nuclear expression of galectins has been studied in cultured stromal cells of human bone marrow and human/porcine keratinocytes. Binding studies revealed positivity for galectin-1, where as galectins-3, -5, and -7 were not reactive with nuclear sites in bone marrow stromal cells and keratinocytes. Presence of binding sites in nucleus adds a new aspect to the functional analysis of these lectins.

Thus far, 15 mammalian galectins have been identified and sequenced (Rabinovich and Gruppi 2005; Elola et al. 2005a, b). Hirabayshi and Kasai (1993) proposed designating galectin subfamilies as: Proto-, tandem-repeat, and Chimera-types based on their domain organization. Galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15, which belong to the mono-CRD type and are found in many cells and tissues, have molecular weights of 14–18 kDa with a single galactoside-binding domain/CRD with a short N-terminal sequence. But the tandem-repeat type galectins (galectin-4, -6, -8, -9, and -12) are composed of two non-identical CRDs joined by a short linker peptide sequence. The single chimera-type galectin (galectin-3) has one CRD and an extended N-terminal tail containing several repeats of proline-tyrosine-glycine-rich motif with a collagenous domain. Galectin-3 with an apparent molecular weight between 26 and 30 kDa is abundant in activated macrophages and epithelial cells. Many of these β-galactoside-binding proteins have also received other names, according to their function, localization, or biochemical features, i.e., galectin-1 (L-14, BHL, or galaptin), galectin-3 [Mac-2, L-29, CBP-35, or εBP for "IgE-binding protein"], galectin-9 (ecalectin), galectin-10 (Charcot-Leyden crystal

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eosinophil protein), galectin-11 (GRIFIN for galectin-related interfiber protein) and galectin-13 (PP13). The vertebrate galectin CRDs are always encoded by three exons with two subtypes and are defined by the exon–intron structure (F4-CRD and F3 CRD). The F4-CRD-linker-F3-CRD gene structure is shared among all vertebrate bi-CRD galectins, one *Ciona intestinalis* galectin (Houzelstein et al. 2004), and the *Strongylocentrotus purpuratus* galectin. The chordate galectins share a common ancestor, and bi-CRD galectins are derived from an ancestral tandem-duplication of mono-CRD galectin either before or in early chordate evolution (Houzelstein et al. 2004).

Analysis of GenBank databases has led to the identification of more galectin-like proteins in mammals, invertebrates, plants, and microorganisms, confirming that these carbohydrate-binding proteins are highly conserved throughout evolution. The structures of several galectin CRDs have been reported, and all exhibit a β-sandwich fold containing two antiparallel β-sheets (Liao et al. 1994; Lobsanov et al. 1993; Seetharaman, et al. 1998; Leonidas et al. 1998). However, their quaternary structures differ. Galectin-1 and -2 form non-covalently associated homodimers through extended β-sheet interactions (Loris 2002). The association state of galectin-3 is regulated by its N-terminal domain, and it can exist in monomeric or oligomeric forms (Birdsall et al. 2001). Finally, because the tandem-repeat type galectins possess two different CRDs, they may adopt more complex assembly states. There are a variety of potential glycoconjugate targets for galectins in mammalian cells, but the molecular mechanisms of carbohydrate recognition remain unclear.

9.3 Galectin Ligands

Galectins have been shown to be involved in multiple biological functions such as cell-matrix and cell-cell interactions, cell proliferation, cell differentiation, cellular transformation, or apoptosis mainly through their binding properties to specific ligands. Galactose is a ligand for galectins. Galactose is part of scaffold structure that synthesizes oligosaccharide ligands for selectins, siglecs and other lectins of the immune system. Galactose residues are added to glycoproteins and glycolipids by members of a large family of galactosyltransferases. The expression of many of these enzymes is regulated by the action of cytokines, and becomes altered in various disease states. Antibodies need to be galactosylated for normal function, and under-galactosylated immunoglobulin (Ig) is associated with rheumatoid arthritis. Thus galectins have potential use as novel anti-inflammatory agents or targets for immunosuppressive drugs, in autoimmune and chronic inflammatory disorders (Bianco et al. 2006). Since galactose containing structures are involved in both the innate and adaptive immune systems, galectins play important roles in host-pathogen interactions, and in tumorigenesis (Rabinovich and Gruppi 2005). Nakahara and Raz (2006) discussed the role of galectins in signal transduction, dividing these proteins into extracellular and intracellular galectins. Galectin-1 is an important link between the sugar signal and the intracellular response. The growth-regulatory interaction between GM1 on human neuroblastoma cells and an endogenous lectin provides an example for glycan functionality.

9.4 Functions of Galectins

Galectins can be found in the extracellular space, in the cell membrane, in the cytoplasm, and even in the nucleus. They can bind to proteins via a carbohydrate interaction or via direct protein-protein interactions. Most galectins have multiple functions and they can mediate (1) receptor cross-linking or lattice formation, (2) cell-extracellular matrix interactions, (3) cell-to-cell interactions, (4) intracellular signaling, and (5) posttranscriptional splicing (Thijssen et al. 2007) (Fig. 9.1). Galectins can regulate inflammation, cell proliferation, the cell cycle, transcription processes, and cell death (Rabinovich et al. 2002a, b). Galectins lack a traditional signal sequence, and several are secreted by an unorthodox mechanism to exert their extracellular functions (Mehul and Hughes 1997). Some galectins are differentially regulated during pre- or post-natal development. Structural studies have shown that the Gal-specific proteins encompass a diverse range of primary and tertiary structures. The binding sites for galactose also seem to vary in different protein-galactose complexes. Sugar-encoded information of glyco-conjugates is translated into cellular responses by endogenous lectins. This remarkable versatility warrants close scrutiny of their emerging network. Cell localization studies have established that these proteins can segregate into multiple intracellular compartments, and the preference for segregation is dependent on the status of the cell. Localization, therefore, likely corresponds to compartmental function. Toscano et al. (2007) reviewed immunoregulatory roles of galectins (particularly galectin-1) and collectins to illustrate the ability of endogenous glycan-binding proteins to act as cytokines, chemokines or growth factors, and thereby modulating innate and adaptive immune responses under physiological or pathological conditions. Understanding the pathophysiologic relevance of endogenous galectins in vivo would reveal novel targets for immunointervention during chronic infection, autoimmunity, transplantation and cancer (Ilarregui et al. 2005; Toscano et al. 2007) (Table 9.1).

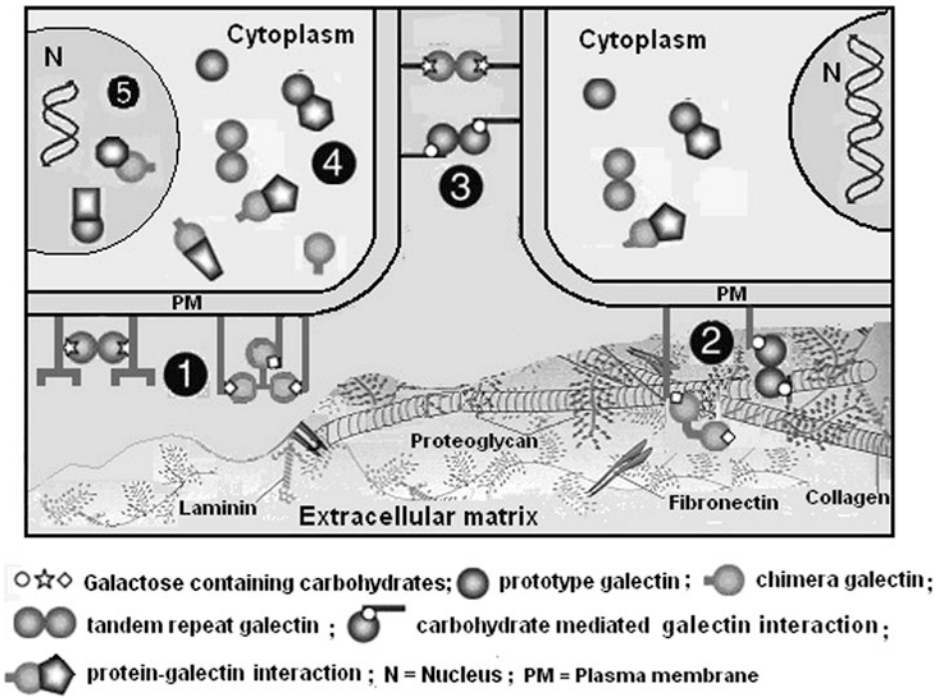

Fig. 9.1 Cellular location and functions of galectins. Galectins can be found in the extracellular space, in the cell membrane, in the cytoplasm, and in the nucleus. Functional interactions of galectins with cell-surface glycoconjugates and extracellular glycoconjugates can lead to cell adhesion and cell signaling. Interactions of galectins with intracellular ligands may also contribute to the regulation of intracellular pathways (Cummings and Liu 2009). They can bind to proteins via a carbohydrate interaction or via direct protein-protein interactions. Most galectins can mediate (1) receptor cross-linking or lattice formation, (2) cell-extracellular matrix interactions, (3) cell-to-cell interactions, (4) intracellular signaling, and (5) posttranscriptional splicing (Thijssen et al. 2007)

9.4.1 Functional Overlap/Divergence Among Galectins

To define the extent of functional overlap/divergence among galectins, a comparative profiling has been made in mouse as a model organism, combining sequence analysis, expression patterns and structural features in homodimeric galectins-1, -2 and -7. Close relationship was apparent at the level of global gene organization. RT-PCR mapping from an array of 17 organs revealed significant differences, separating rather ubiquitous gene expression of galectin-1 from the more restricted individual patterns of galectins-2 and -7. Nuclear presence was seen in case of galectin-1. In addition to nonidentical expression profiles the mapping of CRDs of galectins-1 and -7 by homology modelling and docking of naturally occurring complex tetra- and pentasaccharides disclosed a series of sequence deviations which may underlie disparate affinities for cell surface glycans/glycomimetic peptides (Lohr et al. 2007). A distinct expression profile of Galectin-3 was determined in various murine organs when set into relation to homodimeric galectins-1 and -7. Lohr et al. (2008) demonstrated cell-type specificity and cycle-associated regulation for galectin-3 with increased presence in atretic preantral follicles and in late stages of luteolysis. Staining patterns for galectin-8 were studied in hypo-pharyngeal and laryngeal tumor progression and relate d these parameters to galectins 1, 3 and 7 in the quest to explore the galectin network. Marked upregulation in galectin-8 staining intensity and immunopositive area in malignancy versus dysplasia was seen in hypopharyngeal cancer, in laryngeal cancer for labeling index and high-grade dysplasia/carcinoma. No correlation to recurrence was delineated. Data revealed a divergence within the galectin-1, -3, -7 and -8 network during tumor progression (Cludts et al. 2009).

9.4.2 Cell Homeostasis by Galectins

Galectin are known to participate in cellular homeostasis by modulating cell growth, controlling cell cycle progression, and inducing or inhibiting apoptosis. Both intracellular and extracellular activities of galectins have been described, with the former typically independent of lectin activity, and the latter mediated by lectin activity. Galectin-1 and -3 are recognized as activators and inducers of cell homeostasis in extracellular capacities. By using recombinant proteins, a number of galectins have been shown to interact with

Table 9.1 Schematic representation, biochemical and functional properties of different members of Galectin family (Adapted by permission from Rabinovich et al. (2002c) © Society for Leukocyte Biology)

Galectins/structure	Localisation	Biochemical and functional properties
Galectin-1 	Abundant in most Organs: muscle, heart, liver, prostate, lymph nodes, spleen, thymus, placenta, testis, retina, macrophages, B-cells, T-cells, tumors	Non-covalent homodimer
		Induces apoptosis of activated T cells and immature thymocytes
		Induces a polarized Th2 immune response
		Modulates cell-cell and cell-matrix interactions
		Inhibits acute inflammation:blocks arachidonic acid release, mast cell degranulation and neutrophil extravasation
		Suppresses chronic inflammation and autoimmunity
Galectin-2 	Stomach epithelial cells	Non-covalent homodimers
		Expressed at minor levels in tumor cells
Galectin-3 	Mainly in tumor cells, macrophages, epithelial cells, fibroblasts, activated T cells	Non-lectin domain linked to CRD
		Anti apoptotic and proinfammatory functions
		Modulates cell adhesion and migration
		Induces chemotaxis of monocytes
		Potentiates pro-inflammatory (IL-1) cytokine secretion
		Induces nitric oxide induced apoptosis and ancilkis
		Down regulates IL-5 gene transcription
Galectin-4 	GIT	Compose of two distinct CRD in a single polypeptide chain
		Expressed at sites of tumor cell adhesion
Galectin-5 	Erythrocytes	Prototype galecin: monomer
		No function assigned
Galectin-6 	GIT	Composed of two distinct CRD in a single polypeptide chain
		Closely linked to galectin—4
Galectin-7 	Skin	Prototype galecin: monomer
		Used as a marker of stratified epithelium
		Increases susceptibility of keratinocytes to UVB-induced apoptosis
Galectin-8 	Liver, kidney, Cardiac Muscle, Prostate, Brain	Compose of two distinct CRD in a single polypeptide chain
		Modulates integrin interactions with extracellular matrix
Galectin-9 	Thymus, T-cells, Kidney, Hodgkin's lymphoma	Composed of two distinct CRD in a single polypeptide chain
		Induces eosinophil chemotaxis
		Induces apoptosis of murine thymocytes

(continued)

Table 9.1 (continued)

Galectins/structure	Localisation	Biochemical and functional properties
Galectin-10 	Eosinophils and basophils	Prototype galecin: monomer Mainly expressed by eosinophils, formally called 'Charcot-Leyden Crystal Protein'
Galectin-11 	Lens	Also called 'Grifin' May represent a new lens crystalline Lacks affinity for β–galactoside sugars
Galectin-12 	Adipocytes	Composed of two distinct CRD in a single polypeptide chain Induces apoptosis and cell cycle arrest
Galectin-13 	Human placenta	Similar to proto type 'galectins'? Also called 'pp-13'
Galectin-14 	Eosinophil (Sheep)	Plays a role in eosinophil function and allergic inflammation
Galectin-15 	Uterus (Sheep)	Intracellular gal-15 regulates cell survival, differentiation function Extracellular secreted gal-15 regulates implants and placentation

cell-surface and extracellular matrix glycoconjugates through lectin-carbohydrate interactions. Through this action, they can affect a variety of cellular processes, and the most extensively documented function is induction of apoptosis. Evidences suggest that some of these functions involve binding to cytoplasmic and nuclear proteins, through protein-protein interactions, and modulation of intracellular signaling pathways. Galectin-1, -7, -8, -9 and -12 are characterized as promoters or inducers of apoptosis, while galectin-3 is demonstrated as an inhibitor of apoptosis intracellularly. Thus, some galectins are pro-apoptotic, whereas others are anti-apoptotic; while some galectins induce apoptosis by binding to cell surface glycoproteins, others regulate apoptosis through interactions with intracellular proteins. Involvement of galectin-1, -2, -3, -7, -8, -9, and -12 in apoptosis has been reviewed (Hsu et al. 2006).

9.4.3 Immunological Functions

Ggalectins have attracted the attention of immunologists as novel regulators of host–pathogen interaction (Almkvist and Karlsson 2004; Rabinovich and Gruppi 2005). Galectins participate in a wide spectrum of immunological processes. These proteins regulate the development of pathogenic T-cell responses by influencing T-cell survival, activation and cytokine secretion. Administration of recombinant galectins or their genes into the cells modulate the development and severity of chronic inflammatory responses in experimental models of autoimmunity by triggering different immunoregulatory mechanisms. Galectins are differentially expressed by various immune cells and their expression levels appear to be dependent on cell differentiation and activation. They can interact with cell-surface and extracellular matrix

glycoconjugates (glycoproteins and glycolipids), through lectin-carbohydrate interactions. Current research indicates that galectins play important roles in the immune response through regulating the homeostasis and functions of the immune cells. Galectins have important roles in cancer, where they contribute to neoplastic transformation, tumor cell survival, angiogenesis and tumor metastasis. They can modulate the immune and inflammatory responses and might have a key role helping tumors to escape immune surveillance (Liu and Rabinovich 2005; Liu 2005). Galectins operate at different levels of innate and adaptive immune responses, by modulating cell survival and cell activation or by influencing the Th1/Th2 cytokine balance. The influence of galectins in immunological processes relevant to microbial infection has been explored.

Galectins are novel anti-inflammatory agents or targets for immunosuppressive drugs, and have impact in the development and/or resolution of chronic inflammatory disorders, autoimmunity, and cancer. Inflammation involves the sequential activation of signaling pathways leading to the production of both pro-inflammatory and anti-inflammatory mediators (Ilarregui et al. 2005; Rabinovich and Gruppi 2005). The role of galectins in the initiation, amplification and resolution of the inflammatory response has been reviewed by Rubinstein et al. (2004) who examined the influence of each member of this family in regulating cell adhesion, migration, chemotaxis, antigen presentation, immune cell activation and apoptosis.

Galectin-1 and galectin-3 are upregulated in gastric epithelial cells infected with *Helicobacter pylori*, which suggests that galectins might contribute to bacterial invasion. In addition, galectin-3 has the ability to bind to Gram-negative (G^-) bacteria through the recognition of different bacterial LPSs (Mey et al. 1996) and induce the death of Candida species expressing specific ß-1,2-linked mannans (Kohatsu et al. 2006). The elevation of galectin-9 is involved in the inflammatory response of periodontal ligament cells exposed to *Porphylomonas gingivalis* LPS in vitro and in vivo (Kasamatsu et al. 2005) and can recognize the *Leishmania major*-specific polygalactostosyl epitope (Pelletier et al. 2003).

Role in Chemotaxis: The involvement of galectins in chemotaxis has been reported for galectin-3 which stimulates both monocyte and macrophage chemotaxis (Sano et al. 2000; Papaspyridonos et al. 2008) and galectin- 9 (Hirashima 1999; Matsumoto et al. 1998) which is a selective chemoattractant for eosinophils. Malik et al. (2009) demonstrated a role for galectin-1 in monocyte chemotaxis which differed from galectin-3 in that macrophages were nonresponsive. Study revealed that galectin-1-induced migration of monocytes was mediated by a pertussis toxin (PTX)-sensitive pathway and by the P44/42 MAP kinase pathway.

9.4.4 Signal Transduction by Galectins

9.4.4.1 Galectin-Glycan Lattices Regulate Cell-Surface Glycoprotein Organization and Signaling

The formation of multivalent complexes of soluble galectins with glycoprotein receptors on the plasma membrane helps to organize glycoprotein assemblies on the surface of the cell. In some cell types, this formation of galectin-glycan lattices or scaffolds is critical for organizing plasma membrane domains, such as lipid rafts, or for targeted delivery of glycoproteins to the apical or basolateral surface. Galectin-glycan lattice formation is also involved in regulating the signaling threshold of some cell-surface glycoproteins, including T-cell receptors (TCRs) and growth factor receptors. Finally, galectin-glycan lattices can determine receptor residency time by inhibiting endocytosis of glycoprotein receptors from the cell surface, thus modulating duration of signaling from the cell surface (Garner and Baum 2008). As an example, T-cell activation requires clustering of a threshold number of TCRs at the site of antigen presentation. A deficiency in β1,6 N-acetylglucosaminyltransferase V (Mgat5), an enzyme in the N-glycosylation pathway, lowers T-cell activation thresholds by directly enhancing TCR clustering. Dysregulation of Mgat5 in humans may increase susceptibility to autoimmune diseases, such as multiple sclerosis (Demetriou et al. 2001). Galectin-mediated ligation of glycoproteins on T-cell activation markers induces an increase in the cytosolic calcium concentration $(Ca^{2+})_i$ originating from a transient $(Ca^{2+})_i$ release of internal stores as well as a sustained influx across the plasma membrane (Walzel et al. 1996; Walzel et al. 2002). In transiently transfected Jurkat T-lymphocytes, galectins differentially stimulate the expression of reporter gene constructs, which were activated by the nuclear factor of activated T-cells (NFAT) or the transcription factor, activator protein 1 (AP-1), respectively. Electrophoretic mobility shift assays (EMSAs) provided evidence for gal-1-stimulated increase in the binding of nuclear extracts to a synthetic oligonucleotide with an AP-1 consensus sequence (Walzel et al. 2002). Cha et al. (2008) demonstrated that a galectin-1 lattice is responsible for retaining the renal epithelial Ca^{2+} channel TRPV5 at the plasma membrane, providing evidence for galectin lattice-mediated regulation of ion balance via regulation of channel residency at the cell surface (Cha et al. 2008).

9.4.4.2 Galectins, Integrins and Cell Migration

The interaction of galectins with integrins modulates cell migration as well as other processes. Galectin-1 interacts

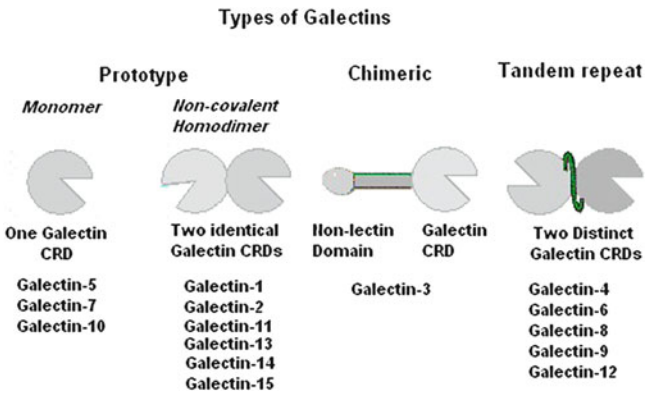

Fig. 9.2 Schematic representation of overall structures of galectins as assembled proteins

with the β1 integrin subunit inducing the phosphorylation of FAK, which modulates cell migration (Moiseeva et al. 2003). Binding of Gal-1 to integrin is involved in cell adhesion (Moiseeva et al. 1999). Moreover, Gal-1 also regulates the expression of the protein ADAM-15 that is involved in integrin-mediated adhesion (Camby et al. 2005). Gal-1 also induces growth inhibition via its interaction with α5β1 (Fischer et al. 2005). This interaction results in the inhibition of the Ras–MEK–ERK pathway and the consecutive transactivation of Sp1, which induces p27 transcription (Fischer et al. 2005). In addition, Gal-1 is involved in the PKCε/vimentin controlled trafficking of integrin β1, a process that is important for cell migration (Fortin et al. 2010). However, it is not known with which molecule(s) Gal-1 is interacting, or in which intra- or extracellular location this interaction is taking place in order to initiate this signaling. Finally, Gal-1 is also involved in cell motility via the Gal-1-induced expression of RhoA and the alteration of the polimerization of the actin cytoskeleton (Camby et al. 2002).

Galectin-3 regulates cell adhesion via binding to α1β1 (Ochieng et al. 1998). Gal-3 also forms a complex with α3β1 and the proteoglycan NG2 (Fukushi et al. 2004). This interaction regulates endothelial cell motility and angiogenesis. In addition, Gal-3 has been shown to regulate the expression of integrin α6β1 and actin cytoskeleton organization (Debray et al. 2004). It is not known with which molecule (s) Gal-3 is interacting to initiate this signaling. Gal-8 interacts with several integrins including α1β1, α3β1, α5β1 and α6β1. These interactions participate in cell adhesion and apoptosis (Hadari et al. 2000).

9.4.5 Common Structural Features in Galectins

Lectins are generally organized as oligomers of noncovalently bound subunits, each displaying a carbohydrate recognition domain (CRD) that binds to the sugar ligand, usually a nonreducing terminal monosaccharide or oligosaccharide. Although lectin-ligand interactions are relatively weak as compared with other immune recognition molecules, high avidity for the target is achieved by association of peptide subunits into oligomeric structures in which multiple CRDs interact with ligand simultaneously. The overall structures of galectins are shown schematically in Fig. 9.2. Galectin-1 and -2 are homodimers composed of subunits of about 130 amino acids. Each subunit folds as one compact globular domain as shown in Fig. 9.3. Galectin-3 and -4 include one or two such domains, as well as others. The shared domain has been referred to as carbohydrate-binding domain. The sequence of each carbohydrate-binding domain has been shown to be mainly encoded by 3 exons (Barondes et al. 1994). Most of the residues that are conserved among galectins are found in the sequence encoded by one of these threee axons. This sequence includes four contiguous β-strands and intervening loops in the structure of galectin-l (Liao et al. 1994) and galectin-2- (Lobsanov et al. 1993) and contains all residues that interact directly with a carbohydrate ligand (Barondes et al. 1994).

The structures of proto-type galectins have been resolved revealing a common jellyroll topology, typical of legume lectins (Liao et al. 1994; Vasta et al. 2004b) (Composed of two distinct CRD in a single polypeptide chain). In galectin dimer, each subunit is composed of an 11-strand antiparallel ß-sandwich that contains a CRD, with the N- and C-termini located at the dimer interface. The structure of the bovine (*Bos taurus*) gal1-LacNAc (N-acetyllactosamine) complex revealed that the amino acids H^{44}, N^{46}, R^{48}, N^{61}, E^{71}, and R^{73} are directly involved in hydrogen bonding with 4 and 6 hydroxyls of galactose unit, and 3 hydroxyl of N-acetylglucosamine group of LacNAc. W^{69} provides a strong hydrophobic interaction with galactose ring of LacNAc (Liao et al. 1994). Thus, galectin-ligand complex has provided a detailed description of lectin-carbohydrate-interactions. The importance of some of these residues for carbohydrate binding activity is also supported by site-directed mutagenesis. Deletion of sequences

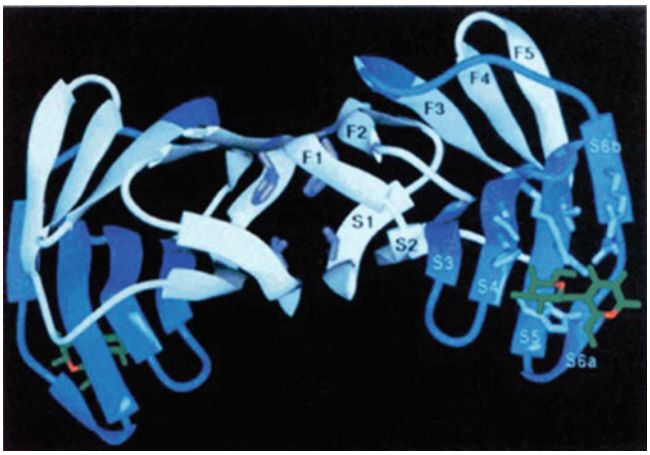

Fig. 9.3 X-ray crystal structure of human galectin-2. The figure shows a dimer of two globular carbohydrate-bindin domains (*blue* and *white ribbon* diagram) with bound lactose (*green stick representation* with *red* ring oxygens). The part of each domain (subunit) encoded by exon III is shown in *blue* and the other parts in *white*. The dimer interface is in the middle and is highlighted by *purple coloring* of the major contributing residues (Val and Ile). Each subunit consists of a sandwich of two β-sheets of 6 and 5 strands. In the subunit to the right the strands of each sheet are labeled Sl-5, S6a, and S6b (strand 6 is interrupted by a β-bulge), and F1-5. The amino acid side chains interacting with the carbohydrate are displayed as *light blue stick* figures. In the right subunit the carbohydrate-binding site is facing toward the viewer, whereas in the left subunit the carbohydrate-binding site is facing away from the viewer (Reprinted by permission from Lobsanov et al. (1993) © American Society for Biochemistry and Molecular Biology)

encoded by the other two exons that encode the carbohydrate-binding domain also impairs activity (Abbott and Feizi 1991). The amino acid identity in the carbohydrate-binding domains among different known galectins from one mammalian species ranges from about 20% to 40%.

9.4.5.1 Binding Sites in Galactose-Specific Proteins

With the assumption that common recognition principles must exist for common substrate recognition, a study was undertaken to identify and characterize any unique galactose-binding site by analyzing the three-dimensional structures of 18 protein-galactose complexes. These proteins belong to seven nonhomologous families; thus, there is no sequence or structural similarity across the families. Within each family, the binding site residues and their relative distances were well conserved, but there were no similarities across families. Sujatha and Balaji (2004) furnished a potential galactose-binding site signature, evaluated by incorporation into the program COTRAN to search for potential galactose-binding sites in proteins that share the same fold as the known galactose-binding proteins. The deduced galactose-binding site signature is strongly validated and can be used to search for galactose-binding sites in proteins. PROSITE-type signature sequences have also been inferred for galectin and C-type animal lectin-like fold families of Gal-binding proteins (Sujatha and Balaji 2004).

9.4.5.2 Role of Aromatic Residue in Galactose-Binding Sites

The presence of an aromatic residue (Trp, Phe, Tyr) facing the nonpolar face of galactose is a common feature of galactose-specific lectins. The interactions such as those between the C-H groups of galactose and the pi-electron cloud of aromatic residues were characterized as weak hydrogen bonds between soft acids and soft bases, largely governed by dispersive and charge transfer interactions. An analysis of the binding sites of several galactose-specific lectins revealed that the spatial position-orientation of galactose relative to the binding site aromatic residue varies substantially. The strength of the C-H. . .pi interactions in galactose-aromatic residue complexes was comparable to that of a hydrogen bond. The study showed that the aromatic residue is important for discriminating galactose from glucose, in addition to its contribution to binding energy (Sujatha et al. 2005).

9.4.5.3 Galectins Bind Multivalent Glycoprotein Asialofetuin

Dam et al. (2002) showed that the binding of multivalent carbohydrates to the Man/Glc-specific lectins ConA and Dioclea grandiflora lectin (DGL) involve negative binding cooperativity that was due to the carbohydrate ligands and not the proteins. The negative cooperativity was associated with the decreasing functional valence of carbohydrates upon progressive binding of their epitopes. Negative cooperativity was also shown in binding of asialofetuin (ASF), a glycoprotein that possesses nine LacNAc epitopes, to galectin-1, -2, -3, -4, -5, and -7, and truncated, monomer versions of galectin-3 and -5. Study indicated that the galectins bind with fractional, high affinities to multivalent glycoproteins such as ASF, independent of the quaternary structures of the galectins. The report has important implications for the binding of galectins to multivalent carbohydrate receptors (Dam et al. 2005).

9.4.6 Galectin Subtypes in Tissue Distribution

9.4.6.1 Galectin Subtypes in Mouse Digestive Tract

Mucosal epithelium showed region/cell-specific localization of each galectin subtype. Gastric mucous cells exhibited intense immunoreactions for galectin-2 and galectin-4/6 with a limited localization of galectin-3 at the surface of the

gastric mucosa. Epithelial cells in the small intestine showed characteristic localizations of galectin-2 and galectin-4/6 in the cytoplasm of goblet cells and the baso-lateral membrane of enterocytes in association with maturation, respectively. Galectin-3 expressed only at the villus tips was concentrated at the myosin-rich terminal web of fully matured enterocytes. Epithelial cells of large intestine contained intense reactions for galectin-3 and galectin-4/6 but not for galectin-2. The stratified squamous epithelium of the forestomach was immunoreactive for galectin-3 and galectin-7. Outside the epithelium, Only galectin-1 was localized in the connective tissue, smooth muscles, and neuronal cell bodies. The subtype-specific localization of galectin suggests its important roles in host-pathogen interaction and epithelial homeostasis such as membrane polarization and trafficking in gut (Nio-Kobayashi et al. 2009).

9.4.6.2 Multiple Galectins in Primary Olfactory Pathway

Primary olfactory neurons project axons from the olfactory neuroepithelium lining the nasal cavity to the olfactory bulb in the brain. These axons grow within large mixed bundles in the olfactory nerve and then sort out into homotypic fascicles in the nerve fiber layer of the olfactory bulb before terminating in topographically fixed glomeruli. The expression of the lactoseries binding galectins in the primary olfactory system and their interactions with carbohydrates on the cell surface have been implicated in axon sorting within the nerve fiber layer. In particular, galectin-3 is expressed by ensheathing cells surrounding nerve fascicles in the submucosa and nerve fiber layer, where it may mediate cross-linking of axons. Galectin-4, -7, and -8 are expressed by the primary olfactory axons as they grow from the nasal cavity to the olfactory bulb. A putative role for NOC-7 and NOC-8 in axon fasciculation and the expression of multiple galectins in the developing olfactory nerve suggest that these molecules may be involved in the formation of this pathway, particularly in the sorting of axons as they converge towards their target (Storan et al. 2004).

9.5 Prototype Galectins (Mono-CRD Type)

Galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15 belong to the mono-CRD type and are found in many cells and tissues, have molecular weights of 14–18 kDa with a single galactoside-binding domain/CRD with a short N-terminal sequence. But the tandem-repeat type galectins (galectin-4, -6, -8, -9, and -12) are composed of two non-identical galactoside-binding domain/CRDs joined by a short linker peptide sequence

9.5.1 Galectin-1

Galectin-1 is differentially expressed by various normal and pathological tissues and appears to be functionally polyvalent, with a wide range of biological activity. The intracellular and extracellular activity of Gal-1 has been described (Chap. 10). Evidence points to Gal-1 and its ligands as one of the master regulators of such immune responses as T-cell homeostasis and survival, T-cell immune disorders, inflammation and allergies as well as host-pathogen interactions. Gal-1 expression or over-expression in tumors and/or the tissue surrounding them must be considered as a sign of the malignant tumor progression that is often related to the long-range dissemination of tumor cells (metastasis), to their dissemination into the surrounding normal tissue, and to tumor immune-escape. Gal-1 in its oxidized form plays a number of important roles in the regeneration of the central nervous system after injury. The targeted over-expression (or delivery) of Gal-1 should be considered as a method of choice for the treatment of some kinds of inflammation-related diseases, neurodegenerative pathologies and muscular dystrophies. A homodimeric (prototype) galectin-1 effectively suppressed symptoms of autoimmune disease in two models at cellular level delineating this lectin as inhibitory (apoptotic) effector for activated T cells (Perillo et al. 1995) (see Chap. 13). Gal-1 has solidly proven its potency to keep inflammatory and autoimmune responses in check in various systems (Gabius 2001; Rabinovich et al. 2002b). Its selection for distinct glycoprotein ligands, including CD3, CD7, CD43, and CD45, and its cross-linking ability appear to be crucial for induction of T cell apoptosis (Nguyen et al. 2001; Rabinovich et al. 2002a).

9.5.2 Galectin-2

Galectin-2, encoded by *LGALS2* gene, is a soluble β-galactoside binding protein. It is found as a homodimer and can bind to lymphotoxin-α. Galectin-2 is structurally related to galectin-1, but has a distinct expression profile primarily confined to gastrointestinal tract (Gitt and Barondes 1991; Gitt et al. 1992; Oka et al. 1999). In fact, Gal-2 and -7 are prototype proteins that are expressed by human tumor cells with cell type specificity (Kopitz et al. 2003). Structurally, Gal-2shares 43% amino acid sequence identity with Gal-1 (Sturm et al. 2004). Gal-2 can bind to T cells and trigger

apoptosis. Prominent differences in the proximal promoter regions between galectins-2 and -1 concern Sp1-, hepatocyte NF-3, and T cell-specific factor-1 binding sites. These sequence elements are positioned equally in respective regions for human and rat galectins-2. Galectin-2 binds to T cells in a β-galactoside-specific manner. In contrast to galectin-1, glycoproteins CD3 and CD7 are not ligands, while β1 integrin (or a closely associated glycoprotein) accounts for a substantial extent of cell surface binding. In general, positivity of galectin-2 was predominantly epithelial without restriction of staining to certain tissue types. Staining was not limited to the cytoplasm but also included nuclear sites (Saal et al. 2005). A single nucleotide polymorphism in an intron of this gene can alter the transcriptional level of the protein, with a resultant increased risk of myocardial infarction (Ozaki and Tanaka 2005; Tanaka and Ozaki 2006).

The brush border of pig small intestine is a local hotspot for galectins. Galectins 3–4, intelectin, and lectin-like anti-glycosyl antibodies have been localized at this boundary. Galectins offer a maximal protection of the brush border against exposure to bile, pancreatic enzymes and pathogens (Thomsen et al. 2009). A lactose-sensitive 14-kDa galectin-2 is enriched in microvillar detergent resistant fraction. Its release from closed, right-side-out microvillar membrane vesicles shows that at least some of the galectin-2 resides at the lumenal surface of brush border, indicating that it plays a role in the organization/stabilization of lipid raft domains.

9.5.2.1 Galectin-2: Inducer of T Cell Apoptosis

The carbohydrate-dependent binding of galectin-2 induces apoptosis in activated T cells. Enhanced cytochrome C release, disruption of the mitochondrial membrane potential, and an increase of the Bax/Bcl-2 ratio by opposite regulation of expression of both proteins add to the evidence that the intrinsic apoptotic pathway is triggered. Notably, galectins-1 and -7 reduce cyclin B1 expression, defining functional differences between the structurally closely related galectins. Cytokine secretion of activated T cells was significantly shifted to the Th2 profile. Thus, galectin-2 acts as proapoptotic effector for activated T cells, suggesting a therapeutic perspective. However, the effects on regulators of cell cycle progression are markedly different between structurally closely related galectins. A single nucleotide polymorphism in an intron of this gene can alter the transcriptional level of the protein, with a resultant increased risk of myocardial infarction (Sturm et al. 2004).

Galectin-2 Induces Apoptosis of Lamina Propria T Lymphocytes: Paclik et al. (2008a) studied the therapeutic effect of galectin-2 in experimental colitis. Galectin-2-constitutively expresses mainly in epithelial compartment of mouse intestine and binds to lamina propria mononuclear cells. During colitis, galectin-2 expression was reduced, but could be restored to normal levels by immunosuppressive treatment. Galectin-2 treatment induced apoptosis of mucosal T cells and thus ameliorated acute and chronic dextran-sodium-sulfate-induced colitis and in a T-helper-cell driven model of antigen-specific transfer colitis. Furthermore, the pro-inflammatory cytokine release was inhibited by galectin-2 treatment. This study provides evidence that galectin-2 induces apoptosis in vivo and ameliorates acute and chronic murine colitis. Furthermore, galectin-2 had no significant toxicity over a broad dose range, suggesting that it may serve as a new therapeutic agent in the treatment of inflammatory bowel disease.

Gal-2 Suppresses Contact Allergy by Inducing Apoptosis in Activated $CD8^+$ T Cells: Galectin-2 regulates cell-mediated inflammatory bowel disease and colitis in mice. Loser et al. (2009) sensitized groups of naive mice to the contact allergen 2,4-dinitro-1-fluorobenzene and systemically treated with galectin-2 to analyze the effects of galectin-2 on contact allergy. Galectin-2 is expressed in murine skin and is up-regulated upon cutaneous inflammation. Interestingly, treatment of mice with galectin-2 significantly reduced the contact allergy response. This effect was long-lasting since rechallenge of galectin-2-treated mice after a 14-day interval still resulted in a decreased ear swelling. Further, galectin-2 induced a reduction of MHC class I-restricted immune responses in the treated animals, which was mediated by the induction of apoptosis specifically in activated $CD8^+$ T cells. Additionally, the galectin-2-binding protein CD29 is up-regulated on the surface of activated $CD8^+$ T cells compared with naive $CD8^+$ T cells or $CD4^+$ T cells, suggesting that increased galectin-2/CD29 signaling might be responsible for proapoptotic effects of galectin-2 on activated $CD8^+$ T cells. Data indicate that galectin-2 may represent a novel therapeutic alternative for the treatment of CD8-mediated inflammatory disorders such as contact allergy (Loser et al. 2009).

9.5.2.2 SNP in *LGALS2* and Lymphotoxin-α as Risk Factor in Cardiovascular Disease

The genotypes for 296 polymorphisms of 202 candidate genes revealed that *LGALS2* is one of the susceptibility loci for atherothrombotic cerebral infarction among Japanese individuals with metabolic syndrome. Genotypes for these polymorphisms may prove informative for the prediction of genetic risk for atherothrombotic cerebral infarction among such individuals (Yamada et al. 2008). Genetic and clinical studies implicate Lymphotoxin-α (LTA), and its binding and regulatory partner galectin-2 as risk factors in the pathogenesis of cardiovascular diseases including myocardial infarction (MI), aortic aneurysm, and cerebral infarction. The LTA gene variability is also

associated with an increased level of C-reactive protein, an inflammatory marker (Naoum et al. 2006). The LTA has multiple functions in regulating the immune system and may contribute to inflammatory processes leading to CHD (coronary heart disease) with susceptibility to MI. (Ozaki et al. 2004). After genotyping approximately 65,000 SNPs in 1,000 patients, Tanaka and Ozaki (2006) found two SNPs located within LTA gene showing significant association with MI. These SNPs seemed to be involved in inflammation by both qualitatively and quantitatively modifying the function of LTA protein, thereby conferring a risk of MI. Ozaki and Tanaka (2005) identified functional SNPs within the LTA located on chromosome 6p21 that conferred susceptibility to myocardial infarction (Tanaka and Ozaki 2006; Topol et al. 2006; Ozaki and Tanaka 2005). The *LGALS2* gene variant is significantly associated with a decreased risk of CHD in women. In addition, the *LGALS2* polymorphism was directly associated with CRP (C-reactive protein) levels in two studies. The LTA gene polymorphisms were directly associated with levels of sTNFRs (soluble tumor necrosis factor receptors) and VCAM-1 in both women and men with CHD (Asselbergs et al. 2007).

The 252GG homozygote variant of LTA is considered as a susceptibility factor for arteriosclerosis and cardiovascular diseases. By contrast, galectin-2-encoding gene *LGALS2* 3279TT homozygote variant has been shown to exert protection against MI by reducing the transcriptional level of galectin-2, thereby leading to a reduced extracellular secretion of LTA. The combination of *LGALS2* 3279TT and LTA 252GG homozygote was significantly less frequent in ischemic stroke group (1.56%) than in controls. This finding suggests a gene-gene interaction (Szolnoki et al. 2009). However, Freilinger et al. (2009) reported that genetic variation in lymphotoxin-α cascade (LTA, LGALS2, and PSMA6) is not a major risk factor. The gene encoding LTA is associated with insulin resistance. Using the *LGALS2* genotype, which affects LTA secretion but is located on another chromosome than the HLA gene cluster or TNF, Christensen et al. (2006) examined the relationship between the LTA pathway and traits of the metabolic syndrome in the British Women for the physically unlinked *LGALS2*. Studies invite attention for further study of *LGALS2* and the LTA pathway for their influence on glucose-insulin regulation (Christensen et al. 2006).

For populations of other genetic background, the relevance of these polymorphisms in the pathogenesis of MI remains controversial. A case–control study on British MI patients and controls showed 98% of power to detect a significant association at OR of 1.57, and 80% power to detect an association with an OR of 1.35 (recessive model). Despite this, there was no significant association of allele frequency with risk of MI. Stratification for age, gender and other cardiovascular risk factors also failed to reveal an association of this polymorphism with MI (Mangino et al. 2007). Kimura et al. (2007) studied SNP of LTA (LTA 252A > G in LTA intron 1) and that of *LGALS2* (*LGALS2* 3279C>T in *LGALS2* intron 1) in Japanese and Korean populations. Although significant associations with MI were not observed in either population, LTA 252GG was significantly associated with severity of the disease for both the Japanese and Korean populations. On the other hand, the polymorphism of *LGALS2* was not associated with the severity of coronary atherosclerosis. These observations showed that, while the LTA 252GG genotype might modify the development of coronary atherosclerosis, the relation of LTA and *LGALS2* to MI itself remained much less certain (Kimura et al. 2007). Moreover, no association could be found in two MI populations of European descent for any of the examined SNPs in the LTA genomic region and *LGALS2* gene. These variants are unlikely to play a significant role in populations of European origin (Sedlacek et al. 2007). Although hypertension, a very prevalent entity in rheumatoid arthritis (RA), is one of the greatest risk factors for MI, the possible association of *LGALS2* 3279C/T and hypertension was genotyped by Panoulas et al. (2009) among 386 RA patients, 272 hypertensives and 114 normotensives. Diastolic blood pressure was significantly lower in TT compared to CC homozygotes even when adjusted for multiple confounders (Panoulas et al. 2009). SNP of genes for LTA and galectin-2 have been implicated as genetic risk factors for RA compared with non-RA controls, thus explaining some of the increased CVD in rheumatoid arthritis, and may be more frequent among patients with RA with prevalent CVD compared with patients with RA without CVD. The LT-A 252GG genotype occurs more frequently among patients with RA than the general population. In RA, this genotype appears to associate with increased likelihood of suffering an myocardial infarction (Panoulas et al. 2008).

BRAP (BRCA1-associated protein) is another galectin-2-binding protein. Association of SNPs in BRAP is risk factor with MI as found in Japanese cohort and in additional Japanese and Taiwanese cohorts. BRAP expression was observed in smooth muscle cells (SMCs) and macrophages in human atherosclerotic lesions. BRAP knockdown by siRNA using cultured coronary endothelial cells suppressed activation of NF-kB, a central mediator of inflammation (Ozaki et al. 2009).

In addition to *LGALS2*, polymorphic sites have been identified within 11-kb region containing the gene encoding galectin-1 (*LGALS1*). The map includes 14 SNPs and two genetic variations of other types detected in a Japanese population sample. Five of the 14 SNPs were not among those deposited in the dbSNP database in NCBI and appeared to be novel. Investigation of haplotype structure within the *LGALS1* locus revealed five common haplotypes covering more than 95% of the test population. One, or a pair, of the SNPs described in this study might serve as a

"tag" for detecting associations between complex diseases and genes in this local segment of chromosome 22q13.1 (Iida et al. 2005).

9.5.3 Galectin-5

The monomeric galectin-5 from extracts of rat lung has been localized to erythrocytes. The deduced amino acid sequence of the cDNA predicts a protein with a M_r of 16,199, with no evidence of a signal peptide. The deduced sequence is identical to the sequences of seven proteolytic peptides derived from purified lectin. Peptide analysis indicated that the N-terminal methionine is cleaved and that serine 2 was acetylated. Galectin-5 is a weak agglutinin of rat erythrocytes, despite its monomeric structure. The gene encoding galectin-5 (*LGALS5*) has been mapped in mouse to chromosome 11, approximately 50 centimorgans from the centromere and 1.8 ± 1.8 centimorgans from the polymorphic marker D11Mit34n, a region syntenic with human chromosome 17q11 (Gitt et al. 1995).

Galectin-5, although mainly cytosolic, is also present on the cell surface of rat reticulocytes and erythrocytes. In reticulocytes, it resides in endosomal compartment. Galectin-5 translocates from cytosol into the endosome lumen, leading to its secretion in association with exosomes. Galectin-5 bound onto the vesicle surface may function in sorting galactose-bearing glycoconjugates. Data imply galectin-5 functionality in the exosomal sorting pathway during rat reticulocyte maturation (Barres et al. 2010). Galectin 1 and galectin 3 are first expressed in the trophectoderm cells of the implanting embryo and have been implicated in the process of implantation. However, presence of galectin 5 in the blastocyst at the time of implantation could be important in the process (Colnot et al. 1998).

9.5.3.1 Interaction with Saccharides and Glycoproteins

Interaction of cell-surface glycans with galectins triggers a wide variety of responses. Among 45 natural glycans tested for binding, galectin-5 reacted best with glycoproteins (gps) presenting a high density of Galβ1-3/4GlcNAc (I/II) and multiantennary N-glycans with II termini. Wu et al. (2006) suggested that (1) Galβ1-3/4GlcNAc and other Galβ1-related oligosaccharides with α1-3 extensions are essential for binding, their polyvalent form in cellular glycoconjugates being a key recognition force for galectin-5; (2) the combining site of galectin-5 appears to be of a shallow-groove type sufficiently large to accommodate a substituted β-galactoside, especially with α-anomeric extension at the non-reducing end (e.g., human blood group B-active II and B-active IIβ1-3 L); (3) the preference within β-anomeric positioning is Galβ1-4 $\geq$ Galβ1-3 $>$ Galβ1-6; and (4) hydrophobic interactions in the vicinity of core galactose unit can enhance binding. Systematic comparison of ligand selection in this family of adhesion/growth-regulatory effectors offers potential for medical applications (Wu et al. 2006). Lensch et al. (2006) studied galectins-5 and -9. After ascertaining species specificity of occurrence of galectin-5, constituted by a short section of rat galectin-9′s N-terminal part and its C-terminal carbohydrate recognition domain, the results on galectin-5 relative to galectin-9 intimated distinct functions especially in erythropoiesis and implied currently unknown mechanisms to compensate its absence from the galectin network in other mammals.

9.5.4 Galectin-7

Galectin-7 is a β-galactoside-binding animal lectin specifically expressed in stratified epithelia. It is expressed in all surface epithelium, glandular epithelium, and connective tissue in human nasal polyps. It contributes to different events associated with the differentiation and development of pluristratified epithelia. It is also associated with epithelial cell migration, which plays a crucial role in the re-epithelialization process of corneal or epidermal wounds. Galectin-7 is a proapoptotic protein, and the ectopic expression of galectin-7 in HeLa cells renders the cells more sensitive to a variety of apoptotic stimuli. In nasal polyposis model for the study of inflammatory processes, Galectin-7 expression coincides with the degree of epithelial stratification, and is subject to upregulation in connective tissue in response to treatment with budesonide. Budesonide modulates galectin-7 expression differently in surface epithelia of polyps from allergic and non-allergic patients (Delbrouck et al. 2005). Expression of galectin-7 is inducible by p53 and is down-regulated in squamous cell carcinomas. Galectin-7 has a suppressive effect on tumor growth, suggesting that galectin-7 gene transfer or other means of specifically inducing galectin-7 expression may be a new approach for management of cancers (Ueda et al. 2004). Studies are consistent with a role for galectin-7 in the regulation of cell growth through a pro-apoptotic effect.

Galectin-7 corresponds to IEF (isoelectric focusing) 17 (12,700 Da; pI, 7.6) in the human keratinocyte protein data base, and is strikingly down-regulated in SV40 transformed keratinocytes (K14). The protein encoded by the galectin-7 clone co-migrated with IEF 17 in 2D gel electrophoresis and bound lactose. The galectin-7 gene was mapped to chromosome 19 (Madsen et al. 1995). The 14-kDa lectin of pI7 predicted by 136-amino-acid ORF is specifically expressed

in keratinocytes. It is expressed at all stages of epidermal differentiation. It is moderately repressed by retinoic acid, a behavior contrasting with those of other keratinocyte markers sensitive to this agent, which, either basal, are induced, or suprabasal, are repressed (Magnaldo et al. 1995). In human, rat and mouse epithelia, Galectin-7 was found to be expressed in interfollicular epidermis and in the outer root sheath of the hair follicle, but not in the hair matrix, nor in the sebaceous glands. It was present in esophagus and oral epithelia, cornea, Hassal's corpuscles of the thymus, but not in simple and transitional epithelia. Galectin-7 can thus be considered as a marker of all subtypes of keratinocytes (Magnaldo et al. 1998). Significant amounts of galectin-7 was detected in trachea and ovaries and localized in pseudostratified epithelium of the trachea and stromal epithelium of ovaries. It suggests that galectin-7 protein might be produced primarily in stratified epithelia, but also in some wet epithelia, and plays a unique role in cell-mucus contact, or the growth of ovarian follicles (Sato et al. 2002). Galectin-7 has been cloned from human, rat and mouse. In the adult, galectin-7 is expressed in all cell layers of epidermis and of other stratified epithelia such as the cornea and the lining of the oesophagus. Galectin-7 mRNA was identified in mouse embryos starting from E13.5, in bilayered ectoderm, and stronger expression was found in areas of embryonic epidermis where stratification was more advanced. In contrast, no expression of galectin-7 was found in epithelia derived from endoderm, such as lining of the intestine, kidney and lung. Thus, galectin-7 is expressed in all stratified epithelia examined so far, and that the onset of its expression coincides with the first visible signs of stratification (Timmons et al. 1999).

Galectin-7 is also associated with epithelial cell migration, which plays a crucial role in the re-epithelialization process of corneal or epidermal wounds. In addition, recent evidence indicates that galectin-7, a product of p53-induced gene 1 (designated as PIG1), is a regulator of apoptosis through JNK activation and mitochondrial cytochrome c release. The increase of galectin-7 is parallel to P53 stabilization. Defects in apoptosis constitute one of the major hallmarks of human cancers, and galectin-7 can act as either a positive or a negative regulatory factor in tumor development, depending on the histological type of the tumor (Saussez and Kiss 2006). UVB irradiation of skin is associated with sunburn/apoptotic keratinocytes. The galectin-7 mRNA and protein are increased rapidly after UVB irradiation of epidermal keratinocytes and involved in UV-induced apoptosis (Bernerd et al. 1999). Galectin-7 acts on a common point in apoptosis signaling pathways. It is a pro-apoptotic protein that functions intracellularly upstream of JNK activation and cytochrome C release (Kuwabara et al. 2002). Galectin-7 was found to be highly inducible by tumor suppressor protein p53 in a colon carcinoma cell line, DLD-1 and its gene is an early transcriptional target of p53 (Polyak et al. 1997).

Galectin-7 has a potential to reduce cell proliferation after carbohydrate-dependent surface binding in neuroblastoma cells (Kopitz et al. 2003). Suppressive effect of galectin-7 on tumor growth suggests that galectin-7 gene transfer or other means of specifically inducing galectin-7 expression may be a new approach for management of cancers (Ueda et al. 2004). Galectin-3 and galectin-7 are potential tumor markers for differentiating thyroid carcinoma from its benign counter part (see Chap. 13). They are supposed to be p53-related regulators in cell growth and apoptosis, being either anti-apoptotic or pro-apoptotic. The immunochemical localisation of Galectin-3 is a useful marker in conjunction with routine haematoylin and eosin staining in differentiating benign from malignant thyroid lesions, while there is no significant adjunct diagnostic value in galectin-7 for thyroid malignancy (Than et al. 2008b).

Leonidas et al. (1998) reported the crystal structure of human galectin-7 in free form and in presence of galactose, galactosamine, lactose, and N-acetyl-lactosamine at high resolution. The galectin-7 structure shows a fold similar to that of prototype galectins -1 and -2, but has greater similarity to galectin-10. Even though the carbohydrate-binding residues are conserved, there are significant changes in this pocket due to shortening of a loop structure. The monomeric human galectin-7 exists as a dimer in the crystals, but adopts a packing arrangement considerably different from that of Gal-1 and Gal-2, which has implications for carbohydrate recognition. N-acetyllactosamine thioureas are good inhibitors of galectin-7 and 9 N (K_D = 23 and 47 μM respectively for 3-pyridylmethyl-thiourea derivative) and represented more than an order of magnitude affinity enhancement over parent natural N-acetyllactosamine (Salameh et al. 2006).

9.5.5 Galectin-10 (Eosinophil Charcot-Leyden Crystal Protein)

The Charcot-Leyden crystal (CLC) protein is a major autocrystallizing constituent of human eosinophils and basophils, comprising ~10% of total cellular protein in these granulocytes. Identification of the distinctive hexagonal bipyramidal crystals of CLC protein in body fluids and secretions has long been considered a hallmark of eosinophil-associated allergic inflam-mation. Although CLC protein possesses lysophospholipase activity, its role(s) in eosinophil or basophil function or associated inflammatory responses has remained speculative. Across mammalian species, human galectin/CLC and ovine galectin-14 are unique in their expression in eosinophils and their release into lung and gastrointestinal tissues following allergen or parasite challenge. The X-ray crystal structure of the CLC protein is very similar to the structure of the galectins, including a partially conserved galectin CRD. Structural

studies on the carbohydrate binding properties of CLC protein demonstrate no affinity for β-galactosides but binds mannose in a manner different from those of other related galectins that have been shown to bind lactosamine. The partial conservation of residues involved in carbohydrate binding led to significant changes in the topology and chemical nature of the CRD. Carbohydrate recognition by the CLC protein in vivo suggests its functional role in the process of inflammation (Swaminathan et al. 1999). Galectin-10 is a novel marker for evaluating celiac disease tissue damage and eosinophils as a possible target for therapeutic approaches (De Re et al. 2009). In sensitive patients, aspirin is associated with nasal and bronchial inflammation, eliciting local symptoms. Galectin-10 mRNA is overexpressed in aspirin-induced asthma, suggesting a novel candidate gene and a potentially innovative pathway for mucosal inflammation in aspirin intolerance (Devouassoux et al. 2008).

$CD4^+CD25^+Foxp3^+$ regulatory T cells ($CD25^+$ T_{reg} cells) direct maintenance of immunological self-tolerance by active suppression of autoaggressive T-cell populations. Galectin-10 is constitutively expressed in human $CD25^+$ T_{reg} cells, while they are nearly absent in resting and activated $CD4^+$ T cells. As expressed in human T lymphocytes, galectin-10 is essential for the functional properties of $CD25^+$ T_{reg} cells (Kubach et al. 2007). The CLC protein possesses weak lysophospholipase activity of eosinophil, but it shows no sequence similarities to any known lysophospholipases. In contrast, CLC protein has moderate sequence similarity, conserved genomic organization, and near structural identity to members of the galectin superfamily, and designated galectin-10. Ackerman et al. (2002) reassessed its enzymatic activity in peripheral blood eosinophils and an eosinophil myelocyte cell line (AML14.3D10). The affinity-purified CLC protein lacked significant lysophospholipase activity. X-ray crystallographic structures of CLC protein in complex with the inhibitors showed that p-chloromercuribenzenesulfonate bound CLC protein via disulfide bonds with Cys-29 and with Cys-57 near CRD, whereas N-ethylmaleimide bound to the galectin-10 CRD via ring stacking interactions with Trp-72, in a manner highly analogous to mannose binding to this CRD. Results clearly showed that CLC protein is not one of the eosinophil's lysophospholipases but it does interact with inhibitors of this lipolytic activity (Ackerman et al. 2002).

Transcriptional Regulation

Analysis of the minimal promoter revealed nine consensus-binding sites for transcription factors, including several that are also found in the minimal promoters of galectins -1, -2, and -3. The decrease in gal-10 promoter activity after disruption of either the GC box (−44 to −50) or the Oct site (−255 to −261) suggests that these sites, along with GATA and EoTF sites, are necessary for full promoter activity. Transcription factors Sp1 and Oct1 bind to the consensus GC box and the Oct site, respectively. Similar to gal-1, gal-10 expression is induced by butyric acid, an effect that is lost upon ablation of GC box. Additionally, AML3 binds to consensus AML site and YY1 binding to the Inr sequence, both elements functioning as silencers in the gal-10 promoter (Dyer and Rosenberg 2001).

Crystal Structure

The crystal structure of the CLC protein at 1.8 A resolution shows overall structural similarity to that of galectins -1 and -2. The CLC protein possesses a CRD comprising most, but not all, of the carbohydrate-binding residues that are conserved among the galectins. The protein exhibits specific (albeit weak) carbohydrate-binding activity for simple saccharides including N-acetyl-D-glucosamine and lactose. Despite CLC protein having no significant sequence or structural similarities to other lysophospholipase catalytic triad has also been identified within the CLC structure, making it a unique dual-function polypeptide. These structural findings suggest a potential intracellular and/or extracellular role(s) for the galectin-associated activities of CLC protein in eosinophil and basophil function in allergic diseases and inflammation (Leonidas et al. 1995).

9.5.6 Galectin-Related Inter-Fiber Protein (Grifin/Galectin-11)

The ability of the vertebrate lens to focus light on retina derives from a number of properties including the expression at high levels of a selection of soluble proteins referred to as the crystallins. Soluble lens protein, though related to the family of galectins, does not bind beta-galactoside sugars and has atypical sequences at normally conserved regions of the carbohydrate-binding domain. Like some galectin family members, it can form a stable dimer. It is expressed only in the lens and is located at the interface between lens fiber cells despite the apparent lack of any membrane-targeting motifs. This protein is designated GRIFIN (galectin-related inter-fiber protein) to reflect its exclusion from the galectin family given the lack of affinity for beta-galactosides. Although the abundance, solubility, and lens-specific expression of GRIFIN would argue that it represents a new crystallin, its location at the fiber cell interface might suggest that its primary function is executed at the membrane. Adipose tissue plays an active role in the development of obesity. Among 29 proteins, differentially expressed between lean and diet-induced obese rats, changes in grifin were associated with obesity (Barceló-Batllori et al. 2005).

In the past decade, several galectin-like proteins such as the lens crystallin protein GRIFIN (Galectin related interfiber protein) and the galectin-related protein GRP (previously HSPC159; hematopoietic stem cell precursor) have been identified (Cooper 2002; Zhou et al. 2006). cDNA sequences have been obtained for guinea pig lens GRIFIN (Simpanya et al. 2008). Although GRIFIN and GRP have close similarity to galectin sequences, due to lack of carbohydrate-binding activity they are not considered to be members of galectin family, but putative products of evolutionary co-option. GRIFIN, described in the rat as a novel crystallin, is expressed at the interface between lens fiber cells, and was proposed to have evolved from a galectin precursor that has lost its capacity to bind lactose. Crystallins are water-soluble structural proteins that account for transparency of the vertebrate lens. They constitute a heterogeneous family, composed of four major groups (α–δ) and several minor groups, with roles as both molecular chaperones and structural proteins (Piatigorsky 1989). The binding interaction between α-crystallin and GRIFIN was enhanced in the presence of ATP. Binding data supported the hypothesis that GRIFIN is a binding partner of α-crystallin in lens (Barton et al. 2009).

The zebrafish (*Danio rerio*) has been established as a useful animal model for studies of early development. Thus, it may constitute the model of choice for gaining further insight into the biological roles of galectins (Vasta et al. 2004a). Ahmed and Vasta (2004c) characterized the zebrafish galectin repertoire as follows: three gal1-like proteins (Drgal1-L1, Drgal1-L2, Drgal1-L3), one chimera type galectin (Drgal3), and two gal9-like proteins (Drgal9-L1 and Drgal9-L2) (Ahmed and Vasta 2004). In silico analysis of the nearly completed genome sequence has enabled the identification of additional members, such as gal4-, gal5-, and gal8-like proteins. Ahmed et al. (2008) identified a homologue of the GRIFIN in zebrafish (Danio rerio) (designated DrGRIFIN), which like the mammalian equivalent is expressed in the lens, particularly in fiber cells of 2 dpf (days post fertilization) embryos. It is weakly expressed in the embryos as early as 21 hpf (hour post fertilization) but strongly at all later stages tested (30 hpf and 3, 4, 6, and 7 dpf). In adult zebrafish tissues, however, DrGRIFIN is also expressed in oocytes, brain, and intestine. Unlike the mammalian homologue, DrGRIFIN contains all amino acids critical for binding to carbohydrate ligands and its activity and confirmed by purification of recombinant DrGRIFIN by affinity chromatography on a lactosyl-Sepharose column. Therefore, DrGRIFIN is a bona fide galectin family member that in addition to its carbohydrate-binding properties, may also function as a crystallin.

9.5.7 Galectin-13 (Placental Protein -13)

Placental protein 13 (PP13) is a galectin expressed by syncytiotrophoblast. PP13 from human term placenta shows structural and functional homology to members of the galectin family. It effectively agglutinates erythrocytes. Similar to human eosinophil Charcot-Leyden crystal protein but not other galectins, galectin-13 possesses weak lysophospholipase activity. N-acetyl-lactosamine, mannose and N-acetyl-glucosamine residues, expressed in human placenta have the strongest binding affinity to galectin-13/PP13. The protein is a homodimer of 16 kDa subunits linked together by disulphide bonds, a process differing from earlier known galectins. The syncytiotrophoblasts show its expression in perinuclear region, while strong labelling of syncytiotrophoblasts' brush border membrane confirmed its externalization activity at the cell surface. PP13/galectin-13 may have special haemostatic and immunobiological functions at the common feto-maternal blood-spaces or developmental role in the placenta (Than et al. 2004). PP-13 has been sequenced and expressed in *E. coli*. The primary structure of PP13 is highly homologous to several members of β-galactoside-binding S-type lectin family. By multiple sequence alignment and structure-based secondary structure prediction, the secondary structure of PP13 was identical with 'proto-type' galectins consisting of a five- and a six-stranded β-sheet, joined by two α-helices. Highly conserved CRD was also present in PP13. Of eight consensus residues in CRD, four identical and three conservatively substituted were shared by PP13. By docking simulations PP13 possessed sugar-binding activity with highest affinity to N-acetyllactosamine and lactose typical of most galectins. All ligands were docked into putative CRD of PP13. Based on several lines of evidence, PP13 was designated as galectin-13 (Visegrády et al. 2001).

PP-13 levels slowly increase during pregnancy. Parallel to its decreased placental expression, an augmented membrane shedding of PP13 contributes to the increased third trimester maternal serum PP13 in women with preterm pre-eclampsia and HELLP syndrome (Nicolaides et al. 2006; Than et al. 2008a). Galectin-13 transcripts have been identified in several normal and malignant tissues. Its possible role in promoting apoptosis has been suggested in U-937 human macrophage cell line. Galectin-13 over-expression facilitated paclitaxel-induced cell death and nuclear translocation of apoptosis-inducing factor (AIF) and endonuclease-G without inducing mitochondrial cytochrome-c release or caspase-3 activation. Galectin-13 promoted apoptosis presumably by activating Ask-1 kinase-JNK and p38-MAPK pro-apoptotic pathways and by suppressing the PI-3 K-Akt and ERK1/2 cytoprotective pathways (Boronkai et al. 2009).

9.5.8 Galectin 14

A full-length cDNA encodes a lectin with subunit M_r 14.6. Like its relative, called L-14-I, the L-14-II exists as a homodimer in solution. The two related human lectins have 43% amino acid sequence identity. The genomic DNA encoding L-14-II (LGALS2) contains four exons with similar intron placement to L-14-I (LGALS1); but the genomic upstream region, which contains several sequences characteristic of regulatory elements, differs significantly from L-14-I (Gitt et al. 1992). The x-ray crystal structure of human dimeric S-Lac lectin, L-14-II, in complex with lactose revealed a twofold symmetry. The twofold symmetric dimer is made up of two extended anti-parallel β-sheets, which associate in a β-sandwich motif. Remarkably, the L-14-II monomer shares not only the same topology, but a very similar β-sheet structure with that of leguminous plant lectins, suggesting a conserved structure-function relationship. Carbohydrate binding by L-14-II was found to involve protein residues that are very highly conserved among all S-Lac lectins. These residues map to a single DNA exon, suggesting a carbohydrate binding cassette common to all S-Lac lectins (Lobsanov et al. 1993).

9.5.8.1 Functional Characterization

A galectin cDNA (galectin-14) from ovine eosinophil-rich leukocytes encodes a prototype galectin that contains one putative CRD and exhibits most identity to galectin-9/ecalectin, a potent eosinophil chemoattractant. The sugar binding properties of recombinant protein were confirmed by a hemagglutination assay and lactose inhibition. The mRNA and protein of galectin-14 are expressed at high levels in eosinophil-rich cell populations. The protein localizes to the cytoplasmic, but not the granular, compartment of eosinophils. Galectin-14 was not detected in neutrophils, macrophages, or lymphocytes. But it is released from eosinophils into the lumen of the lungs after challenge with house dust mite allergen. The restricted expression of this galectin to eosinophils and its release into the lumen of the lung in a sheep asthma model indicates that it may play an important role in eosinophil function and allergic inflammation (Dunphy et al. 2002).

Galectin-14 secretion from peripheral blood eosinophils can be induced by same stimuli that induce eosinophil degranulation. Recombinant galectin-14 can bind in vitro to eosinophils, neutrophils and activated lymphocytes. Glycan array screening indicated that galectin-14 recognizes terminal N-acetyllactosamine residues which can be modified with α1-2-fucosylation and, uniquely for a galectin, prefers α2- over α2-sialylation. Galectin-14 showed greatest affinity for lacto-N-neotetraose, an immunomodulatory oligosaccharide expressed by helminths. Galectin-14 binds specifically to laminin in vitro, and to mucus and mucus producing cells on lung and intestinal tissue sections. In vivo, galectin-14 is abundantly present in mucus scrapings collected from either lungs or gastrointestinal tract following allergen or parasite challenge, respectively. In vivo secretion of eosinophil galectins may be specifically induced at epithelial surfaces after recruitment of eosinophils by allergic stimuli, and that eosinophil galectins may be involved in promoting adhesion and changing mucus properties during parasite infection and allergies (Young et al. 2009).

9.5.8.2 Chicken Galectins

Two avian galectins have been detected in chicken liver (chicken galectin-16 CG-16) and intestine (chicken galectin-14; CG-14) with different developmental regulation. Epithelial cells of the mesonephric proximal tubules of kidney were immunoreactive for CG-14 from day 5 onwards. For CG-16 a rather similar pattern of staining was seen, additional positivity in early glomerular podocytes being notable. Results demonstrate quantitative differences in the developmental regulation of the two avian galectins with obvious similarities in the cell-type pattern but with a separate intracellular localisation profile (Stierstorfer et al. 2000). Kaltner et al. (2008) reported the cloning and expression of a third prototype CG. It has deceptively similar electrophoretic mobility compared with recombinant C-14, the protein first isolated from embryonic skin, and turned out to be identical with the intestinal protein. Hydrodynamic properties unusual for a homodimeric galectin and characteristic traits in the proximal promoter region set it apart from the two already known CGs. Their structural vicinity to galectin-1 prompts their classification as CG-1A (CG-16)/CG-1B (CG-14), whereas sequence similarity to mammalian galectin-2 gives reason to refer to the intestinal protein as CG-2. Overall results reveal a network of three prototype galectins in chicken (Kaltner et al. 2008).

To examine how sequence changes affect carbohydrate specificity, the two closely related proto-type chicken galectins CG-14 and CG-16 were selected as models and tested for binding of 56 free saccharides and 34 well-defined glycoproteins. The two galectins share preference for the II (Galβ1-4GlcNAc) versus I (Galβ1-3GlcNAc) version of β-galactosides. A pronounced difference was found owing to the reactivity of CG-14 with histo-blood group ABH active oligosaccharides and A/B active glycoproteins. This study identifies activity differences and provides information on their relation to structural divergence (Wu et al. 2007).

9.5.9 Galectin-15

Secretions of the uterus support survival and growth of the conceptus (embryo/fetus and associated membranes) during

pregnancy. Galectin-15, also known as OVGAL11 and a member of the galectin family of secreted β-galactoside lectins containing a conserved CRD and a separate putative integrin binding domain, is present in the uterus of sheep. In endometria of cyclic and pregnant sheep, galectin-15 mRNA was expressed specifically in the endometrial luminal epithelium but not in the conceptus. The intracellular role of galectin-15 is to regulate cell survival, differentiation and function, while the extracellular role of secreted galectin-15 is to regulate implantation and placentation by functioning as a heterophilic cell adhesion molecule between the conceptus trophectoderm and endometrial LE (Gray et al. 2005). In the uterine lumen, secreted galectin-15 protein increased between days 14 and 16 of pregnancy. Galectin-15 protein was functional in binding lactose and mannose sugars and immunologically identical to the unnamed Mr 14 kDa protein from the ovine uterus that forms crystalline inclusion bodies in endometrial epithelia and trophectoderm (Tr) conceptus. Based on the functional studies of other galectins, galectin-15 is hypothesized to function extracellularly to regulate Tr migration and adhesion to the endometrial epithelium and intracellularly to regulate Tr cell survival, growth, and differentiation (Gray et al. 2005; Satterfield et al. 2006).

9.6 Evolution of Galectins

During past two decades, substantial progress has been made in elucidation of structural diversity of lectin repertoires of invertebrates, protochordates and ectothermic vertebrates, providing particularly valuable information on those groups that constitute the invertebrate/vertebrate 'boundary'. Although representatives of lectin families typical of mammals, such as C-type lectins, galectins and pentraxins, have been described in these taxa, the detailed study of selected model species has yielded either novel variants of structures described for mammalian lectin representatives or novel lectin families with unique sequence motifs, multidomain arrangements and a new structural fold. Along with high structural diversity of lectin repertoires in these taxa, a wide spectrum of biological roles is starting to emerge, underscoring the value of invertebrate and lower vertebrate models for gaining insight into structural, functional and evolutionary aspects of lectins (Vasta et al. 2004a).

9.6.1 Phylogenetic Analysis of Galectin Family

Galectins are widely distributed from higher vertebrates to lower invertebrates, including mammals, amphibians, fish, birds, nematodes, and sponges, having even been found in the mushroom *Coprinus cinereus*. Although the identification of many galectin relatives in widely divergent organisms (including *Arabidopsis, Drosophila, Caenorhabditis, Danio, Xenopus*, and human) has added significantly to the size and complexity of this intriguing protein family, several common themes arise, which suggest promising new research targets (Cooper 2002). Galectins have been found in the genome of the insects *Drosophila* and *Anopheles*, of viruses and of the plant *Arabadopsis*. To elucidate the evolutionary history of galectin-like proteins in chordates, Houzelstein et al. (2004) exploited three independent lines of evidence: (1) location of galectin encoding genes (*LGALS*) in the human genome; (2) exon-intron organization of galectin encoding genes; and (3) sequence comparison of CRDs of chordate galectins. Results of Houzelstein et al. (2004) suggest that a duplication of a mono-CRD galectin gene gave rise to an original bi-CRD galectin gene, before or early in chordate evolution. The N-terminal and C-terminal CRDs of this original galectin subsequently diverged into two different subtypes, defined by exon-intron structure (F4-CRD and F3-CRD). It was inferred that all vertebrate mono-CRD galectins known belong to either the F3- or F4- subtype. A sequence of duplication and divergence events of the different galectins in chordates was proposed (Houzelstein et al. 2004). Therefore, the galectin gene family must have evolved from the start of multicellular organisms, at least a million of years ago. In shrimp, investigations of the penaeidins, which are constitutively expressed peptides, have highlighted the importance of hemocytes and hematopoiesis as major elements of the immune response, providing both local and systemic reactions. The activation of hematopoiesis must be regarded as a regulatory way for the expression and distribution of constitutively expressed immune effectors (Bachère et al. 2004).

9.6.2 Galectins in Lower Vertebrates

First reptilian galectin was isolated from the skin of the lizard *Podarcis hispanica* (Solis et al. 2000). Up to five lactose-binding proteins were isolated from the lizard *Podarcis hispanica* on asialofetuin-Sepharose. The main component, abundantly expressed in skin, and under native conditions behaved as a monomer with a molecular mass of 14.5 kDa and an isoelectric pH 6.3. Based on sequence homology of the 58 *N*-terminal amino acid residues with galectins, and on its demonstrated galactoside-binding activity, this lectin was named LG-14 (from *Lizard Galectin* and 14 kDa). LG-14 falls into and strengthens the still thinly populated category of monomeric prototype galectins.

The crystal structures of congerin I and -II, the galectins from conger eel, have been determined. The congerin I revealed a fold evolution via strand swap; however, the structure of congerin II resembles other prototype galectins. A comparison of the two congerin genes with that of several

other galectins suggests acceralated evolution of both congerin genes following gene duplication. The presence of a Mes (2-[N-morpholino]ethanesulfonic acid) molecule near the carbohydrate-binding site in the crystal structure points to the possibility of an additional binding site in congerin II. The binding site consists of a group of residues that had been replaced following gene duplication suggesting that the binding site was built under selective pressure. Congerin II may be a protein specialized for biological defense with an affinity for target carbohydrates on parasites' cell surface (Shirai et al. 2002).

The bi-CRD *Branchiostoma belcheri tsingtauense* galectin (BbtGal)-L together and its alternatively spliced mono-CRD isoform BbtGal-S from amphioxus intestine are encoded by a 9488-bp gene with eight exons and seven introns. BbtGal is a member of the galectin family. Phylogenetic analysis suggested that the BbtGal gene was the primitive form of chordate galectin family. BbtGal-L mRNA was mainly expressed in the immunity-related organs, such as hepatic diverticulum, intestine, and gill, but BbtGal-S was ubiquitously expressed in all tissues (Yu et al. 2007).

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Galectin-1: Forms and Functions

10

Anita Gupta

Galectin-1 (Gal-1) is an animal lectin ranging from *Caenorhabditis elegans* to humans, which is defined by the affinity for β-galactosides and by significant sequence similarity in carbohydrate-binding site. Gal-1 is differentially expressed by various normal and pathological tissues and appears to be functionally polyvalent, with a wide range of biological activity. Evidences indicate that Gal-1 and its ligands are important regulators of immune responses such as T-cell homeostasis and survival, inflammation and allergies as well as host–pathogen interactions. Overexpression of Gal-1 in tumors is considered as a sign of the malignant tumor progression that is often related to the long-range dissemination of tumoral cells (metastasis), to their dissemination into the surrounding normal tissue, and to tumor immune-escape. Gal-1 in its oxidized form plays a number of important roles in the regeneration of the central nervous system after injury. The targeted overexpression of Gal-1 should be considered as a method of choice for the treatment of some kinds of inflammation-related diseases, neurodegenerative pathologies and muscular dystrophies. In contrast, the targeted inhibition of Gal-1 expression is what should be developed for therapeutic applications against cancer progression. Gal-1 is thus a promising molecular target for the development of new and original therapeutic tools.

10.1 The Subcellular Distribution

Galectin-1 shows both intracellular and extracellular functions. Hence it shows characteristics of typical cytoplasmic protein as well as an acetylated N-terminus and a lack of glycosylations (Clerch et al. 1988). It is present in cell nuclei and cytosol and also translocates to the intracellular side of cell membranes. Though Gal-1 lacks a secretion signal sequence and does not pass through endoplasmic reticulum/Golgi pathway (Hughes 1999), it is secreted and found on the extracellular side of all cell membranes as well as in the extracellular matrices of various normal and neoplastic tissues (van den Brule et al. 2003; Camby et al. 2006; von Wolff et al. 2005). Evidence suggests that this protein is secreted like FGF-2 via inside-out transportation involving direct translocation across the plasma membrane of mammalian cells (Nickel 2005). The ß-galactoside-binding site may constitute the primary targeting motif for galectin export machinery using ß-galactoside-containing surface molecules as export receptors for intracellular Gal-1 (Seelenmeyer et al. 2005). Nuclear presence of galectins suggests a role of these endogenous lectins in the regulation of transcription, pre-mRNA splicing and transport processes. Stromal cells of human bone marrow and human/porcine keratinocytes revealed positive reaction for galectin-1, whereas galectins-3, -5, and -7 were not reactive with nuclear sites under identical conditions (Purkrábková et al. 2003).

10.2 Molecular Characteristics

10.2.1 Galectin-1 Gene

Gal-1 is encoded by *LSGALS1* gene located on chromosome 22q12. Although it is not yet clear whether the 15 galectins identified so far have functions in common, a striking common feature among all galectins is their strong modulation of expression during development, differentiation and under different physiological or pathological conditions. This suggests that the expression of different galectins is finely tuned and possibly coordinated. Among galectin genes, a small region spanning over initial transcription start site (−63/+45) is sufficient in the promoter region of Gal-1 gene for its transcriptional activity in mice (Chiariotti et al. 2004). Both an upstream and a downstream position-dependent cis-element are necessary for efficient transcriptional activity; an additional start-up site has been mapped at position-31; and an Sp1-binding site (−57/–48) and a consensus initiator (Inr) element (which

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DOI 10.1007/978-3-7091-1065-2_10,

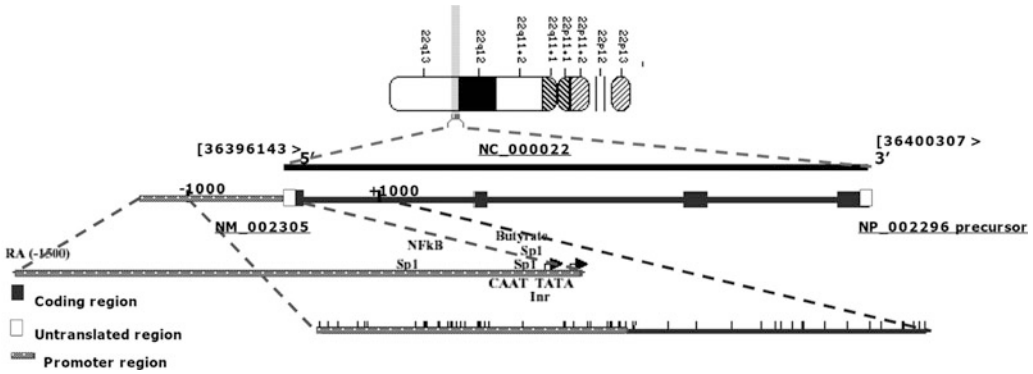

Fig. 10.1 Map of the Gal-1 gene on the human chromosome 22q12. The sequences were retrieved using the MapViewer program and the Entrez genome database on the NCBI website and were analyzed using the GeneWorks program produced by IntelliGenetics Inc. The *curved arrows* indicate the initial transcription start-up sites (Chiariotti et al. 2004). The 0.6 kb transcript results from the splicing of four exons (the *boxes* indicate four coding regions) and encodes for a protein of 135 aminoacids (Reprinted by permission from Camby et al. 2006 © Oxford University Press)

partially overlaps a non-canonical TATA box) direct RNA initiation (Chiariotti et al. 2004). The upstream transcripts contribute to more than half of the Gal-1 mRNA population (Fig. 10.1). The 5′-end of this transcript is extremely GC-rich and may fold into a stable hairpin structure which can influence translation (Chiariotti et al. 2004). The approximate position of the other putative and/or characterized regulatory elements is indicated in Fig. 10.1 and relates to the CAAT box, to NF-kB binding site and to the sodium butyrate and retinoic acid response sequences. Full length cDNAs coding for a 14-kDa was isolated from HL-60 cells and human placenta.

The cDNA clones for lectins had identical sequences with short 5′- and 3′-untranslated regions and coded for 135-amino acid protein which lacks a hydrophobic signal peptide sequence. Biochemical data showed that, despite the presence of a possible N-linked glycosylation site, the protein was not found glycosylated. The 14-kDa lectin is encoded by a single gene. The lectin expressed in *E. coli* was biologically active. Another full-length cDNA for this protein from a HepG2 cDNA library encodes a protein with subunit molecular weight of 14,650. The *E. coli* expressed product bound to a lactose affinity column and specifically eluted with lactose. Like L-14-I, the new lectin, L-14-II, exists as a homodimer in solution. The two related human lectins have 43% amino acid sequence identity. The genomic DNA encoding L-14-II (*LGALS2*) contains four exons with similar intron placement to L-14-I (*LGALS1*); but the genomic upstream region, which contains several sequences characteristic of regulatory elements, differs significantly from L-14-I.(Gitt et al. 1992) Gal-1 occurs as a monomer as well as a non-covalent homodimer consisting of subunits of one CRD (dGal-1, 29.5 kDa) (Barondes et al. 1994; Cho and Cummings 1995). Each form is associated with different biological activities. Gal-1 with a subunit molecular mass of 14.5 kDa contains six cysteine residues per subunit. Non-covalent homodimer of 29.5-kDa is widely expressed in many tissues. The cysteine residues should be in a free state in order to maintain a molecular structure that is capable of showing lectin activity. The 0.6 kb transcript is the result of splicing of four exons encoding a protein with 135 amino acids (Fig. 10.1).

10.2.2 X-Ray Structure of Human Gal-1

The overall folding of Gal-1 involves a β sandwich consisting of two anti-parallel β-sheets (Fig. 10.2). This jelly-roll topology of the CRD constitutes typical folding patterns of galectins. Human Gal-1 exists as a dimer in solution. The integrity of this dimer is maintained principally by interactions between the monomers at the interface and through the well-conserved hydrophobic core, a factor which explains the observed stability of dimer in molecular terms (Lopez-Lucendo et al. 2004). Single-site mutations introduced at some distance from the CRD can affect the lectin fold and influence sugar binding. Both the substitutions introduced in C2S and R111H mutants altered the presentation of loop, harbouring Asp123 in common "jelly-roll" fold. The orientation of side-chain was inverted 180° and positions of two key residues in sugar-binding site of R111H mutant were shifted, i.e. His52 and Trp68. The decrease in ligand affinity in both mutants and a significant increase in the entropic penalty were found to outweigh a slight enhancement of enthalpic contribution. The position of SH-groups in galectin appeared to considerably restrict the potential to form intra-molecular disulphide bridges and was assumed to be the reason for unstable lectin activity in absence of reducing agent. However, this offers no obvious explanation for the improved stability of the C2S mutant

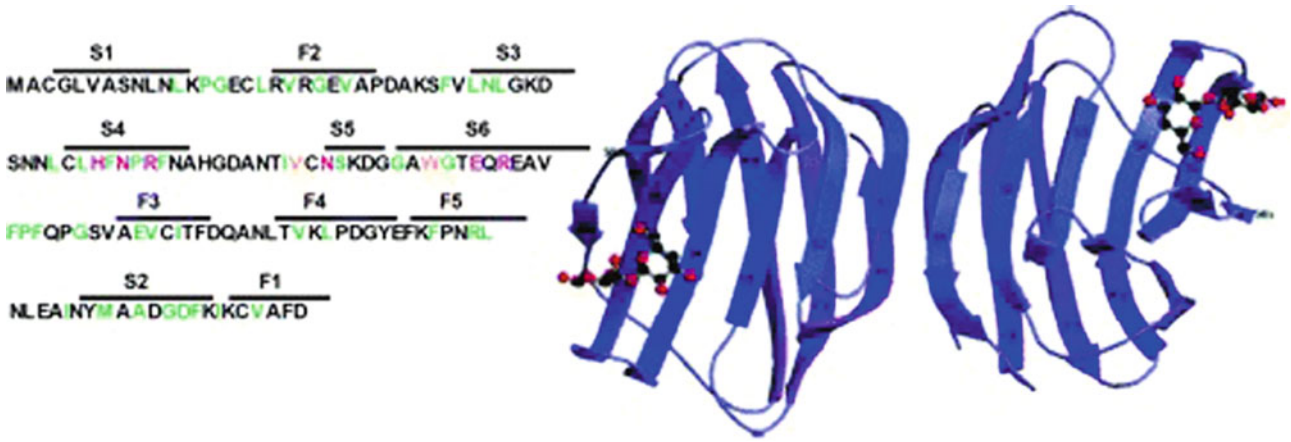

Fig. 10.2 *Right Panel*: **Ribbon diagram of the homodimeric human galectin-1.** The β-strands in the five-stranded (F1–F5) and six-stranded (S1–S6a/S6b) β-sheets are indicated by the letter-number code. The overall folding of Gal-1 involves a ß sandwich consisting of two antiparallel ß-sheets of five (F1–F5) and six (S1–S6a/b) strands respectively. The N and the C termini of each monomer are positioned at the dimer interface and the CRDs are located at the far ends of the same face, a configuration which constitutes a long, negatively charged cleft in the cavity. The 3D ribbon diagram of the homodimeric human Gal-1 was designed with MOLSCRIPT by Lopez-Lucendo et al. (2004). *Left Panel*: **Sequence of human galectin-1**. Residue numbers of Leg1_Human are indicated. The positions of the β-strands for Leg1_Human are indicated by *horizontal arrows*. The *green* amino acid symbols illustrate highly conserved residues. The key residues of the CRD, which is known to interact directly with bound carbohydrate by means of hydrogen bonds, are *colored pink*, while those interacting with carbohydrates via van der Waals interactions are *orange* (Cooper 2002) and include His^{44}, Asn^{46}, Arg^{48}, Val^{59}, Asn^{61}, Trp^{68}, Glu^{71}, and Arg^{73}. A lactose (Galß1–4Glc) is illustrated in the CRD (Reprinted by permission from Lopez-Lucendo et al 2004 © Elsevier)

under oxidative conditions. The noted long-range effects in single-site mutants are relevant for the functional divergence of closely related galectins and in more general terms, the functionality definition of distinct amino acids (Lopez-Lucendo et al. 2004). The main characteristics of dGal-1 is that it spontaneously dissociates at low concentrations (K_D ~7 μM) into a monomeric form that is still able to bind to carbohydrates (Cho and Cummings 1995), but with a lower affinity (Leppanen et al. 2005).

10.2.2.1 Solution Structures of Galectins

Ligand presence and a shift to an aprotic solvent typical for bioaffinity chromatography might alter the shape of homodimeric human lectin in solution. Using small angle neutron and synchrotron x-ray scattering, the radius of gyration of human galectin-1 decreased from 19.1 ± 0.1 Å in absence of ligand to 18.2 ± 0.1 Å after ligand interaction. In presence of aprotic solvent dimethyl sulfoxide, which did not impair binding capacity, Gal-1 formed dimers of a dimer, yielding tetramers with a cylindrical shape. Surprisingly, no dissociation into subunits occurred. NMR monitoring was in accord with these data. In contrast, an agglutinin from mistletoe sharing galactose specificity showed a reduced radius of gyration from ~62 Å in water to 48.7 Å in dimethyl sulfoxide. Evidently, the solvent caused opposite responses in two tested galactoside-binding lectins with different folding patterns. Thus, ligand presence and an aprotic solvent significantly affect the shape of galectin-1 in solution (He et al. 2003).

10.2.2.2 Cross-Linking Properties of Galectins

Evidence suggests that biological activities of galectins are related to their multivalent binding properties since most galectins possess two carbohydrate recognition domains and are therefore bivalent (Chap. 9). For example, Gal-1, which is dimeric, binds and cross-links specific glycoprotein counter-receptors on the surface of human T-cells leading to apoptosis. Different galectin-1 counter-receptors associated with specific phosphatase or kinase activities form separate clusters on the surface of the cells as a result of the lectin binding to the carbohydrate chains of the respective glycoproteins. Importantly, monovalent Gal-1 is inactive in this system. This indicates that the separation and organization of signaling molecules that result from Gal-1 binding is involved in the apoptotic signal. The separation of specific glycoprotein receptors induced by Gal-1 binding was modeled on the basis of molecular and structural studies of the binding of lectins to multivalent carbohydrates resulting in the formation of specific two- and three-dimensional cross-linked lattices. Brewer (2002) reviewed the binding and cross-linking properties of Gal-1 and other lectins as a model for the biological signal transduction properties of the galectin family of animal lectins. Bovine heart Gal-1 forms stable dimers. In contrast, recombinant murine Gal-3, as well as its proteolytical derived C-terminal domain is predominantly monomeric (Morris et al. 2004).

Gal-1 regulates leukocyte turnover by inducing cell surface exposure of phosphatidylserine (PS), a ligand that targets cells for phagocytic removal, in absence of apoptosis.

Gal-1 monomer-dimer equilibrium appears to modulate Gal-1-induced PS exposure. Monomer-dimer equilibrium regulates Gal-1 sensitivity to oxidative inactivation and provides a mechanism whereby ligand partially protects Gal-1 from oxidation (Stowell et al. 2009).

10.2.3 Gal-1 from Toad (*Bufo arenarum Hensel*) Ovary

A galectin from toad (*Bufo arenarum Hensel*) ovary consists of identical single-chain polypeptide subunits composed of 134 amino acids (calculated mass, 14,797 Da), and its N-terminal residue, alanine, is N-acetylated. In comparison to sequences of known galectins, the *B. arenarum galectin* exhibits highest identity (48% for whole molecule and 77% for CRD) with the bovine spleen Gal-1, but surprisingly less identity (38% for whole molecule and 47% for CRD) with a Gal from *Xenopus laevis* skin (Marschal et al. 1992). Unlike the *X. laevis* galectin, the binding activity of the *B. arenarum* galectin for N-acetyllactosamine, the human blood group A tetrasaccharide and Gaβ1,3GalNAc relative to lactose, was in agreement with that observed for Gal-1 subgroup and those galectins having "conserved" (type I) CRDs (Ahmed and Vasta 1994). Moreover, the toad galectin shares three of the six cysteine residues that are conserved in all mammalian Gals-1, but not in the galectins from *X. laevis*, fish, and invertebrates described so far (Hirabayashi and Kasai 1993). Furthermore, galectins with conserved (type I) CRDs, represented by the *B. arenarum* ovary galectin, and those with "variable" (type II) CRDs, represented by *X. laevis* 16-kDa galectin, clearly constitute distinct subgroups in the extant amphibian taxa and may have diverged early in the evolution of chordate lineages (Ahmed et al. 1996).

Crystal structures of *B. arenarum* Gal-1 in complex with two related carbohydrates, LacNAc and TDG (TDG, thiodigalactoside (ß-D-galactopyranosyl-1-thio-ß-D-galactopyrano-side) showed that the topologically equivalent hydroxyl groups in two disaccharides have identical patterns of interaction with Gal-1. Groups that are not equivalent between two sugars present in second moiety of disaccharide, interact differently with the protein, but use the same number and quality of interactions. Structures showed additional protein-carbohydrate interactions not present in other reported lectin-lactose complexes. These contacts provide an explanation for the enhanced affinity of Gal-1 for TDG and LacNAc relative to lactose. Comparison of *B. arenarum* with other Gal-1 structures shows that among different galectins there are significant changes in accessible surface area buried upon dimer formation, providing a rationale for the variations observed in free-energies of dimerization. The structure of *B. arenarum* Gal-1 has a large cleft with a strong negative potential that connects two binding sites at the surface of the protein. Such a striking characteristic suggests that this cleft is probably involved in interactions of the galectin with other intra or extra-cellular proteins (Bianchet et al. 2000).

10.2.4 GRIFIN Homologue in Zebrafish (DrGRIFIN)

Galectin-like proteins such as rat lens crystalline protein GRIFIN (Galectin-related inter fiber protein) and the galectin-related protein GRP (previously HSPC159; hematopoietic stem cell precursor) lack carbohydrate-binding activity. Their inclusion in the galectin family has been debated as they are considered products of evolutionary co-option. A homologue of GRIFIN is present in zebrafish (Danio rerio) (designated DrGRIFIN), which like the mammalian equivalent is expressed in the lens, particularly in the fiber cells, as revealed in dpf (days post fertilization) embryos. As evidenced, it is weakly expressed in the embryos as early as 21 hpf (hour post fertilization) but strongly at all later stages tested (30 hpf and 3, 4, 6, and 7 dpf). In adult zebrafish tissues, however, DrGRIFIN is also expressed in oocytes, brain, and intestine. Unlike the mammalian homologue, DrGRIFIN contains all amino acids critical for binding to carbohydrate ligands and its activity was confirmed as the recombinant DrGRIFIN could be purified to homogeneity by affinity chromatography on a lactosyl-Sepharose column. Therefore, DrGRIFIN is a bona fide galectin family member that in addition to its carbohydrate-binding properties may also function as a crystalline (Ahmed and Vasta 2008).

10.3 Regulation of *Gal-1* Gene

10.3.1 Gal-1 in IMP1 Deficient Mice

Various physicochemical agents are known to modulate the expression of Gal-1 (Table 10.1). The methylation status of Gal-1 promoter is also a very important mechanism that controls the expression of the gene (Chiariotti et al. 2004). Downregulation of *Igf2* translation and the postnatal intestine showed reduced expression of transcripts encoding ECM components, such as galectin- 1, lumican, tenascin-C, procollagen transcripts, and the Hsp47 procollagen chaperone. It demonstrates that Insulin-like growth factor II mRNA-binding protein 1(IMP1) is essential for normal growth and development and may facilitate intestinal morphogenesis via regulation of ECM formation (Hansen et al. 2004).

Table 10.1 The regulation of Gal-1 expression[a]

Agent/factor	Tissues/cells	Effects	References
Budesonide (glucocorticoid)	Human nasal polyps	Increase	Delbrouck et al. 2002; Goldstone and Lavin 1991
Progesterone	Mouse uterine tissue	Increase	Choe et al 1997
Estrogen	Mouse uterine tissue/Rat1a embryo cells	Increase	Choe et al 1997; Tahara et al 2003
TSH	Rat thyroid	Increase	Chiariotti et al 1994a
Retinoic acid (all-trans)	1. Embryonal carcinomas, mouse myoblastic cells	Increase	Lu et al 2000; Choufani et al 2001
	2. Transformed rat neural cells	Decrease	Chiariotti et al. 1994a
TGFβ1	Metastatic mammary adenocarcinoma (LM3) cells, murine lung adenocarcinoma LP07 cells, and the human breast adenocarci noma (MCF-7) cells	Increase	Daroqui et al 2007
IL-12	Peripheral blood mononuclear cells and cord blood $CD4^+$ cells	Decrease	Filén et al. 2006
δFosB	Quiescent neuronal precursor cells	Increase	Kurushima et al 2005; Miura et al 2005
5-Azacytidine	Human hepatic carcinomas, osteosarcoma cells; human T leukemia cells; rat liver and thyroid cells	Increase	Kondoh et al 2003; Chiariotti et al. 2004; Poirier et al 2001a
Sodium butyrate	Human colon carcinomas; HNSCCs, globelet cells; prostate cancers; mouse embryonal carcinomas	Increase	Ellerhorst et al 1999a; Gaudier et al 2004; Gillenwater et al 1998; Lu and Lotan 1999; Ohannesian et al 1994
Cyclophosphamide	Rat lymphomas	Decrease	Rabinovich et al. 2002b
Benzodiazepine	Jurkat T lymphoma cells	Decrease	Rochard et al. 2004
Valproic acid	Mouse embryo	Increase	Kultima et al 2004
Oxidized low-density lipoprotein	Human endothelial cells	Increase	Baum et al 1995; Perillo et al 1995

[a]Modified after permission from Camby et al. 2006 © Oxford University Press

10.3.2 Blimp-1 Induces Galectin-1 Expression

B lymphocyte-induced maturation protein-1 (Blimp-1), a master regulator for plasma cell differentiation, was necessary and sufficient to induce Gal-1 expression. Blimp-1 is a transcriptional repressor (Keller and Maniatis 1992). Microarray study revealed that Gal-1 is up-regulated upon ectopic expression of Blimp-1 in mature human B cell lines (Shaffer et al. 2002). The ectopic expression of Gal-1 in mature B cells increased Ig μ-chain transcript levels as well as the overall level of Ig production. The effect of Gal-1 on promoting Ig production appears to be mediated through an extracellular receptor(s) and to depend on the binding of β-galactosides before terminal differentiation of B cells (Tsai et al. 2008). The jck murine model, which results from a double point mutation in *nek8* gene, suggests that *Nek8* mutation causes over-expression of Gal-1, sorcin, and vimentin and accumulation of major urinary protein in renal cysts of jck mice in PKD proteome relative to wild type (Valkova et al. 2005).

Regulation by Ovarian Steroids: Gal-1 mRNA is abundantly expressed in mouse reproductive organs such as uterus and ovary. Uterine expression of Gal-1 mRNA is specifically regulated during embryo implantation. Its expression increased on fifth day post coitum (dpc 5) when embryos hatched into endometrial epithelial cells. In absence of embryos, however, Gal-1 in mouse uterus decreased on dpc 5. In delayed implantation mice, Gal-1 mRNA level was augmented by termination of delay of implantation. Ovarian steroids progesterone and estrogen differentially regulated Gal-1 mRNA level in uterine tissues (Choe et al. 1997).

Induction by TSH in Thyroid Cells: Expression of Gal-1 is low in normal and very high in transformed thyroid cells. Gal-1 gene expression is transiently induced after TSH stimulation of normal quiescent FRTL-5 rat thyroid cells. Permanent activation of Gal-1 gene expression was obtained in same cells infected with a wild-type and a temperature sensitive mutant of Kirsten murine sarcoma virus, both at the permissive and non permissive temperature for transformation (Chiariotti et al. 1994a).

10.3.3 Regulation by Retinoic Acid

Growth stimulation and induction of cell differentiation are accompanied by strong modulation of Gal-1 gene expression. Gal-1 mRNA is undetectable in rat brain but abundantly present in rat oligodendrocytes precursors transformed by polyoma middle T oncogene. Retinoic acid (RA) treatment of these transformed cells leads to acquisition of a differentiated phenotype accompanied by a 30-fold

decrease of Gal-1 mRNA. Removal of RA restored both the transformed undifferentiated phenotype and high Gal-1 expression (Chiariotti et al. 1994a). A RA responsiveness region was found within sequence from −1,578 to −1,448 upstream of transcription start site (+1). In contrast, high constitutive Gal-1 expression in C2C12 cells appeared to be mediated by a sequence within the promoter region from −62 to +1, which contains a Sp1 consensus sequence. EMSA indicated that transcription factor SP1 bound to Gal-1 Sp1 site; and mutagenesis of this Sp1 site abolished both the binding of nuclear proteins to the mutated Sp1 site and the high constitutive expression of Gal-1 gene.

RARβ Receptors and Expression of Galectin-1, -3 and -8 in Human Cholesteatomas: In human cholesteatomas, there is predominant expression of RARβ and Gal-1. Furthermore, the level of RARβ expression correlated highly with the level of galectin-8 expression, which also correlated with the level of RARα and RARγ. In addition, this parameter also correlated with the level of Gal-1 and Gal-3 expression. Simon et al. (2001) suggest that cholesteatomas may originate in an undifferentiated population of keratinocytes, and that a relation may exist between retinoid activity and galectins.

Macrophage migration inhibitory factor (MIF) expression is significantly higher in the epithelium and vessels of connective tissues of recurrent as opposed to non-recurrent cholesteatoma and significantly correlated to RARβ expression in non-infected cholesteatomas and to MMP-3 and anti-apoptotic Gal-3 in infected cholesteatomas. Together with galectin-3, MIF could cooperate to form an anti-apoptotic feedback loop (Choufani et al. 2001).

10.3.4 Regulation by TGF-β, IL-12 and FosB Gene Products

Daroqui et al. (2007) suggested a potential cross talk between TGF-β and Gal-1 in highly metastatic mammary adenocarcinoma (LM3) cells, murine lung adenocarcinoma LP07 cells, and the human breast adenocarcinoma MCF-7 cell lines. TGF-β1-mediated upregulation of Gal-1 expression was specifically mediated by TGFβRI and TGFβRII. The presence of three putative binding sites for Smad4 and Smad3 transcription factors in Gal-1 gene was consistent with the ability of TGF-β1 to trigger a Smad-dependent signaling pathway in these cells. Thus, TGF-β1 may control Gal-1 expression, suggesting that distinct mechanisms might cooperate in tilting the balance toward an immunosuppressive environment at the tumor site. IL-12 enhances the generation of Th1 lymphocytes and inhibits the production of Th2 subset. IL-12 regulated proteins were studied in microsomal fraction of Th cells. Gal-1 and CD7 are known to interact with each other, and regulate immunity through influencing apoptosis and cytokine production. Filén et al. (2006) indicated that IL-12 down-regulates the expression of both Gal-1 and CD7 in the microsomal fraction of peripheral blood mononuclear cells and cord blood $CD4^+$ cells. The down-regulation of these proteins is likely to have a role in specific Th cell selection and cytokine environment creation.

In rat 3Y1 embryo cell lines expressing FosB and δFosB as fusion proteins (ER-FosB, ER-δFosB) with ligand-binding domain of human estrogen receptor (ER) (Nishioka et al. 2002), the binding of estrogen to the fusion proteins resulted in their nuclear translocation. Among several proteins whose expression was affected after estrogen administration, one of the proteins was galectin-1 (Tahara et al. 2003).

10.3.5 Regulation by Metabolites/Drugs/Other Agents

The expression of Gal-1 can be induced in cultured hepatoma-derived cells by treatment with 5-AzaCytidine (AzaC), a DNA demethylating drug. Interestingly, Gal-1 and AzaC individually have been shown to affect similar cell processes in cancer cells, including differentiation, and growth inhibition (Chiariotti et al. 1994b; c/r Camby et al. 2006). The sensitivity to Gal-1 is associated with repression of endogenous Gal-1 gene whereas non-sensitive cells express high levels of Gal-1. Repression of Gal-1 gene in sensitive cells is associated with hyper-methylation of promoter region. Transient treatment of non-expressing cells with demethylating agent AzaC led to irreversible demethylation and subsequent reactivation of galectin-1 gene (Salvatore et al. 2000). The expression of Gal-1, accompanied by its secretion and its binding to cell surface receptors, could be involved in AzaC observed effects in hematopoietic cells where Gal-1 modulates differentiation or apoptosis.

Poirier et al. (2001a) investigated the effect of AzaC and Gal-1 on human lymphoid B cells phenotype. Treatment of lymphoid B cells with AzaC resulted in: (1) a decrease in cell growth with an arrest of cell cycle at Go/G1 phase, (2) phenotypic changes consistent with a differentiated phenotype, and (3) the expression of p16, a tumor-suppressor gene whose expression was dependent of its promoter demethylation, and of Gal-1. The targeting of Gal-1 to the plasma membrane followed its cytosolic expression. Recombinant Gal-1 added to BL36 cells displayed growth inhibition and phenotypic changes consistent with a commitment toward differentiation. It seems that AzaC-induced Gal-1 expression and consequent binding of Gal-1 on its cell

membrane receptor may, in part, be involved in AzaC-induced plasmacytic differentiation. Poirier et al. (2001a) proposed that the released Gal-1 was immediately recruited to modulate cell activity. Gal-1 may do this by interacting with and modulating cell receptors via its carbohydrate recognition domains because the Gal-1-receptor interaction is abrogated by thiodigalactoside (Fouillit et al. 2000). While Gal-1 binds to T and B lymphoblastoid cells (Fouillit et al. 2000; Baum et al. 1995), studies have demonstrated that galectins are immunosuppressive in animal models of autoimmune diseases (Rabinovich et al. 1999). Whereas the full role of Gal-1 in modulating immune function is not yet understood, the increase in Gal-1 expression by AzaC in BL cells suggests that Gal-1 may play a role in the behaviour of normal leukocytes and of tumor cells.

The underlying mechanism, triggered by demethylating stimulus probably involves the stimulation of a signaling cascade that regulates cell proliferation and viability. Demethylating stimulus may modify a pathway activated by the membrane-anchored protein-tyrosine phosphatase CD45. Engagement of CD45 is known to regulate Src tyrosine kinases phosphorylation, phospholipase Cγ regulation, inositol phosphate production, diacylglycerol production, PKC activation, and calcium mobilisation (c/r Poirier et al. 2001a). Increased synthesis and secretion of Gal-1 by the cell could account for part of the phenotypic alterations detected in AzaC treated cells. Gal-1-induced dimerisation and/or segregation might inhibit the catalytic site in CD45, thereby blocking tyrosine phosphates activity. Because Gal-1 binding to cell surface receptors results in tyrosine phosphorylation (Fouillit et al. 2000; Vespa et al. 1999), it may allow a kinase-dependent signal to be transduced. Several studies have linked Gal-1 expression with growth inhibition and cell death (Goldstone and Lavin 1991; Delbrouck et al. 2002; Perillo et al. 1995). However, the reports that some growth inhibitory agents did not induce Gal-1 expression indicated that Gal-1 expression is not dependent on the cell's growth state in general, though it may be involved in growth suppression (Gillenwater et al. 1998). Moreover, it is likely that Gal-1 acts in a manner to regulate specific signal transduction processes that is determined by the cell type and by the state of cell differentiation, since, exogenous rGal-1 added to BL cells inhibited cell growth. Besides, Gal-1 as well as AzaC induced an expression of the cell surface plasma cell antigen, CD138, a phenotypic marker that identifies cells with plasmacytic differentiation (Kopper and Sebestyen 2000). This is consistent with the hypothesis that AzaC and Gal-1 share similar signals for differentiation, however, since there was a significant difference in the expression of CD19 and CD23 after AzaC or Gal-1 treatments it is likely that some pathways are specifically modified by AzaC. The mechanisms involved in these different pathways, important in clinical therapy, remain to be elucidated in the future (Poirier et al. 2001b).

Cyclophosphamide Modulates Gal-1 Expression: Gal-1 has been shown to contribute to tumor cell evasion of immune responses by modulating survival and differentiation of effector T cells. A single low dose of cyclophosphamide has an antimetastatic effect on lymphoma (L-TACB)-bearing rats by modulating the host immune response. Galectin-1 has potent immunomodulatory properties by regulating cell-matrix interactions and T-cell apoptosis. A single low dose of cyclophosphamide modulates the expression of Gal-1 and Bcl-2 by tumors, which could in turn influence the apoptotic threshold of spleen mononuclear cells. Conversely, Gal-1 expression was significantly reduced in spleen cells and lymph node metastasis through period of study. Cyclophosphamide treatment restored the basal levels of Gal-1 expression in primary tumors and spleens. Results suggest that, in addition to other well-known functions of cyclophosphamide, Cy may also modulate Gal-1 expression and function during tumor growth and metastasis with critical implications for tumor-immune escape and immunotherapy (Rabinovich et al. 2002b; ZacarÃas Fluck et al. 2007).

Effects of Butyrate and Valproic Acid: Colonic mucin glycosylation can be modified by butyrate. In HT29-Cl.16E cells, the most striking effect of butyrate was on galectin-1 gene, which increased 8- to 18-fold, with a central and apical intracellular localization. Butyrate effects have possible link with mucins expressed by HT29-Cl.16E cells (Gaudier et al. 2004). The antiepileptic drug valproic acid (VPA) is a potent inducer of neural tube defects (NTDs) in human and mouse embryos. Some VPA-responsive genes have been associated with NTDs or VPA effects, whereas others provide putative VPA targets, associated with processes relevant to neural tube formation such as, galectin-1, fatty acid synthase (Fasn), annexins A5 (Kultima et al. 2004). The ErbB protein family or epidermal growth factor receptor (EGFR), a family of four structurally related receptor tyrosine kinases is associated with genes involved in cell-matrix interactions including galectin 1 and galectin 3. These data represent profiles of transcriptional changes associated with ErbB2-related pathways in the breast, and identify potentially useful targets for prognosis and therapy (Mackay et al. 2003).

Global gene expression profiles were analyzed in European wild boar naturally infected with *Mycobacterium bovis*. While some proteins overexpressed in infected animals, lower expression was observed for galectin-1, complement component C1qB, certain HLA class I and MHC class II antigens and Ig chains in infected animals (Galindo et al. 2009).

Effect of Hypoxia: The expression of Gal-1 has been shown to be regulated by hypoxia. Ectopically expressed hypoxia-inducible factor (HIF) 1α protein, an oxygen-sensitive subunit of HIF-1 that is a master factor for cellular response to hypoxia, significantly increases galectin-1 expression at both m-RNA and protein levels in colorectal cancer (CRC) cell lines. The knockdown of Gal-1 by its specific shRNA can significantly reduce hypoxia-induced invasion and migration of CRC cell line, proposing that Gal-1 mediates the HIF-1-induced migration and invasion of CRC cells during hypoxia. The CCAAT/enhancer binding protein-α (C/EBPα), a critical transcriptional factor for hematopoietic cell differentiation, can directly activate galectin-1 through binding to the −48 to −42 bp region of its promoter. Moreover, knockdown or chemical inhibition of galectin-1 partially blocks the differentiation induced by HIF-1α or C/EBPα, which can be rescued by recombinant galectin-1. These observations give new insights on the mechanisms for Gal-1 expression regulation and HIF-1α- and C/EBPα-induced leukemic cell differentiation (Zhao et al. 2011).

10.4 Gal-1 in Cell Signaling

Gal-1 is present both inside and outside cells, and has both intracellular and extracellular functions. The extracellular functions require the carbohydrate-binding properties of dGal-1 while the intracellular ones are associated with carbohydrate-independent interactions between Gal-1 and other proteins. The Gal-1 induced growth inhibition requires functional interactions with α5β1 integrin (Fischer et al. 2005). The antiproliferative effects result from inhibition of Ras-MEK-ERK pathway and consecutive transcriptional induction of p27: two Sp1-binding sites in p27 promoter are crucial to Gal-1 responsiveness (Fischer et al. 2005). The inhibition of the Ras-MEK-ERK cascade by Gal-1 increases Sp1 transactivation, with DNA binding relating to the reduced threonine-phosphorylation of Sp1 (Fischer et al. 2005). Furthermore, Gal-1 induces p21 transcription and selectively increases p27 protein stability. The Gal-1-mediated accumulation of p27 and p21 inhibits Cdk2 activity and, ultimately, results in G1 cell cycle arrest and growth inhibition (Fischer et al. 2005). The Gal-1-induced increase in cell motility involves Gal-1-induced increase in rhoA expression and the alteration of polymerization of actin cytoskeleton (Camby et al. 2002). Gal-1 is recruited from cytosol to cell membrane by H-Ras-GTP in a lactose-independent manner with the resulting stabilization of H-Ras-GTP, the clustering of H-RAS-GTP and Gal-1 in non-raft microdomains (Prior et al. 2003), the subsequent binding to Raf-1 (but not to PI3Kinase) and the activation of ERK signaling pathway (Elad-Sfadia et al. 2002). Nuclear Gal-1 interacts with Gemin4 and is co-immunoprecipitated with the nuclear SMN complexes involved in the splicing pathway (Vyakarnam et al. 1997)

10.5 Ligands/Receptors of Gal-1

10.5.1 Each Galectin Recognizes Different Glycan Structures

Human galectins have divergent roles and each galectin recognizes different glycan receptors (Camby et al. 2006; Ilarregui et al. 2005) although most of the members of galectin family bind weakly to simple disaccharide lactose (Galβ1-4Glc). While Gal-1 inhibits mast cell degranulation (Rubinstein et al. 2004b), Gal-3 induces degranulation in mast cells independently of IgE-mediated antigen stimulation (Frigeri et al. 1993). Gal-1 blocks leukocyte chemotaxis (La et al. 2003), whereas Gal-3 has opposite effect, inducing leukocyte chemotaxis (Sano et al. 2000) and release of preformed IL-8 from neutrophils (Jeng et al. 1994), which further augments chemotaxis of leukocytes. In addition, Gal-1 inhibits acute inflammatory responses through various mechanisms, including suppression of phospholipase A_2-induced edema (Rabinovich et al. 2000) and inhibition of neutrophil extravasation (La et al. 2003). Gal-3 enhances the extravasation of neutrophils, and Gal-3 null mice also exhibit attenuated leukocyte infiltration following challenge (Hsu et al. 2000). Interestingly, patients with reduced Gal-2 expression were found to have reduced risk for myocardial infarction, suggesting that Gal-2 may also have pro-inflammatory roles (Ozaki et al. 2004). Furthermore, Gal-1, Gal-2, and Gal-3 have all been reported to signal T cells through different receptors (Hahn et al. 2004; Sturm et al. 2004; Stillman et al. 2006). These types of studies suggest that Gal-1, Gal-2, and Gal-3 recognize distinct receptors on leukocytes.

Functional studies indicated that Gal-1, Gal-2, and Gal-3 recognized distinct receptors on leukocytes although different galectins could also recognize related receptors. For example, Gal-3 attenuates Gal-1 inhibition of growth in neuroblastoma cells at the receptor level, and both Gal-1 and Gal-3 induce superoxide production in human neutrophils (Almkvist et al. 2001). Gal-1 and Gal-2 both induce surface exposure of phosphatidylserine (PS) in activated human neutrophils in the absence of apoptosis through a Ca^{2+}-dependent pathway (Karmakar et al. 2005). In this way, galectins likely exhibit unique versatility in a wide range of biological functions (Ilarregui et al. 2005). Although some differences have been reported in glycan recognition by these galectins (Hirabayashi

et al. 2002; Brewer 2004), there are many questions remaining about their glycan recognition and subsequent effects on galectin binding. It has been suggested that differences in biological effects of Gal-1 and Gal-3 result from differences in tertiary structure, rather than ligand binding properties (Perillo et al. 1995; Ahmad et al. 2004), because Gal-3 was thought to behave primarily as a monomer (Massa et al. 1993). However, Gal-3 can form homo-oligomeric structures (Nieminen et al. 2007), which supports the likelihood that the major differences in biological functions by these lectins are because of differences in glycan recognition.

10.5.2 Interactions of Galectin-1

Although Gal-1 was first described to bind ß-galactosides, it is now clear that besides its interaction with ß-galactosides it is also engaged in protein–protein interactions (Camby et al. 2006). Infact, lectin activity of Gal-1 is observed when it is extracellular, while the protein–protein interactions of Gal-1 participate in its intracellular functions. As a result, Gal-1 has large number of binding partners.

10.5.2.1 Protein–Carbohydrate Interactions

The lectin activity of Gal-1 relates to its carbohydrate-binding site (Fig. 10.2). Thermodynamic calculations indicate that sugar binding to Gal-1 is enthalpically driven, and suggest the concept that weak interactions constitute the main forces in stability of complex formation (Lopez-Lucendo et al. 2004). The dissociation constant of dGal-1 with various glycoproteins is ~5 μM (Symons et al. 2000). Galectin-1 preferentially recognizes multiple Galβ1,4GlcNAc units, which may be presented on the branches of *N*- or *O*-linked glycans on cell surface glycoproteins. Although dGal-1 binds preferentially to glycoconjugates containing disaccharide N-acetyllactosamine (Gal-ß1–3/4 GlcNAc/LacNAcII), its binding to individual lactosamine units is characterized by relatively low affinity (K_D ~50 μM). However, the arrangement of lactosamine disaccharides in multiantennary repeating chains increases the binding avidity (K_D ~4 μM) (Ahmad et al. 2004). In contrast, there is no increase in avidity when recognition unit is repeated in a string (poly-N-lactosamine) (Ahmad et al. 2004). In polysaccharides, dGal-1 does not bind glycans that lack a terminal non-reducing unmodified N-acetyllactosamine (Stowell et al. 2004). In search of molecular mechanism of Gal-1 secretion, a Gal-1 export mutant identified 26 single amino acid changes that cause a defect of both export and binding to counter receptors. It was indicated that β-galactoside binding site represents the primary targeting motif of galectins defining a galectin export machinery that makes use of β-galactoside-containing surface molecules as export receptors for intracellular galectin-1 (Seelenmeyer et al. 2005). Isothermal titration calorimetry measurements determined affinities of five galactose-containing ligands for Gal-1. Although the galactose moiety of each oligosaccharide is necessary for binding, it is not sufficient by itself. The nature of both the reducing sugar in the disaccharide and the interglycosidic linkage play essential roles in binding to human Gal-1 (Meynier et al. 2009).

The specificity of Gal-1 for glycans depends not only on the structure of glycans but also on the mode of their presentation viz., solid phase or in solution. In solution-based assays (Blixt et al. 2004; Hirabayashi et al. 2002; Leppanen et al. 2005), Gal-1 binds glycans with a single *N*-acetyllactosamine (LacNAc) unit (Galβ1-4GlcNAc) equivalently to those with poly-*N*-acetyllactosamine [poly(LacNAc)] sequences (Galβ1-4GlcNAc)$_n$; however, the dimeric form of Gal-1 showed a significant preference for poly(LacNAc)-containing glycans in solid phase assays. Human recombinant homodimer (dGal-1) binds with high affinity (K_D ~ 2–4 μM) to immobilized extended glycans containing terminal N-acetyllactosamine (LacNAc; Galβ1-4GlcNAc) sequences on poly-N-acetyllactosamine [poly(LacNAc)/(3Galβ1-4GlcNAcβ1-)$_n$] sequences, complex-type biantennary N-glycans, or novel chitin-derived glycans modified to contain terminal LacNAc. Although terminal Gal residues are important for dGal-1 recognition, dGal-1 bound similarly to α3-sialylated and α2-fucosylated terminal LacNAc, but not to α6-sialylated and α3-fucosylated terminal LacNAc. Results suggested that dGal-1 functions as a dimer to recognize LacNAc units on extended poly(LacNAc) on cell surfaces (Leppanen et al. 2005). Gal-1 also exists in an oxidized form, which lacks lectin activity (Outenreath and Jones 1992).

Significantly, Gal-1 does not recognize internal Lac-NAc units within poly(LacNAc) (Leppanen et al. 2005; Stowell et al. 2004), suggesting that this preference likely reflects favorable poly(LacNAc) conformational constraints of terminal LacNAc unit that are enhanced by immobilization. But Gal-1 recognized poly-(LacNAc)-containing glycans on leukocyte surfaces with a similar affinity as observed for immobilized poly(LacNAc) glycans (Leppanen et al. 2005). Likewise, frontal affinity chromatography or isothermal calorimetry, in which glycans are free in solution, also did not reveal significant difference in carbohydrate recognition (Hirabayashi et al. 2002; Ahmad et al. 2004). This suggests that galectin-glycan interactions may be tested in context of immobilized glycan presentation. Stowell et al. (2008a, b) and others evaluated Gal-1, Gal-2, and Gal-3 interactions using immobilized glycans in a glycan microarray format that included hundreds of structurally diverse glycans, and compared the results to binding determinants toward promyelocytic HL60 cells, which respond to signals by these galectins resulting in exposure of surface PS (Stowell et al. 2007; Fukumori et al. 2003) and tested binding to

human erythrocytes. These galectins exhibited differences in glycan binding and demonstrated that each of these galectins mechanistically differed in their binding to glycans on the microarrays and that these differences were reflected in the determinants required for cell binding and signaling. The specific glycan recognition by each galectin underscores the basis for differences in their biological activities. Gal-1 binds to a number of ECM components in ß-galactoside-dependent manner in following order: laminin > cellular fibronectin > thrombospondin > plasma fibronectin > vitronectin > osteopontin (Moiseeva et al. 2000, 2003). Laminin and cellular fibronectin are glycoproteins which are highly N-glycosylated with bi- and tetra-antennary poly-N-lactosamines (Carsons et al. 1987).

Gal-1 is a receptor for the angiogenesis inhibitor anginex, and is crucial for tumor angiogenesis. Gal-1 is overexpressed in endothelial cells of different human tumors. The role of gal-1 in tumor angiogenesis is demonstrated in zebrafish model and in gal-1-null mice, in which tumor growth is markedly impaired because of insufficient tumor angiogenesis. Thus, gal-1 regulates tumor angiogenesis and is a target for angiostatic cancer therapy (Thijssen et al. 2006).

N-Glycans on CD45 Negatively Regulate Galectin-1: Although terminal galactose residues are important for dGal-1 recognition, dGal-1 binds similarly to α3-sialylated and α2-fucosylated terminal N-acetyllactosamine, but not to α6-sialylated or α3-fucosylated terminal N-acetyllactosamine (Amano et al. 2003; Leppanen et al. 2005). The addition of sialic acid to T cell surface glycoproteins influences essential T cell functions such as selection in the thymus and homing in the peripheral circulation. Addition of α2,6-linked sialic acid to Galβ1,4GlcNAc sequence, the preferred ligand for galectin-1, inhibits recognition of this saccharide ligand by Gal-1. SAα2,6Gal sequences, created by ST6Gal I enzyme (sialyltransferase), are present on medullary thymocytes resistant to Gal-1-induced death but not on galectin-1-susceptible cortical thymocytes. Because the ST6Gal I preferentially utilizes N-glycans as acceptor substrates, it was shown that N-glycans are essential for galectin-1-induced T cell death. Expression of ST6Gal I specifically resulted in increased sialylation of N-glycans on CD45 which is a T cell receptor for galectin-1. ST6Gal I expression abrogated the reduction in CD45 tyrosine phosphatase activity that results from galectin-1 binding. Sialylation of CD45 by the ST6Gal I also prevented galectin-1-induced clustering of CD45 on the T cell surface, an initial step in galectin-1 cell death. Thus, regulation of glycoprotein sialylation may control susceptibility to cell death at specific points during T cell development and peripheral activation (Amano et al. 2003) (see Camby et al. 2006).

Galectin-1 Interacts with Subunit of Integrin: The activity of integrin adhesion receptors is essential for normal cellular function and survival. N-glycosylations of ß-integrins regulate ß1 integrin functions by modulating their heterodimerization with α chains and ligand-binding activity (Gu and Taniguchi 2004). By direct binding to ß1 integrins (without cross-linking them) dGal-1 increases the amounts of partly activated ß1 integrins, but does not induce dimerization with α subunits (Moiseeva et al. 2003). In case of vascular smooth muscle cells this interaction of Gal-1 with α1ß1 integrin has been reported both as transiently phosphorylating the focal adhesion kinase (FAK) and as modulating attachment of cells and their spreading and migration on laminin, but not on cellular fibronectin (Moiseeva et al. 2003). Gal-1 is secreted during skeletal muscle differentiation and accumulates with laminin in the basement membrane surrounding each myofiber (Gu and Taniguchi 2004). The coincidence of Gal-1 secretion with the onset of myoblast differentiation and fusion and the transition in myoblast adhesion and mobility on laminin are regulated by the interaction of Gal-1 with laminin and the α7ß1 integrin. Fischer et al. (2005) have shown that Gal-1-induced growth inhibition requires functional interactions with α5ß1 integrin. Gal-1 from mouse macrophages has been found to specifically associate with other integrins such as α_Mß2 (the CR3) (Avni et al. 1998).

IgA1 as Premier Serum Glycoprotein Recognized by Human Galectin-1: Endogenous glycoproteins co-purified with human heart galectin-1 (HHL) are excellent ligands for HHL. These glycoproteins are rich in T antigen (Galβ1→3 GalNAc-) of O-linked oligosaccharides. It seems that galectin-1 plays important role in anchoring of microbial and cancer cells known to be rich in T antigen, in high serum IgA1 turn over and in tissue sequestering of IgA1 immune complexes especially after their microbial desialylation in IgA nephropathy and other immune complex-mediated disorders (Sangeetha and Appukuttan 2005).

CD2, CD3, CD7 CD43, CD45, CA125 as Gal-1 Receptors: Elola et al. (2005a) reviewed galectin-1 receptors, and some of the mechanisms by which this lectin affects different cell types. Several Gal-1 receptors are discussed such as CD45, CD7, CD43, CD2, CD3, CD4, CD107, CEA, actin, extracellular matrix proteins such as laminin and fibronectin, glycosaminoglycans, integrins, a β-lactosamine glycolipid, GM1 ganglioside, polypeptide HBGp82, glycoprotein 90 K/MAC-2BP, CA125 ovarian cancer antigen, and pre-B cell receptor (*R:* Elola et al. 2005a). A number of T-cell glycoproteins from MOLT-4 and Jurkat human T cells have

been shown to be specific receptors for mammalian Gal-1 binding (Pace et al. 1999; Walzel et al. 1999; Symons et al. 2000; Fajka-Boja et al. 2002; Seelenmeyer et al. 2003). Primary structure of CA125 is a giant mucin-like glycoprotein present on cell surface of ovarian tumor. CA125 is a counter receptor for Gal-1, as both soluble and membrane-associated fragments of CA125 from HeLa cell lysates bind specifically to human Gal-1 with high efficiency. This interaction depends on β-galactose-terminated, O-linked oligosaccharide chains of CA125, and has preference for Gal-1 versus Gal-3. Results suggest that CA125 might be a factor involved in the regulation of Gal-1 export to the cell surface (Seelenmeyer et al. 2003). CD45 can positively and negatively regulate Gal-1-induced T cell death, depending on the glycosylation status of the cells. The CD45 inhibitory effect involved the phosphatase domain. Oligosaccharide-mediated clustering of CD45 facilitated galectin-1-induced cell death (Nguyen et al. 2001).

Gal-1 is a major receptor for the carbohydrate portion of the ganglioside GM1 exposed on the surface of human neuroblastoma cells (Kopitz et al. 1998; Andre et al. 2004). Cell confluence increases the surface presentation of dGal-1. Under these circumstances Gal-1 acts as a negative growth regulator of neuroblastoma cells, though without being pro-apoptotic (Kopitz et al. 1998, 2001).

10.5.2.2 Protein–Protein Interactions

The proteins identified so far that interact in a carbohydrate-independent manner with Gal-1 are not structurally related to each other and do not seem to share any common domains or motifs. The galectin sites that are involved in protein–protein interactions have not been fully established. Gemin4, a cytoplasmic and also found in the nucleus of cells, is a member of the survival of motor neuron protein (SMN) complex and the miRNP particle (microRNA [ribonucleoprotein [RNP]). Nuclear Gal-1 interacts with Gemin4 and is co-immunoprecipitated with nuclear SMN complexes involved in splicing pathway (Vyakarnam et al. 1997). The interactions of Gal-1 with Gemin4 and nuclear SMN complex (Park et al. 2001) are loci of action in spliceosome assembly.

10.6 Functions of Galectin-1

10.6.1 Role of Galectin-1 in Apoptosis

A growing body of evidence indicates that Gal-1 functions as a homeostatic agent by modulating innate and adaptative immune responses. A high level of expression is found in lymphatic organs, which feature high rates of apoptosis. Furthermore, Gal-1 can initiate T cell apoptosis and potetiates apoptosis in the epithelial tumor cell lines (MCF7 and BeWo). It induces the inhibition of cell growth and cell-cycle arrest and promotes apoptosis of activated, but not resting immune cells (He and Baum 2004; Rabinovich et al. 2002b). The suggested-resting T cells are sensitized to CD95/Fas-mediated cell death by Gal-1 (Matarrese et al. 2005). Furthermore, the Gal-1 expressed by thymic epithelial cells promotes the apoptosis of immature cortical thymocytes in vitro (Perillo et al. 1997), suggesting a potential role for this protein in the processes of positive and/or negative selection within thymic microenvironment. Gal-1 also suppresses the secretion of the pro-inflammatory cytokine interleukin-2 (IL-2) and favors the secretion of the anti-inflammatory cytokine IL-10 (van der Leij et al. 2004). Battig et al. (2004) have shown that the dimeric form of Gal-1 is a dramatically more potent inducer of apoptosis in T cells than wild-type Gal-1. Evidence indicates that the amount of Gal-1 secreted by different cell types in the ECM is sufficient to kill T cells (Perillo et al. 1995; He and Baum 2004).

A number of T-cell glycoproteins from human MOLT-4 and Jurkat T cells have been shown to be specific receptors for mammalian Gal-1 and are involved in Gal-1-mediated T-cell death: CD45, CD43, CD7 (c/r Pace et al. 1999; Walzel et al. 1999; Symons et al. 2000; Fajka-Boja et al. 2002). However, although the deletion mutants of the glycoproteins confirm their importance in the apoptotic response to Gal-, the role of CD45 in T-cell apoptosis mediated by Gal-1 remains controversial since Gal-1 induces apoptosis in CD45-deficient T cells (Walzel et al. 1999; Fajka-Boja et al. 2002). Earl et al. (2010) showed that Gal-1 signaling through CD45, which carries both N- and O-glycans, is regulated by CD45 isoform expression, core 2 O-glycan formation and the balance of N-glycan sialylation. Regulation of Gal-1 T cell death by O-glycans is mediated through CD45 phosphatase activity. As for CD7, it seems that only specific spliced isoforms or glycoforms of CD45 may be important in signaling Gal-1-induced cell death (Nguyen et al. 2001; Xu and Weiss 2002; Amano et al. 2003; See Camby et al. 2006).

Gal-1 interferes with the Fas-associated apoptosis cascade in T-cell lines Jurkat and MOLT-4. Gal-1 and an Apo-1 mAb induced DNA-fragmentation in Jurkat T-cells whereas MOLT-4 cells were resistant. Gal-1 stimulated DNA-fragmentation could be inhibited by caspase-8 inhibitor II (Z-IETD-FMK) and a neutralizing Fas mAb. Fas could be identified as a target for Gal-1 recognition. Gal-1 stimulates the activation and proteolytic processing of procaspase-8 and downstream procaspase-3 in Jurkat-T cells. Inhibition of Gal-1 induced procaspase-8 activation by a neutralizing Fas mAb strongly suggests that Gal-1 recognition of Fas is associated with caspase-8 activation.

This report provides experimental evidence for targeting of Gal-1 to glycotopes on Fas and the subsequent activation of the apoptotic death-receptor pathway (Brandt et al. 2008).

Galectin-Induced Activation of NFAT and AP-1 in Human Jurkat T-Lymphocytes: Galectin-mediated ligation of glycoepitopes on T-cell activation markers induces an increase in the cytosolic $(Ca^{2+})_i$ originating from a transient Ca^{2+} release of internal stores as well as a sustained influx across the plasma membrane. The signal transduction events that lead to galectin-induced cell death in activated T cells involve several intracellular mediators including the induction of specific transcription factors (i.e., NFAT, AP-1), the activation of Lck/ZAP-70/MAPK signaling pathway, the modulation of Bcl-2 protein production, the depolarization of the mitochondrial membrane potential and cytochrome C release, the activation of caspases and the participation of the ceramide pathway (Rabinovich et al. 2000; Walzel et al. 2000; Hahn et al. 2004; Ion et al. 2005; Matarrese et al. 2005). Electrophoretic mobility shift assays (EMSAs) provided evidence for Gal-1-stimulated increase in the binding of nuclear extracts to a synthetic oligonucleotide with an AP-1 consensus sequence (Walzel et al. 2002). However, the Gal-1-induced apoptosis in human T leukemia MOLT-4 cells deficient in Fas-induced cell death is not dependent on the activation of caspase-3 or on cytochrome c release—two hallmarks of apoptosis—but involves the rapid nuclear translocation of EndoG from mitochondria (Hahn et al. 2004), implying that Gal-1-induced cell death might also relate to one of the other types of cell death (Broker et al. 2005). In addition, in vivo studies on experimental autoimmunity models have revealed the ability of Gal-1 to skew the balance toward a T2-type cytokine response by reducing the levels of IFNγ, TNFα, IL-2, and IL-12 and increasing the level of IL-5 secretion (Santucci et al. 2000, 2003). Galectin-1 tunes TCR binding and signal transduction to regulate CD8 burst size (Liu et al. 2009a).

Induction of Apoptosis in Galectin-1-Stimulated Jurkat T Cells: Galectin-1 induces death of diverse cell types including lymphocytes and tumor cells. Gal-1 sensitizes human resting T cells to Fas (CD95)/caspase-8-mediated cell death. The treatment of Jurkat E6.1 cells with N-glycan processing inhibitors (1-deoxymannojirimycin and swainsonine) strongly reduced the cell binding of Gal-1-biotin, conjugate binding to cell lysate glycoproteins, and to CD3 immunoprecipitates as well as the binding of CD2 and CD3 to immobilized Gal-1. Study provided evidence that Gal-1 triggers through binding to N-linked glycans a Ca^{2+}-sensitive apoptotic pathway (Walzel et al. 2006) and that the JNK/c-Jun/AP-1 pathway plays a key role for T-cell death regulation in response to gal-1 stimulation (Brandt et al. 2010).

Galectin-1 is highly expressed in thymus and induces apoptosis of specific thymocyte subsets and activated T cells. Galectin-1 binds to N- and O- glycans on several glycoprotein receptors, including CD7, CD43, and CD45. Galectin-1 kills immature thymocytes and activated peripheral T cells by binding to glycans on T cell glycoproteins including CD7, CD45, and CD43. Role for CD43 in galectin-1-induced death and the effects of O-glycan modification on galectin-1 binding to CD43 has been demonstrated. It appeared that CD43 bearing either core 1 or core 2 O-glycans can positively regulate T cell susceptibility to galectin-1, identifying a function for CD43 in controlling cell death. Studies also demonstrated that different T cell glycoproteins on the same cell have distinct requirements for glycan modifications that allow recognition and cross-linking by galectin-1 (Hernandez et al. 2006).

Galectin-1-Induced Apoptosis of Immature Thymocytes is Regulated by NF-kB: CD7, one of the galectin-1 receptors, has crucial roles in Gal-mediated apoptosis of activated T-cells and T-lymphoma progression in peripheral tissues. CD7 promoter activity was increased by NF-kB and this activity was synergistic when Sp1 was co-expressed in immature T-cell line L7. Furthermore, the regulation of CD7 gene expression through NF-kB activation induced by TCR/CD28 might have significant implications for T-cell homeostasis (Koh et al. 2008). However, while understanding regulatory pathway in functional mature T-cells it was revealed that CD7 expression was downregulated by Twist2 in Jurkat cells, a human T-cell lymphoma cell line, and in EL4 cells, a murine T-cell lymphoma cell line. Furthermore, ectopic expression of Twist2 in Jurkat cells reduced galectin-1-induced apoptosis. Based on these results, it was concluded that upregulation of Twist2 increases the resistance to galectin-1-mediated-apoptosis, which may have significant implications for the progression of some T-cells into tumors such as Sezary cells (Koh et al. 2009).

Cell Death via Mitochondrial Pathway: A high level of expression is found in lymphatic organs, which feature high rates of apoptosis. However, the galectin-1 T cell death pathway is distinct from other death pathways, including those initiated by Fas and corticosteroids. Galectin-1-induced cell death proceeds via a caspase-independent pathway that involves a unique pattern of mitochondrial events, and different galectin family members can coordinately regulate susceptibility to cell death (Hahn et al. 2004). Furthermore, Gal-1 triggers an apoptotic program involving an increase of mitochondrial membrane potential and participation of the ceramide pathway. In addition, Gal-1 induces mitochondrial coalescence, budding, and fission accompanied by an increase and/or redistribution of fission-associated molecules h-Fis and DRP-1. Importantly, these changes are

detected in both resting and activated human T cells, suggesting that Gal-1-induced cell death might become an excellent model to analyze the morphogenetic changes of mitochondria during the execution of cell death. This suggests the association among Gal-1, Fas/Fas ligand-mediated cell death, and the mitochondrial pathway, providing a rational basis for the immunoregulatory properties of Gal-1 in experimental models of chronic inflammation and cancer (Matarrese et al. 2005).

Galectin-1 initiates the acid sphingomyelinase mediated release of ceramide, which is critical in further steps. Elevation of ceramide level coincides with exposure of phosphatidylserine on the outer cell membrane. The downstream events such as decrease of Bcl-2 protein, depolarization of the mitochondria and activation of the caspase 9 and caspase 3 depend on production of ceramide. All downstream steps, including production of ceramide, require the generation of membrane rafts and the presence of two tyrosine kinases, p56 (lck) and ZAP70. Acid sphingomyelinase mediated release of ceramide is essential to trigger the mitochondrial pathway of apoptosis by galectin-1. Based on these findings a model of the mechanism of galectin-1 triggered cell has been suggested (Ion et al. 2006).

Galβ1-4GlcNAc and Galβ1-3GalNAc Epitopes on BeWo Cells Have Regulatory Effects on Cell Proliferation: Galectin-1 shows apoptotic potential in the epithelial tumor cell lines (MCF7 and BeWo) in vitro, only with additional stress stimuli (Wiest et al. 2005). Gal-1 inhibited BeWo cell proliferation in a concentration-dependent manner. The lectin decreased cellular hCG and progesterone production as well as hCGβ gene transcription by BeWo cells. Gal-1 mediated inhibition of cellular progesterone production was reduced in presence of a Thomsen-Friedenreich (TF)-polyacrylamide conjugate. Therefore, ligation of Galβ1-4GlcNAc and Galβ1-3GalNAc epitopes on BeWo cells may have regulatory effects on cell proliferation and hCG and progesterone production (Jeschke et al. 2004, 2006).

Dimeric Form of Gal-1 is a Powerful Inducer of Apoptosis: Since the affinity of the monomers for each other is rather low, the in vivo efficacy of galectin-1 is limited because the equilibrium is shifted towards the inactive monomeric form at lower concentrations. A covalently linked form of the dimer based on the galectin-1 crystal structure is a potent inducer of apoptosis in murine thymocytes as well as murine mature T cells at concentrations 10-fold lower than wild-type galectin-1. This structurally optimized form of galectin-1 may be a powerful tool to treat chronic inflammatory diseases (Battig et al. 2004). Moreover, recombinant human galectin-1 shows biphasic effect on the growth and death of early hematopoietic cells (Vas et al. 2005).

Surface Exposure of Phosphatidylserine in Activated Human Neutrophils: Cell turn-over depends on the surface exposure of phosphatidylserine (PS) in apoptotic cells, leading to their phagocytic recognition and removal. Galectins induce PS exposure in a carbohydrate-dependent fashion in activated, but not resting, human neutrophils and in several leukocyte cell lines. Apoptotic cells redistribute PS to the cell surface by both Ca^{2+} -dependent and -independent mechanisms. Binding of dGal-1 to glycoconjugates on N-formyl-Met-Leu-Phe (fMLP)-activated neutrophils exposes PS and facilitates neutrophil phagocytosis by macrophages, yet it does not initiate apoptosis. It appeared that dGal-1 initiated Ca^{2+} fluxes are required to redistribute PS to the surface of activated neutrophils. Results suggest that transient Ca^{2+} fluxes contribute to a sustained redistribution of PS on neutrophils activated with fMLP and dGal-1 (Karmakar et al. 2005). Hence surface PS exposure is not always associated with apoptosis in activated neutrophils. The exposure of PS in cell lines treated with galectins is sustained and does not affect cell viability. Unexpectedly, galectins-1, -2, and -4 bind well to activated T lymphocytes, but do not induce either PS exposure or apoptosis, indicating that galectin's effects are cell specific. These results suggest immunoregulatory contribution of galectins in regulating leukocyte turnover independently of apoptosis (Stowell et al. 2007).

10.6.2 Gal-1 in Cell Growth and Differentiation

Regulation of Cell Growth: While extracellular Gal-1 has no effect on the growth rates of naïve T cells (Endharti et al. 2005) or of astrocytic (Camby et al. 2002) or colon (Hittelet et al. 2002) tumor cell lines, Gal-1 is mitogenic for various types of normal or pathological murine and human cells, such as murine Thy-1-negative spleen or lymph node cells (Symons et al. 2000), mammalian vascular cells (Moiseeva et al. 2000), and hepatic stellate cells (Maeda et al. 2003). Gal-1 inhibits the growth of other cell types such as neuroblastoma (Kopitz et al. 2001) and stromal bone marrow cells (Andersen et al. 2003). Interestingly, it seems that depending on the dose involved, Gal-1 causes the biphasic modulation of cell growth. While high doses (~1 μM) of recombinant Gal-1 inhibit cell proliferation independently of Gal-1 sugar-binding activity, low doses (~1 nM) of Gal-1 are mitogenic and are susceptible to inhibition by lactose (Adams et al. 1996; Vas et al. 2005). While the knock-down of Gal-1 expression in murine melanomas (Rubinstein et al. 2004a) and human glioma cells (Camby et al. 2006) does not affect

their growth rate in vitro, it does decrease it in 9 L rat gliosarcomas (Yamaoka et al. 2000). Furthermore, Gal-1 can also regulate cell cycle progression in human mammary tumor cells (Wells et al. 1997). The paradoxical biphasic effects of Gal-1 on cell growth are highly dependent on cell type and cell activation state, and might also be influenced by the relative distribution of monomeric versus dimeric, or intracellular versus extracellular forms. However, Gal-1 signaling in leukocytes requires expression of complex-type N-glycans (Karmakar et al. 2008)

Regulation of Cell Motility: While cell migration is the net result of adhesion, motility, and invasion (Lefranc et al. 2005), Gal-1 modifies each of these three steps in cell migration-related processes. Gal-1 increases adhesion of various normal and cancer cells to ECM via cross-linking of integrins exposed on cell surfaces with carbohydrate moieties of ECM components such as laminin and fibronectin (Ellerhorst et al. 1999b; Moiseeva et al. 1999; van den Brule et al. 2003). In addition, Gal-1 can also mediate homotypical cell interaction, so favoring the aggregation of human melanoma cells (Tinari et al. 2001) and heterotypical cell interactions such as the interaction between cancer and endothelial cells, which, in its turn, favors the dispersion of tumor cells (Clausse et al. 1999; Glinsky et al. 2000).

Gal-1 causes the increased motility of glioma cells and the reorganization of the actin cytoskeleton associated with an increased expression of RhoA, a protein that modulates actin polymerization and depolymerization (Camby et al. 2002). Conversely, the knock-down of Gal-1 expression in glioma cells reduces motility and adhesiveness (Camby et al. 2002, 2005). Oxidized Gal-1 stimulates the migration of Schwann cells from both proximal and the distal stumps of transected nerves and promotes axonal regeneration after peripheral nerve injury (Fukaya et al. 2003). In colon carcinomas a Gal-1-enriched ECM decreases colon carcinoma cell motility (Hittelet et al. 2002). Using a proteomic approach based on the comparison of highly and poorly invasive mammary carcinoma cell lines, Harvey et al. (2001) identified the membrane expression of Gal-1 as a signature of cell invasiveness.

Regulation of Chemotaxis: Gal-1 blocks leukocyte chemotaxis (La et al. 2003), whereas Gal-3 has the opposite effect, inducing leukocyte chemotaxis (Sano et al. 2000) and the release of pre-formed IL-8 from neutrophils (Jeng et al. 1994), which further augments chemotaxis of leukocytes (Baggiolini et al. 1992). Galectin-1 stimulates monocyte migration but is not chemotactic for macrophages. Galectin-1-induced monocyte chemotaxis is blocked by lactose and inhibited by an anti-galectin-1 antibody. Furthermore, galectin-1-mediated monocyte migration is significantly inhibited by MEK inhibitors suggesting that MAP kinase pathways are involved in galectin-1. Migration involves G-protein in galectin-1-induced chemotaxis. A role for galectin-1 in monocyte chemotaxis differs from galectin-3 in that macrophages are nonresponsive. Furthermore, observations suggest that galectin-1 may be involved in chemoattraction at sites of inflammation in vivo and may contribute to disease processes such as atherosclerosis (Malik et al. 2009).

Differentiation of the Myogenic Lineage: During the course of myoblast differentiation intracellular Gal-1 is externalized as myoblasts fused into myotubes (Cooper and Barondes 1990). The role of Gal-1 in the case of myoblast fusion may be explained by the fact that the adherence of the myoblast to the extracellular component laminin is disrupted in the presence of Gal-1 via the selective modulation by Gal-1 of the interaction between the α7ß1 integrin and fibronectin and laminin (Gu et al. 1994). Although the exact role of Gal-1 in myogenesis remains to be seen, this galectin has been shown to induce non-committed myogenic cells in the dermis to express myogenic markers. It increases the terminal differentiation of committed myogenic cells and has a role to play in the development and regenerative ability of muscles (Cooper and Barondes 1990; Harrison and Wilson 1992; Goldring et al. 2002). Gal-1 may thus be regarded as a potentially important tool in the treatment of cases of human muscular dystrophy (Goldring et al. 2002).

Differentiation of Hematopoietic Lineage: Mesenchymal cells give rise to the stromal marrow environment that supports hematopoiesis. These cells constitute a wide range of differentiation potentials (e.g., adipocytes, osteoblasts, chondrocytes, lymphocytes, erythrocytes, macrophages) as well as a complex relationship with hematopoietic and endothelial cells. Numerous reports have suggested that Gal-1 may be a key element in the course of hematopoietic cell differentiation (Andersen et al. 2003; Silva et al. 2003; Wang et al. 2004; Vas et al. 2005). The K562 human leukemia cell line expresses Gal-1 in the cytosol, but upon treatment with erythropoietin these cells develop an erythroid phenotype that leads to the externalization of cytosolic Gal-1 (Lutomski et al. 1997). Similarly, Gal-1 is externalized during adipocyte differentiation (Wang et al. 2004) and is able to modulate osteoblastic differentiation (Andersen et al. 2003) as well as the proliferation and death of hematopoietic stem and progenitor cells (Vas et al. 2005).

Gal-1 During Lineage Commitment of $CD4^+CD8^+$ Thymocyte Cell: During linage commitment of $CD4^+CD8^+$ thymocytes, $CD4^+CD8^+$ show skewed differentiation of into $CD4^-CD8^+$ thymocytes in presence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). DPK cells, a $CD4^+CD8^+$ thymic lymphoma cell line can differentiate into $CD4^+CD8^-$ thymocytes after antigen stimulation. Among the 10 up-regulated proteins, S100A4, S100A6, and galectin-1 were

highly up-regulated and dramatically increased after antigen stimulation, similar to transcription factors intimately associated with lineage commitment. In thymus S100A4, S1006, and galectin-1 were most prominently expressed in $CD4^+CD8^+$ thymocytes, but not at all in $CD4^-CD8^+$ and $CD4^-CD8^-$ thymocytes. In spleen, expression of S100A4, S1006, and galectin-1 was greater in CD4 than in CD8 splenocytes. On addition of TCDD to antigen-stimulated DPK cells, antigen-induced up-regulation of S100A4, S1006, and galectin-1 was remarkably reduced, probably accounting for the skewed differentiation of $CD4^+CD8^+$ into $CD4^-CD8^+$ thymocytes induced by TCDD (Jeon et al. 2009).

In the thymus, during T-cell differentiation, the expression of peripheral benzodiazepine receptor (PBR) modulates. Rochard et al. (2004) studied role of PBR in Jurkat cells, which are immature T lymphocytes. These cells are PBR negative and were stably transfected to achieve PBR levels similar to that in mature T cells. A majority of modulated genes encode proteins playing direct or indirect roles during lymphocyte maturation process. In particular, PBR expression induced several differentiation markers, or key regulating elements. By contrast, some regulators of TCR signaling were reduced. PBR expression also affected the expression of critical apoptosis regulators: the proapoptotic lipocortin I, galectin-1, and galectin-9 were reduced while the antiapoptotic Bcl-2 was induced. Results supported the hypothesis that PBR controls T-cell maturation and suggested mechanisms through which PBR may regulate thymocyte-positive selection (Rochard et al. 2004).

Menstrual Cycle, Early Gestation and Embryogenesis: Gal-1 expression has been reported in male and female gonads (Timmons et al. 2002; Dettin et al. 2003). In testis, Gal-1 was identified in Leydig cell. Exogenously added Gal-1 has an inhibitory effect both on the steroidogenic activity of Leydig cells in testicles (Martinez et al. 2004) and on the granulosa cells in ovary (Jeschke et al. 2004; Walzel et al. 2004). Gal-1 influences rat Leydig cells morphology, decrease in cell viability in culture. Testosterone production was reduced after addition of Gal-1, reaching a minimum of 26% after 24 h. Study indicated that the reduction in viability and in steroidogenesis was caused by apoptosis induced by Gal-1. Besides, addition of Gal-1 caused Leydig cell detachment. The CRD is involved in inducing apoptosis. These findings indicated that Gal-1 and laminin-1 interactions form the molecular basis of Leydig cell function and survival. Galectin-1 plays a role in biphasic growth of Leydig tumor cells (Biron et al. 2006).

In the uterus Gal-1 expression is restricted to the endometrium and varies during the menstrual cycle and the early phases of gestation (von Wolff et al. 2005). The expression of Gal-1 increases significantly in late secretory phase endometrium and in decidual tissue (von Wolff et al. 2005), and shows a specific pattern of expression in trophoblastic tissue (Maquoi et al. 1997; Vicovac et al. 1998). During first trimester of embryogenesis Gal-1 is expressed in connective tissue, in smooth and striated muscles, and in some epithelia such as skin, gonads, thyroid gland, and kidneys (Hughes 2004; von Wolff et al. 2005).

10.6.3 Gal-1 and Ras in Cell Transformation

H-Ras–Gal-1 interactions establish an essential link between Gal-1 and Ras associated with cell transformation and human malignancies that can be exploited to selectively target oncogenic Ras proteins. In fact, H-Ras-GTP recruits Gal-1 from the cytosol to the cell membrane with the resulting stabilization of H-Ras-GTP, the clustering of H-Ras-GTP and Gal-1 in non-raft microdomains (Prior et al. 2003), the subsequent binding to Raf-1 (but not to PI3Kinase), the activation of the ERK signaling pathway and, finally, increased cell transformation (Elad-Sfadia et al. 2002). So, in addition to increasing and prolonging H-Ras activation, the Gal-1-H-Ras complex renders the activated molecule selective toward Raf-1, but not toward PI3K (Ashery et al. 2006). Fischer et al. (2005) have observed that the antiproliferative potential of Gal-1 in a number of carcinoma cell lines requires functional interaction with the α5ß1 integrin. Antiproliferative effects result from the inhibition of the Ras-MEK-ERK pathway and the consecutive transcriptional induction of p27, whose promoter contains two Sp1-binding sites crucial for Gal-1 responsiveness (Fischer et al. 2005). The inhibition of the Ras-MEK-ERK cascade by Gal-1 increases Sp1 transactivation and DNA binding due to the reduced threonine phosphorylation of Sp1. In addition, Gal-1 induces p21 transcription and selectively increases p27 protein stability, while the Gal-1-mediated accumulation of p27 and p21 inhibits cyclin-dependent kinase 2 activity, a process which ultimately results in G1 cell cycle arrest and growth inhibition (Fischer et al. 2005).

Gal-1 interacts in a lactose-independent manner with H-Ras-guanosine triphosphate (H-Ras-GTP) through its farnesyl cystein carboxymethylester and so strengthens its membrane association (Paz et al. 2001). Farnesylthiosalicylic acid (FTS) disrupted H-Ras(12V)-galectin-1 interactions. Overexpression of Gal-1 increased membrane-associated Ras, Ras-GTP, and active ERK resulting in cell transformation, which was blocked by dominant negative Ras. H-Ras(12V)-galectin-1 interactions establish an essential link between two proteins associated with cell transformation and human malignancies that can be exploited to selectively target oncogenic Ras proteins. Rotblat et al. (2004) identified a hydrophobic pocket in Gal-1, analogous to Cdc42 geranylgeranyl-binding cavity in RhoGDI, having homologous isoprenoid-binding residues, including critical L11, whose RhoGDI L77 homologue changes on Cdc42

binding. By substituting L11A, Rotblat et al. (2004) obtained a dominant interfering Gal-1 that possessed normal carbohydrate-binding capacity but inhibited H-Ras GTP-loading and ESR-kinase activation, dislodged H-Ras (G12V) from cell membrane, and attenuated H-Ras(G12V) fibroblast transformation and PC12-cell neurite outgrowth. Thus, independent of carbohydrate binding, Gal-1 cooperates with Ras, whereas galectin-1inhibits its activity (Rotblat et al. 2004). The binding of Gal-1 to Ras is an interesting and potentially significant function of Gal-1. These functions have been discussed in following sections.

10.6.4 Development of Nerve Structure

Galectin-1 seems to have a variety of biological functions, which vary according to time and the site at which biological function is taking place. In addition, these functions could vary according to the structure of Gal-1 by which a particular biological function is taking place. Disulfide bond formation alters the structure of Gal-1 that confers the ability to promote axonal regeneration. However, structural analysis revealed that the axonal regeneration-promoting factor exists as an oxidized form of Gal-1, containing three intramolecular disulfide bonds. The Gal-1 subunits are not covalently linked but the monomers are in a dynamic equilibrium with dimeric form. Since the affinity of monomers for each other is rather low (in range of 10^{-5} M), the in vivo efficacy of Gal-1 is limited because the equilibrium is shifted towards the inactive monomeric form at lower concentrations.

10.6.4.1 Galectin-1 in Sensory and Motor Neurons

Galectin-1 is widely distributed in central and peripheral nervous systems of rodents during their development. Galectin-1 is important for the embryonic development of primary sensory neurons and their synaptic connections in spinal cord. Galectin-1 is colocalized c-Ret mRNA in small DRG neurons. About 20% of the DRG neurons showed intense galectin-1-reactivity (IR). On the other hand, only 6.8% displayed TrkA mRNA positive signals. Galectin-1-IR was increased in the dorsal horn at 1 to 2 weeks after axotomy. The endogenous galectin-1 may potentiate neuropathic pain after the peripheral nerve injury at least partly by increasing SPR in the dorsal horn (Imbe et al. 2003).

Gal-1 plays a number of important roles in the formation of neural network of olfactory bulb of mice (Puche et al. 1996). Gal-1 homozygous null mutant (Gal-$1^{-/-}$) mice are viable and can grow into adults without any phenotypical abnormalities except for a deficiency in olfactory network (Tenne-Brown et al. 1998) and a reduced thermal sensitivity (McGraw et al. 2005). In these mice the neuronal subpopulation in olfactory bulb, which normally expresses Gal-1, does not reach appropriate targets in olfactory glomeruli. During its development into adulthood, a rat's sensory neurons from the dorsal root ganglion express Gal-1, as do some spinal motor neurons. The initial expression in sensory neurons begins as they finish their final mitotic division and begin their growth toward their targets in the dorsal horn of spinal cord. When Gal-1-expressing neurons reach their targets, Gal-1 expression remains high, albeit at lower levels (Hynes et al. 1990; Sango et al. 2004). In addition to neurons, Gal-1 mRNAs are also detected in non-neuronal cells such as the pia mater, the choroid plexus, and the pineal gland as well as in reactive astrocytic and Schwann cells (Akazawa et al. 2004; Sango et al. 2004; Egnaczyk et al. 2003; Gaudet et al. 2005; c/r Camby et al. 2006; Gaudet et al. 2005).

Gal-1 has been shown to promote axonal regeneration through the activation of macrophages. Also, Gal-1 may act within the injured neuron to enhance re-growth: the injury-induced regulation of Gal-1 in numerous types of peripherally- and centrally-projecting neurons correlates positively with the regenerative potential of their axons. Kopitz et al. (2004) used two chicken proto-type galectins, i.e., monomeric CG-14 and dimeric CG-16, with very similar carbohydrate affinities, and rat hippocampal neurons in culture to assess the involvement of carbohydrate-protein interaction in axonal growth and directionality, neurite sprouting and axon regenerative capacity after section. In view of the concept of sugar code, Kopitz et al. (2004) indicated that biological effects triggered by glycan binding engaging an endogenous lectin can be modulated by carbohydrate affinity and/or by other factors like differential cross-linking capacity.

Galectin-1 regulates neural progenitor cell proliferation in two neurogenic regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus. The temporal profile of endogenous Gal-1 expression and the effects of human recombinant Gal-1 on neurogenesis and neurological functions in an experimental focal ischemic model suggest that Gal-1 is one of the principal regulators of adult SVZ neurogenesis through its carbohydrate-binding ability and provide evidence that Gal-1 protein has a role in improvement of sensorimotor function after stroke (Ishibashi et al. 2007).

Neural Stem Cells: In the subventricular zone of the adult mammalian forebrain, neural stem cells (NSCs) reside and proliferate to generate young neurons. Sakaguchi et al. (2006) identified galectin-1, which is expressed in a subset of slowly dividing subventricular zone astrocytes, which include NSCs. Based on results from intraventricular infusion experiments and phenotypic analyses of knockout mice, it was demonstrated that galectin-1 is an endogenous factor that promotes the proliferation of NSCs in the adult brain. Further experiments revealed that mouse adult NSCs as well

as OP9 cells express galectin-1. The molecular mechanism by which galectin-1 enhances proliferation of NSCs is unknown. It is tempting, however, to suggest that galectin-1 binds to the carbohydrate chains of signaling molecules and modulates signal transduction as do HSPGs and/or cystatin C.

Plachta et al. (2007) indicated that galectin-1 actively participates in the elimination of neuronal processes after lesion, and that engineered embryonic stem cells (ESC) are a useful tool for studying relevant aspects of neuronal degeneration that have been hitherto difficult to analyze (Plachta et al. 2007). Akama et al. (2008) identified seven proteins with increased expression and one protein with decreased expression from ESC to neural stem cells (NSC), and eight proteins with decreased expression from NSC to neurons. Laminin-binding protein, galectin 1, increased in NSC, and Gal-1 and a cell adhesion receptor, laminin receptor (RSSA), decreased in neurons. The mRNA of Gal-1 was also up-regulated in NS cells and down-regulated in neurons, implying an important role of Gal-1 in regulating the differentiation. The differentially expressed proteins provide insight into the molecular basis of neurogenesis from ES cells to NSC and neurons.

Proangiogenic and Promigratory Effects of Gal-1 in Hs683 Cells: Galectin 1 is involved in Hs683 oligodendroglioma chemoresistance, neoangiogenesis, and migration. Down-regulating Gal-1 expression in Hs683 cells through targeted siRNA provokes a marked decrease in expression of brain-expressed X-linked gene: BEX2. Decreasing BEX2 expression in Hs683 cells increases the survival of Hs683 orthotopic xenograft-bearing mice. Furthermore, the decrease in BEX2 expression impairs vasculogenic mimicry channel formation in vitro and angiogenesis in vivo, and modulates glioma cell adhesion and invasive features through modification of several genes, reported to play a role in cancer cell migration, including MAP2, plexin C1, SWAP70, and integrin β_6 (Le Mercier et al. 2009).

Altered Primary Afferent Anatomy and Reduced Thermal Sensitivity in Gal-1$^{-/-}$ Mice: The transmission of nociceptive information occurs along non-myelinated, or thinly myelinated, primary afferent axons. During neuronal development and following injury, trophic factors and their respective receptors regulate their survival and repair. Reports show that Gal-1, which is expressed by nociceptive primary afferent neurons during development and into adulthood, is involved in axonal path finding and regeneration. McGraw et al. (2005) characterized anatomical differences in dorsal root ganglia (DRG) of Gal-1 homozygous null mutant mice (Gal-1$^{-/-}$), as well as behavioural differences in tests of nociception. While there was no difference in the total number of axons in the dorsal root of Gal-1$^{-/-}$ mice, there were an increased number of myelinated axons, suggesting that in the absence of Gal-1, neurons that are normally destined to become IB4-binding instead become NF200-expressing. In addition, mice lacking Gal-1 had a decreased sensitivity to noxious thermal stimuli. The exogenous application of rGal-1 has been shown to promote the rate of peripheral nerve regeneration. Galectin-1 null mutant mice showed an attenuated rate of functional recovery of whisking movement after a facial nerve. Thus, Gal-1 is involved in nociceptive neuronal development and that the lack of this protein results in anatomical and functional deficits in adulthood (McGraw et al. 2004a; 2005).

Mouse Gal-1 Inhibits Toxicity of Glutamate by Modifying NR1 NMDA Receptor Expression: Neuronal death in neurodegenerative disease is caused by the neurotransmitter glutamate. This cell death can arise from either excess levels of glutamate due to decreased astrocyte clearance or due to increased susceptibility. However, galectin-1 is a potential neuroprotective factor secreted by astrocytes and protects mouse and rat cerebellar neurons from the toxic effects of glutamate. The effect is mediated through increased expression of NMDA receptor NR1 and increased proportion of the NR1a subunit subtype. Galectin-1 also decreased the expression of PKC associated with increased resistance to glutamate toxicity. Results suggested that the astrocytic galectin-1 can protect neurons against the effects of excito-toxicity as seen in stroke and ischemic injury (Lekishvili et al. 2006).

δFosB Together with Galectin-1 Mediate Neuroprotection and Neurogenesis in Response to Brain Insult: Jun and Fos family proteins are components of an AP-1 (activator protein-1) complex, and are known to regulate the transcription of various genes involved in cell proliferation, differentiation and apoptosis. δFosB, one of the AP-1 subunits, triggers one round of proliferation in quiescent rat embryo cell lines, followed by a different cell fate such as morphological alteration or delayed cell death. Transient forebrain ischemia causes selective induction of δFosB subunit, in cells within the ventricle wall or those in the dentate gyrus in the rat brain prior to neurogenesis, followed by induction of nestin (a marker for neuronal precursor cells) or galectin-1. Different lines of approaches suggested that δFosB can promote the proliferation of quiescent neuronal precursor cells, thus enhancing neurogenesis after transient forebrain ischemia (Kurushima et al. 2005). Miura et al. (2005) demonstrated that the expression of galectin-1 is required for the proliferative activation of quiescent rat1A cells by δFosB, thus indicating that galectin-1 is one of functional targets of δFosB in rat3Y1 cell line. This is supported by the facts that the expression of δFosB is highly inducible in the adult brain in response to various insults

such as ischemic reperfusion injury, seizure induced by electric stimulation or cocaine administration. On the other hand, galectin-1 has also been shown to be involved in the regeneration of damaged axons in the peripheral nerve, as well as in neurite outgrowth or synaptic connectivity in the olfactory system during development. Miura et al. (2005) proposed that δFosB together with galectin-1 mediate neuroprotection and neurogenesis in response to brain damage (**R:** Miura et al. 2005)

Miura et al. (2004) indicated that N-terminally processed form of galectin-1, galectin-1β (Gal-1β), a natural monomeric form of galectin-1 lacking its six amino-terminal residues promotes axonal regeneration but not cell death. Expression of Gal-1β was induced by δFosB. The properties and biological functions of Gal-1β have been compared with the full-length form of galectin-1 (Gal-1α). The rmGal-1α exists as a monomer under oxidized conditions and forms a dimer under reduced conditions, while the rmGal-1β exists as a monomer regardless of redox conditions. The affinity of rmGal-1β to β-lactose was two-fold lower than that of rmGal-1α under reduced conditions. In contrast, both rmGal-1α and rmGal-1β exhibited an equivalent capacity to promote axonal regeneration from the dorsal root ganglion explants. Results suggest that the biochemical properties of rmGal-1β determine its biological functions.

Galectin-1 Induces Expression of BDNF in Astrocytes: Astrocytes, in CNS, are considered to act in cooperation with neurons and other glial cells and to participate in the development and maintenance of functions of the CNS. Immature astrocytes possess a polygonal shape and have no processes, and continue to proliferate, while mature astrocytes have stellate cell morphology, and proliferate slowly. Stellate astrocytes, which immediately appear at the site of brain lesions caused by ischemia or other brain injuries, are known to produce several neurotrophic factors to protect neurons from delayed post-lesion death. Brain-derived neurotrophic factor (BDNF), a neuroprotective polypeptide, is considered to be responsible for neuron proliferation, differentiation, and survival. BDNF is known to promote neuronal survival, guide axonal path finding, and participate in activity-dependent synaptic plasticity during development. An agent that enhances production of BDNF is expected to be useful for the treatment of neurodegenerative diseases. In this context, galectin-1 induces astrocyte differentiation and strongly inhibits astrocyte proliferation, and then the differentiated astrocytes greatly enhance the production of BDNF. The effect of galectin-1 is astrocyte-specific and does not have any effect on neurons. Prevention of neuronal loss during CNS injuries is important to maintain brain function. Induction of neuroprotective factors in astrocytes by an endogenous mammalian lectin may be a new mechanism for preventing neuronal loss after brain injury, and may be useful for the treatment of neurodegenerative disorders (**R:** Endo 2005; Sasaki et al. 2004).

10.6.4.2 Oxidized Gal-1 in Promotion of Axonal Regeneration

Although many factors have been implicated in the regenerative response of peripheral axons to nerve injury, the signals that prompt neurons to extend processes in peripheral nerves after axotomy are not well-understood. The oxidized galectin-1 shows no lectin activity and exists as a monomer in a physiological solution. Oxidized galectin-1 has been shown to promote axonal regeneration from transected-nerve sites in an in vitro dorsal root ganglion (DRG) explant model as well as in in vivo peripheral nerve axotomy model. It advances the restoration of nerve function after peripheral nerve injury. Oxidized galectin-1 likely acts as an autocrine or paracrine factor to promote axonal regeneration, functioning more like a cytokine than as a lectin (Review: Kadoya and Horie 2005; Kadoya et al. 2005). In an in vitro peripheral nerve regeneration model of adult rats, the exogenous oxidized recombinant human galectin-1 (rGal-1/Ox) increased the number and diameter of regenerating myelinated fibers. At a similarly low concentration, rGal-1/Ox also was effective in enhancing axonal regeneration in vivo. Since Gal-1 is expressed in the regenerating sciatic nerves as well as in both sensory and motor neurons, results indicated that Gal-1, which is secreted into the extra-cellular space, is subsequently oxidized and then may regulate initial repair after axotomy. It was proposed that axonal regeneration occurs in axotomized peripheral nerves as a result of cytosolic reduced Gal-1 being released from Schwann cells and injured axons, which then becomes oxidized in the extracellular space. Gal-1/Ox in the extracellular space stimulates macrophages to secrete a factor that promotes axonal growth and Schwann cell migration, thus enhancing peripheral nerve regeneration and functional recovery (**R:** Horie et al. 2005).

Oxidized Galectin-1 as Therapy of ALS: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that affects almost selectively motor neurons in the CNS. Most ALS patients die within 5 years of onset. One of the neuropathological features of ALS is an "axonal spheroid," a large swelling of a motor axon within the anterior horn of the spinal cord; this abnormal structure seems to be related to the pathogenesis of motor neuron degeneration in ALS. In 2001, acumulation of galectin-1 was observed in ALS spheroids in close association with neurofilaments. A marked depletion of galectin-1 is another abnormality frequently observed in the skin of ALS patients. Findings, therefore, suggest that galectin-1 may be involved in the pathogenesis of ALS. Oxidized galectin-1 may have a beneficial effect on the pathophysiology of ALS since administered oxidized

galectin-1 to transgenic mice with H46R mutant SOD1, an ALS model mouse, improved the motor activity, delayed the onset of symptoms, and prolonged the survival of the galectin-1-treated mice. Furthermore, the number of remaining motor neurons in the spinal cord was more preserved in the galectin-1-treated mice than in the non-treated mice. Administration of rhGAL-1/ox to the mice delayed the onset of their disease and prolonged the life of the mice and the duration of their illness. Motor function was improved in rhGAL-1/ox-treated mice. Studies suggested that rhGAL-1/ox administration could be a new therapeutic strategy for ALS (Chang-Hong et al. 2005; Kadoya et al. 2005; Kato et al. 2005).

Macrophage Activation Enables Cat Retinal Ganglion Cells to Regenerate Injured Axons into the Mature Optic Nerve: Retinal ganglion cells (RGCs) in mature mammals are generally unable to regenerate injured axons into the optic nerve. However, an intravitreal injection of either of two macrophage activators, oxidized galectin-1 or zymosan, strongly enhanced the regeneration of transected RGC axons beyond an optic nerve crush site in adult cats. Data indicated that RGCs of adult cats are capable of reverting to an active growth state and at least partially overcoming an inhibitory CNS environment as a result of intravitreal macrophage activation (Okada et al. 2005).

Gal-1 in its oxidized form promotes neurite outgrowth (Outenreath and Jones 1992) and enhances axonal regeneration in peripheral (Inagaki et al. 2000; Fukaya et al. 2003; Kadoya et al. 2005) and central (McGraw et al. 2004a; Rubinstein et al. 2004b; McGraw et al. 2005) nerves even at relatively low concentrations (picoM range) (Horie and Kadoya 2000). The marked axonal regeneration-promoting activity of oxidized Gal-1 is likely to be paracrine. Indeed, Gal-1 is expressed in dorsal root ganglion neurons and motor neurons, with immunoreactivity restricted to the neuronal cell bodies, the axons, and the Schwann cells of adult rodents (Hynes et al. 1990; Fukaya et al. 2003; Horie and Kadoya 2000). After axonal injury, cytosolic reduced Gal-1 is likely to be externalized from growing axons and reactive Schwann cells to an extracellular space where some of the molecules may be converted into an oxidized form and may enhance axonal regeneration (Horie and Kadoya 2000; McGraw et al. 2004a; Rubinstein et al. 2004b; Miura et al. 2004; Sango et al. 2004). Miura et al. (2004) have identified a novel, naturally occurring, N-terminally processed form of Gal-1 that lacks the six amino-terminal residues of full length Gal-1. This isoform of Gal-1, which is monomeric under both reducing and oxidizing conditions, promotes axonal regeneration (Miura et al. 2004). Since oxidized Gal-1-induced neurite outgrowth is not observed on isolated neurons, the secreted Gal-1 probably influences the non-neuronal cells surrounding the axons, including the Schwann cells (Fukaya et al. 2003), and in so doing recruits macrophages, fibroblasts, and perineuronal cells (Horie et al. 2004). In this respect, macrophages are potential candidates since they secrete an axonal regeneration-promoting factor when stimulated by oxidized Gal-1 (Horie et al. 2004). A preclinical study using rats with surgically transected sciatic nerves has shown that the administration by an osmotic pump of oxidized Gal-1 at the site of surgery restores nerve function (Kadoya et al. 2005). In contrast to the effect of oxidized Gal-1 on axonal regeneration as suggested, the effects of Gal-1 on astrocyte differentiation and BDNF production depend on carbohydrate-binding activity and are astrocyte-specific since no effects on neurons have been observed (Sasaki et al. 2004). Thus, Gal-1 may thus be considered as a means for the prevention of neuronal loss in cases of injury to CNS (Egnaczyk et al. 2003).

Galectin-1 in Rat Nervous System: Proteomic approach identified two members of galectin family, namely galectin-1 and galectin-3 in primary rat cerebellar astrocytes (Yang et al. 2006). In rat nervous system, galectin-1 mRNA is predominantly observed in the cell bodies of neurons such as oculomotor nucleus (III), trochlear nucleus (IV), trigeminal motor nucleus (V), abducens nucleus (VI), facial nucleus (VII), hypoglossal nucleus (XII), red nucleus, and locus ceruleus. Neurons in pineal gland and dorsal root ganglia expressed galectin-1 mRNA. In the adult rats, the axotomy of facial nerve induced transient up-regulation of galectin-1 mRNA around 6 h after axotomy. These results indicated that galectin-1 may play roles in the early event of nerve injury and regeneration through the transient change of its expression level (Akazawa et al. 2004). The differential expression pattern of Gal-1 following peripheral axotomy and dorsal rhizotomy suggested that endogenous Gal-1 might be important to the regenerative response of injured axons (McGraw et al. 2005).

Galectin-1 from Bovine Brain: A β-galactoside-specific soluble 14-kD lectin from sheep brain was isolated, sequenced, and compared with similar galectins from other species. The isolated galectin shares all the absolutely preserved and critical residues of the mammalian galectin-1 subfamily. The isolated sheep brain galectin (SBG) showed more than 90% amino acid sequence (92%) and carbohydrate recognition domain identity (96%) with human brain galectin-1. Conformational changes were found induced by interaction of the protein with its specific disaccharide and oxidizing agent (hydrogen peroxide). Upon oxidation a drastic change in the environment of aromatic residues and conformation of the galectin was observed with the loss of

hemagglutination activity, while no significant change was observed upon addition of D-lactose (Gal(β1-4)Glc) in the far-UV and near-UV spectra, suggesting no significant modification in the secondary as well as tertiary structures of sheep brain galectin (Shahwan et al. 2004). The galectin-1 from buffalo brain is a dimeric protein of 24.5 kDa with subunit mass of 13.8. The most potent inhibitor of the galectin activity was lactose, giving complete inhibition of hemagglutination at 0.8 mM. Galectin showed higher specificity towards human blood group A (Shamsul et al. 2007).

10.6.5 Skeletal Muscle Development

Regulation of Myotube Growth in Regenerating Skeletal Muscles: Galectin-1 is highly expressed in skeletal muscle and has been implicated in skeletal muscle development and in adult muscle regeneration, but also in the degeneration of neuronal processes and/or in peripheral nerve regeneration. Galectin-1 is involved in muscle stem cell behavior and in tissue regeneration after muscle injury in adult mice. The Gal-1 mRNA is expressed in the extrasynaptic and perisynaptic regions of the muscle, and its immunoreactivity can be detected in both regions (Svensson and Tågerud 2009). Muscle satellite cells play a critical role in skeletal muscle regeneration. In intact adult muscles, galectin-1 was associated with basement membranes of myofibers. Kami and Senba (2005) suggest that Gal-1 is a novel factor that promotes both myoblast fusion and axonal growth following muscle injury, and consequently, regulates myotube growth in regenerating skeletal muscles (**R:** Kami and Senba 2005; Cerri et al. 2008). Galectin-1 is an early marker of myogenesis as the transcripts and protein are initially confined to the somites, starting from day 9.0 of embryogenesis. By comparing the spatio-temporal distribution of Galectin-1 transcripts in control and Myf5 null mutant embryos, it was established that it acts downstream of Myf5. However, early myogenesis did not seem to be affected in Galectin-1 null mutant embryos indicating that, unlike in adult, Galectin-1 does not play a role in muscle fate acquisition during development (Shoji et al. 2009).

Knockdown experiments in zebrafish embryos targeted to the 5′-UTR sequence of DrGal-1-L2 resulted in a phenotype with a bent tail and disorganized muscle fibers. However, DrGal-1-L1 knockdown embryos showed no similar morphological defects, indicating that the observed effects are sequence-specific, and not due to the toxicity of the morpholino-modified oligos. Further, ectopic expression of native DrGal-1-L2 specifically rescued the phenotype. These results suggest that galectins produced by notochord play a key role in somatic cell differentiation and development (Ahmed et al. 2009b).

Incorporation of Vitronectin and Chondroitin Sulfate B in ECM of SMC: Gal-1 binds to a number of ECM components in ß-galactoside-dependent manner in following order: laminin > cellularfibronectin > thrombospondin > plasma fibronectin > vitronectin > osteopontin (Moiseeva et al. 2000, 2003). Laminin and cellular fibronectin are glycoproteins which are highly N-glycosylated with bi- and tetra-antennary poly-N-lactosamines (Carsons et al. 1987). Gal-1 is also involved in ECM assembly and remodeling: it inhibits the incorporation of vitronectin and chondroitin sulphate B into ECM of vascular smooth muscle cells (Moiseeva et al. 2003); the interaction seems to depend on vitronectin conformation since it preferentially recognizes unfolded vitronectin multimers rather than inactive folded monomers (Moiseeva et al. 2003). Some of the major cell surface-binding partners of Gal-1 have been reviewed (Camby et al. 2006).

10.6.6 Gal-1 and the Immune System

10.6.6.1 Galectin-1 Modulates Immune System in a Number of Ways

Galectin-1 produced by thymic epithelial cells causes apoptosis in human thymocytes (Perillo et al. 1997). In peripheral blood, galectin-1 causes apoptosis of activated T cells, but it supports the survival of naive T cells (Vespa et al. 1999; Perillo et al. 1995; Endharti et al. 2005). Galectin-1 has also been proposed to shift the T cell polarization reaction from Th1 to Th2 by triggering apoptosis in Th1 cells (Rabinovich et al. 2004; Toscano et al. 2007). Galectin-1 also promotes surface exposure of phosphatidylserine (PS) without accompanying apoptosis in human T cell lines (Stowell et al. 2007). Galectins in general and Gal-1 in particular, are known to be deeply involved in the initiation, amplification, and resolution of inflammatory responses (Almkvist and Karlsson 2004).

Galectin-1 has pleiotropic immunomodulatory functions, including regulation of lymphocyte survival and cytokine secretion in autoimmune, transplant disease, and parasitic infection models. Galectins are differentially expressed by various immune cells and their expression levels appear to be dependent on cell differentiation and activation. Galectin-1 inhibits clonal expansion and induces apoptosis of antigen-primed T lymphocytes and suppresses the development of T-cell-mediated autoimmune diseases in vivo. Since galectin-1 is expressed in activated but not resting T cells, it has been hypothesized that Gal-1-induced apoptosis may constitute an autocrine suicide mechanism to eliminate activated T cells leading to the termination of an effector immune response. Gal-1 plays a key role in human platelet activation and function. It binds to human platelets in a carbohydrate-dependent manner and synergizes with ADP or thrombin to induce

platelet aggregation and ATP release. Furthermore, Gal-1 induces F-actin polymerization, up-regulation of P-selectin, and GPIIIa expression; promotes shedding of microvesicles and triggers conformational changes in GPIIb/IIIa (Pacienza et al. 2008).

Innate Immune Functions for Galectin-1: Inhibition of Cell Fusion by Nipah Virus Envelope Glycoproteins: Nipah virus (NiV) is an emerging pathogen that causes severe febrile encephalitis. The primary targets of NiV are endothelial cells. NiV envelope-mediated cell-cell fusion is blocked by Gal-1. This inhibition is specific to the Paramyxoviridae family because Gal-1 did not inhibit fusion triggered by envelope glycoproteins of other viruses. The physiologic dimeric form of Gal-1 was required for fusion inhibition since a monomeric Gal-1 mutant had no inhibitory effect on cell fusion. Gal-1 binds to specific N-glycans on NiV glycoproteins and aberrantly oligomerizes NiV. Gal-1also increases pro-inflammatory cytokines such as IL-6, known to be protective in the setting of other viral diseases such as Ebola infections. Thus, Gal-1 may have direct antiviral effects and may also augment the innate immune response against this emerging pathogen (Levroney et al. 2005).

Oxidative Burst and Degranulation of Porcine Neutrophils by Galectin-1: Local galectin-1 concentrations under physiological conditions might reach suitable levels for pig PMN stimulation, and might be a natural inducer of O_2^- formation or degranulation. Porcine galectins might produce enhanced responses in vivo when they stimulate neutrophils in combination with some other stimuli (Elola et al. 2005b). Galectin-1 tunes TCR binding and signal transduction to regulate CD8 burst size. T cell burst is regulated by duration of TCR engagement and balanced control of Ag-induced activation, expansion, and apoptosis. Galectin-1-deficient CD8 T cells undergo greater cell division in response to TCR stimulation, with fewer dividing cells undergoing apoptosis. TCR-induced ERK signaling was sustained in activated galectin-1-deficient CD8 T cells and antagonized by recombinant galectin-1, indicating galectin-1 modulates TCR feed-forward/feedback loops involved in signal discrimination and procession. Therefore, galectin-1, inducibly expressed by activated CD8 T cells, functions as an autocrine negative regulator of peripheral CD8 T cell TCR binding, signal transduction, and burst size. Together with recent findings demonstrating that Gal-1 promotes binding of agonist tetramers to the TCR of OT-1 thymocytes, these studies identify galectin-1 as a tuner of TCR binding, signaling, and functional fate determination that can differentially specify outcome, depending on the developmental and activation stage of the T cell (Liu et al. 2009b).

10.6.6.2 Activity of Galectin-1 at the Crossroad of Innate and Adaptive Immunity

Gal-1 can act as a link between innate and adaptive immunity by modulating the physiology of neutrophils, monocytes, and dendritic cells. The naturally occurring population of dedicated regulatory T cells that co-express $CD4^+$ and $CD25^+$ plays a key role in the maintenance of peripheral T-cell tolerance, though their mechanism of action has remained obscure. Galectin-1 shows immunoregulatory activity in vivo in experimental models of autoimmunity and cancer. Galectin-1 affects T cell fate and regulates monocyte and macrophage physiology. Treatment with galectin-1 in vitro differentially regulates constitutive and inducible FcγRI expression on human monocytes and FcγRI-dependent phagocytosis. In addition, galectin-1 inhibits IFN-γ-induced MHC class II expression and MHC-II-dependent Ag presentation in a dose-dependent manner. These regulatory effects were also evident in mouse macrophages following treatment with r-galectin-1 and in galectin-1-deficient mice. In these functions galectin-1 does not appear to affect survival of human monocytes, but rather influences FcγRI- and MHC-II-dependent functions through active mechanisms involving modulation of an ERK1/2-dependent pathway (Barrionuevo et al. 2007).

Gal-1-Matured Human Monocyte-Derived DCs: Galectin-1 induces a phenotypic and functional maturation in human monocyte-derived DCs (MDDCs) similar to but distinct from the activity of the exogenous pathogen stimuli, LPS. Immature human MDDCs exposed to galectin-1 up-regulated cell surface markers characteristic of DC maturation (CD40, CD83, CD86, and HLA-DR), secreted high levels of IL-6 and TNF- α, stimulated T cell proliferation, and showed reduced endocytic capacity, similar to LPS-matured MDDCs. However, unlike LPS-matured DCs, galectin-1-treated MDDCs did not produce the Th1-polarizing cytokine IL-12. In addition to modulating many of the same DC maturation genes as LPS, galectin-1 also uniquely up-regulated a significant subset of genes related to cell migration through the ECM. Findings suggested that galectin-1 is an endogenous activator of human MDDCs that up-regulates a significant subset of genes distinct from those regulated by an exogenous stimulus (LPS). The unique effect of galectin-1 on increase in DC migration through ECM suggests that galectin-1 is an important component in initiating an immune response (Fulcher et al. 2006).

Tolerogenic Signals Delivered by DCs to T Cells Through a Gal-1-Driven Immunoregulatory Circuit: Gal-1-DC represent a novel tool to control differentially the afferent and efferent arms of the T cell response (Perone et al. 2006b). Galectin-1 can endow DCs with tolerogenic

potential. After exposure to Gal-1, DCs acquired an IL-27--dependent regulatory function, promoted IL-10-mediated T cell tolerance and suppressed autoimmune neuroinflammation. Consistent with its regulatory function, Gal-1 had its highest expression on DCs exposed to tolerogenic stimuli and was most abundant from the peak through the resolution of autoimmune pathology. DCs lacking Gal-1 had greater immunogenic potential and an impaired ability to halt inflammatory disease. These studies identify a tolerogenic circuit linking Gal-1 signaling, IL-27-producing DCs and IL-10-secreting T cells, which has broad therapeutic implications in immunopathology (Ilarregui et al. 2009).

Regulation of BCR Signaling Through an Association OCA-B with Galectin-1: OCA-B is a B cell-specific transcriptional co-activator for OCT factors during the activation of immunoglobulin genes. In addition, OCA-B is crucial for B cell activation and germinal center formation. Yu et al. (2006) identified galectin-1, and related galectins as a OCA-B-interacting protein. The galectin-1 binding domain in OCA-B is localized to the N terminus of OCA-B. In B cells lacking OCA-B expression, increased galectin-1 expression, secretion, and cell surface association are observed. Galectin-1 is shown to negatively regulate B cell proliferation and tyrosine phosphorylation upon BCR stimulation. Results raised the possibility that OCA-B may regulate BCR signaling through an association with galectin-1.

Galectin-1-Dependent pre-B Cell Receptor Relocalization and Activation: Interactions between B cell progenitors and bone marrow stromal cells are essential for normal B cell differentiation. It has been noticed that an immune developmental synapse is formed between human pre-B and stromal cells in vitro, leading to the initiation of signal transduction from the pre-BCR. This process relies on the direct interaction between the pre-BCR and the stromal cell-derived Gal-1 and is dependent on Gal-1 anchoring to cell surface glycosylated counter-receptors, present on stromal and pre-B cells (Gauthier et al. 2002, Vas et al. 2005). Rossi et al. (2006) identified α4β1 (VLA-4), α5β1 (VLA-5), and α4β7 integrins as major Gal-1-glycosylated counter-receptors involved in synapse formation. Pre-B cell integrins and their stromal cell ligands (ADAM15/fibronectin), together with the pre-BCR and Gal-1, form a homogeneous lattice at the contact area between pre-B and stromal cells. Results suggest that during pre-B/stromal cell synapse formation, relocalization of pre-B cell integrins mediated by their stromal cell ligands drives pre-BCR clustering and activation in a Gal-1-dependent manner.

In late-stage B cell activation and maturation, soluble galectin-1 produced by activated B cells resulting from *Trypanosoma cruzi* infection in mice causes T cell apoptosis and affects IFN- production (Zuniga et al. 2001). Galectin-1 regulates apoptotic pathways in human naive and IgM^+ memory B cells through altering balances in Bcl-2 family proteins (Tabrizi et al. 2009). Intracellular galectin-1 may associate with B cell-specific Oct-1-associated coactivator, OCA-B, to negatively regulate BCR signaling (Yu et al. 2006).

Galectin-1: A Key Effector of Regulation Mediated by $CD4^+CD25^+$ T Cells: Galectin-1 is over-expressed in regulatory T cells, and its expression is increased after activation. Recent evidence indicates that Gal-1 contributes to the immunosuppressive activity of $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells. *In vivo*, direct administration of Gal-1 suppresses chronic inflammation in experimental models of autoimmunity by skewing the balance of the immune response toward a T_H2 cytokine profile. Analysis of the mechanistic basis of this anti-inflammatory effect revealed that T_H1 and T_H17-differentiated cells share a common glycan motif, which can be specifically targeted by Gal-1, providing a novel link between differential glycosylation, susceptibility to cell death, and the regulation of the inflammatory response. Most importantly, blockade of Gal-1 binding significantly reduced the inhibitory effects of human and mouse $CD4^+CD25^+$T cells. Reduced regulatory activity was observed in $CD4^+CD25^+$ T cells obtained from Gal-1-homozygous null mutant mice. These results suggested that Gal-1 is the key effector of the regulation mediated by these cells (Garin et al. 2007). In addition, expression of Gal-1 at sites of tumor growth and metastasis can influence tumor progression by regulating cell-cell and cell-matrix interactions, tumor cell invasiveness, and angiogenesis. Furthermore, Gal-1 can also function as a soluble mediator employed by tumor cells to evade the immune response (c/r Pacienza et al. 2008).

Galectin-1 Co-clusters CD43/CD45 on DCs and Induces Cell Activation: Galectin-1 activates human monocyte-derived dendritic cells (MDDCs) and triggers a specific genetic program that up-regulates DC migration through the extracellular matrix, an integral property of mucosal DCs. Fulcher et al. (2009) identified the Gal-1 receptors on MDDCs and immediate downstream effectors of Gal-1-induced MDDC activation and migration. Galectin-1 binding to surface CD43 and CD45 on MDDCs induced an unusual unipolar co-clustering of these receptors and activates calcium flux that is abrogated by lactose. Syk and protein kinase C tyrosine kinases are effectors of the DC activation by Gal-1. Galectin-1, but not lipopolysaccharide, stimulated Syk phosphorylation and recruitment of phosphorylated Syk to the CD43 and CD45 co-cluster on MDDCs. Inhibitors of Syk and protein kinase C signaling abrogated galectin-1-induced DC activation as monitored by interleukin-6 production; and MMP-1, -10, and -12 gene up-regulation; and enhanced migration through the extracellular

matrix. The latter two are specific features of galectin-1-activated DCs. Interestingly, galectin-1 can also prime DCs to respond more quickly to low dose lipopolysaccharide stimulation (Fulcher et al. 2009).

Cross-Linking of GM1 Ganglioside by Gal-1 Mediates Regulatory T Cell Activity: Several autoimmune disorders are suppressed in animal models by treatment with GM1 cross-linking units of certain toxins such as B subunit of cholera toxin (CtxB). GM1 being a binding partner for Gal-1, which is known to ameliorate symptoms in certain animal models of autoimmune disorders, such as murine experimental autoimmune encephalomyelitis (EAE) and further highlighted the role of GM1 in demonstrating enhanced susceptibility to EAE. Results indicate GM1 in murine $CD4^+$ and $CD8^+$ effector T (T_{eff}) cells to be primary target of Gal-1 expressed by T_{reg} cells, the resulting co-cross-linking and TRPC5 channel activation contributing to the mechanism of autoimmune suppression (Wang et al. 2009).

Galectin-1 Affects the Cross-Liking of HIV-1 Infection: The HIV-1 infection is initiated by the stable attachment of the virion to the target cell surface. Although this process relies primarily upon interaction between virus-encoded gp120 and cell surface CD4, a number of other interactions may influence binding of HIV-1 to host cells. For example, galectin-1 acts as a soluble adhesion molecule by facilitating attachment of HIV-1 to the cell surface. Experiments using fusion inhibitor T-20 confirmed that galectin-1 is primarily affecting HIV-1 attachment. Therefore, it was proposed that galectin-1, which is released in an exocrine fashion at HIV-1 replication sites, can cross-link HIV-1 and target cells and promote a firmer adhesion of the virus to the cell surface, thereby augmenting the efficiency of the infection process. Overall, findings suggest that galectin-1 might affect the pathogenesis of HIV-1 infection (Ouellet et al. 2005). Galectin-1 is known to interact for example with ganglioside GM1 and also the hydrophobic tail of oncogenic H-Ras. Observations indicate the potential of galectin-1 to affect membrane properties beyond the immediate interaction with cell surface epitopes (Gupta et al. 2006).

10.6.7 Role of Galetin-1 and Other Systems

Galectin-1 in Keratinocytes

Multipotent stem cells are localized in the bulge region of the outer root sheath of hair follicles, while stem cells giving rise to interfollicular epidermis reside in its basal. Galectin-1 reactivity is present in squamous epithelial cells (Chovanec et al. 2004; Klima et al. 2005). Since keratin 19 and nuclear reactivity to galectin-1 are potential markers of epidermal stem cells, Dvorankova et al. (2005) detected the expression of these markers in adult cells migrating from the hair follicle, where cells expressing keratin 19 are located in the bulge region. Observations indicated the transient expression of keratin 19 and nuclear galectin-1 binding sites in originally negative interfollicular epidermal cells induced by adhesion. Studies on expression of the lysosome-associated membrane protein 1 (Lamp-1) and expression of the epidermal galectins-1, -3 and -7, in human keratinocytes indicated that the up-regulated Lamp-1 expression at confluence could be involved in keratinocyte differentiation, but apparently not through interaction with galectin-3 (Sarafian et al. 2006).

Reproductive Tissues

Gal-1 has been detected in pig granulosa cell lysates. The lectin stimulated the proliferation of granulose cells from pig ovaries in culture and inhibited the FSH-stimulated progesterone synthesis of granulosa cells. It appeared to interfere with the receptor-dependent mechanism of FSH-stimulated progesterone production. It was suggested that Gal-1 exerts its inhibitory effect on steroidogenic activity of granulosa cells by interfering with the hormone-receptor interaction resulting in decreased responses to FSH stimulation (Walzel et al. 2004). In human endometrium, Gal-1 was localized mainly in stromal cells, whereas Gal-3 was predominantly found in epithelial cells. Cycle-dependent expression of Gal-1 in stromal cells and Gal-3 in epithelial cells suggested these lectins to be involved in the regulation of different endometrial cellular functions (von Wolff et al. 2005). A possible implication of galectins-1 and -3 has been indicated in the invasiveness of the transformed trophoblastic cell (Bozic et al. 2004).

Galectin-1 in Fetomaternal Tolerance

Phylogenetic footprinting and shadowing unveiled conserved cis motifs, including an estrogen responsive element in the 5′ promoter of *LGALS1* that could account for sex steroid regulation of *LGALS1* expression, suggesting a role of Gal-1 in immune-endocrine cross-talk and emergence of hormonal and redox regulation of Gal-1 in placental mammals at maternal-fetal immune tolerance. Galectin-1 is expressed in immune privileged sites and is implicated in establishing maternal-fetal immune tolerance, which is essential for successful pregnancy in eutherian mammals. A successful pregnancy requires synchronized adaptation of maternal immune-endocrine mechanisms to the fetus. Gal-1 has a pivotal role in conferring fetomaternal tolerance. Consistently with a marked decrease in Gal-1 expression during failing pregnancies, Gal-1-deficient mice showed higher rates of fetal loss compared to wild-type mice in allogeneic matings, whereas fetal survival was unaffected in syngeneic matings. Thus, Gal-1 is a pivotal regulator of fetomaternal tolerance that has potential therapeutic implications in threatened pregnancies (Blois et al. 2007;

Ilarregui et al. 2009). Gal-1 may be involved in the regulation of the inflammatory responses to chorioamniotic infection (Than et al. 2008a, b). Gal-1, Gal-1 ligand, Thomsen-Friedenreich (TF) (Galβ1-3GalNAc-) and Gal-3 are expressed and up-regulated on the membrane of extravillous trophoblast (EVT) in preeclamptic placentas. In addition, the expression of Gal-1 is significantly up-regulated in decidual tissue of pre-eclamptic placentas and villous trophoblast tissue of HELLP placentas (Than et al. 2008b). Jeschke et al. (2007) speculated that expression of both galectins and TF on the membrane of preeclamptic EVT and up-regulation of Gal-1 in preeclamptic decidual cells may at least in part compensate for the apoptotic effects of maternal immune cells.

Interaction Between Chondrocytes and a Lactose-Modified Chitosan

Gal-1 plays an important role in enhancing cell adhesion to extracellular matrix and inducing cell proliferation. Chitosan is a derivative of chitin extracted from lobsters, crabs and shrimps' exoskeletons. Although chitosan membranes show no cytotoxicity, some cell types (e.g. 3 T3 cells) fail to attach and proliferate on their surface. Over-expression of Gal-1 does not enhance 3 T3 cell proliferation on chitosan membranes. However, coating the chitosan membrane with recombinant Gal-1 proteins significantly expedites 3 T3 cells proliferation. Findings support a role for altered levels of protein phosphorylation in Gal-1-mediated cell attachment and proliferation on chitosan membranes (Chang et al. 2004). The Chitlac glycopolymer has been shown to promote pig chondrocyte aggregation and to induce extracellular matrix production. Recombinant Galectin-1 interacts in a dose-dependent manner with Chitlac. Expression level of galectin-1 gene was significantly higher in chondrocytes cultivated on Chitlac. Data indicated the role of Galectin-1 as a bridging agent between Chitlac and chondrocyte aggregates (Marcon et al. 2005).

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Tandem-Repeat Type Galectins

11

Anita Gupta

Hirabayashi and Kasai (1993) proposed designating galectin subfamilies as proto-, tandem-repeat, and chimera- types based on their domain organization. The prototype galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) consist of a single CRD with a short N-terminal sequence and have been discussed in Chap. 9, The tandem-repeat type galectins (galectin-4, -6, -8, -9, and -12) are composed of two non-identical CRDs joined by a "hinge" region (short linker peptide sequence) and form a sub-family of galectins (see Fig. 9.2). The single chimera-type galectin (galectin-3) has one CRD and an extended N-terminal tail containing several repeats of proline-tyrosine-glycine-rich motif and will be discussed in Chap. 12. Alternative splicing leads to the formation of distinct splice variants (isoforms) of galectin-8 and galectin-9 with tandem-repeat-type structures. Galectin-8- consists of several isoforms, each made of two domains of approximately 140 amino-acids, both having a CRD. These domains are joined by a 'link peptide' of variable length. The isoforms share identical CRDs and differ only in linker region.

11.1 Galectin 4

11.1.1 Localization and Tissue Distribution

Galectin-4 belongs to a subfamily of galectins composed of two CRD within the same peptide chain. The two domains have all conserved galectin signature amino acids, but their overall sequences are only ~40% identical. Both domains bind lactose with a similar affinity as other galectins, but their respective preferences for other disaccharides, and larger saccharides, are distinctly different. Thus galectin-4 has a property of a natural cross-linker, but in a modified sense each domain prefers a different subset of ligands. Similarly to other galectins, galectin-4 is synthesized as a cytosolic protein, but can be externalized. During development and in adult normal tissues, galectin-4 is expressed only in alimentary tract, from the tongue to the large intestine. It is often found in relatively insoluble complexes, as a component of either adherens junctions or lipid rafts in the microvillus membrane, and it has been proposed to stabilize these structures. Strong expression of galectin-4 can be induced, however, in cancers from other tissues including breast and liver. Within a collection of human epithelial cancer cell lines, galectin-4 is over-expressed and soluble in those forming highly differentiated polarized monolayers, but absent in less differentiated ones. In cultured cells, intracellular galectin-4 may promote resistance to nutrient starvation, whereas—as an extracellular protein—it can mediate cell adhesion. Because of its distinct induction in breast and other cancers, it may be a valuable diagnostic marker and target for the development of inhibitory carbohydrate-based drugs (Huflejt and Leffler 2004). EM examination of galectin 4 confirmed highly elevated levels of the protein in endocrine, parietal, and chief cells in weaned rats than in suckling rats. Galectin 4 was strongly localized in weaned rats than in suckling rats in the nuclei of all cell types and in secretory granules in endocrine and chief cells, suggesting that galectin 4 is implicated in nuclear events and perhaps in secretory processes (Niepceron et al. 2004).

Galectin-4 has been found to be localized in the epithelium of the alimentary tract, including oral mucosa, esophagus, and intestinal mucosa (Chiu et al. 1994; Wooters et al. 2005a). In gastric mucosa, galectin 4 was present at lower levels in suckling than in weaned rats, but mRNA levels did not change significantly during postnatal development. It was more strongly localized in the nuclei of all cell types and in or over secretory granules in endocrine and chief cells of weaned rats compared to suckling rats, suggesting that galectin 4 is implicated in nuclear events and perhaps in secretory processes (Niepceron et al. 2004). Chiu et al (1994) found that galectin-4 in porcine oral and upper esophageal epithelium is water-insoluble as a component of adherens junction complexes. Danielsen and Deurs (1997) reported that galectin-4 forms detergent-insoluble complexes with apically sorting brush-border enzymes in

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porcine small intestine, when the isolation was performed at low temperature. On the contrary, Huflejt et al. (1997) showed the localization of galectin-4 on the basal membrane of human colon adenocarcinoma T84 cells in a confluent and polarized condition, whereas galectin-3 tends to be concentrated in granular inclusions mostly localized on the apical side. They also showed that galectin-4 was concentrated at the leading edges of lamellipodia in semiconfluent T84 cells. These results suggest that galectin-4 is located in a different area of cells dependently on tissues and culture conditions and that it plays a role in cell adhesion or cell migration.

Mouse galectin-4 is expressed in small intestine, colon, liver, kidney, spleen, heart and P19X1 cells in BW-5147 and 3T3 cell lines. Galectin-4 is expressed in spermatozoons and oocytes and its expression during early mouse embryogenesis appears in eight-cell embryos and remains in later stages. Mature epithelial cells at the villous tips stained the most intensely with the mAb, with progressively less intense staining observed along the sides of villi and into the crypts. Galectin-4 was also associated with nuclei in villous tip cells, indicating that some galectin-4 may migrate to the nucleus during terminal maturation of these cells. In intestinal crypts, a specific subset of cells, which may be enteroendocrine cells, expressed galectin-4 at a relatively high level (Wooters et al. 2005a). Lactose-binding proteins with molecular masses of 14-, 17-, 18-, 28-, and 34-kDa are present in extracts from porcine small intestinal mucosa. Thirty four-kilodalton protein, galectin-4 was the most abundant of these proteins. RT-PCR identified two galectin-4 isoforms that differed in the length of their linker region. The larger isoform, galectin-4.1, is nine amino acids longer in its linker region than the smaller isoform, galectin-4.2. Based on nucleotide sequence similarities, the two isoforms are likely splice variants of galectin-4 pre-mRNA and not products of separate genes like murine galectins-4 and -6 (Wooters et al. 2005b).

Nio et al. (2005) detected signals for five galectin subtypes (galectin-2, -3, -4/6, and -7) exclusively in the epithelia of gut. In the glandular stomach, galectin-2 and -4/6 were predominantly expressed from gastric pits to neck of gastric glands. The small intestine exhibited intense, maturation-associated expressions of galectin-2, -3, and -4/6 mRNAs. Galectin-2 was intensely expressed from crypts to the base of villi, whereas transcripts of galectin-3 gathered at villous tips. Signals for galectin-4/6 were most intense at the lower half of villi. Galectin-2 was also expressed in goblet cells of the small intestine but not in those of the large intestine. In the large intestine, galectin-4/6 predominated, and the upper half of crypts simultaneously contained transcripts of galectin-3. Stratified epithelium from the lip to fore-stomach and anus intensely expressed galectin-7 with weak expressions of galectin-3. To assess intestinal lipid rafts functions through the characterization of their protein markers, Nguyen et al. (2006) isolated lipid rafts of rat mucosa. Membrane preparations were enriched in cholesterol, ganglioside GM1, and N aminopeptidase (NAP) known as intestinal lipid rafts markers. Together results indicated that some digestive enzymes, trafficking and signaling proteins may be functionally distributed in the intestine lipid rafts. Mucosal epithelium in mouse showed region/cell-specific localization of each galectin subtype. Gastric mucous cells exhibited intense immunoreactions for galectin-2 and galectin-4/6 with a limited localization of galectin-3 at the surface of gastric mucosa. Epithelial cells in small intestine showed characteristic localizations of galectin-2 and galectin-4/6 in the cytoplasm of goblet cells and the basolateral membrane of enterocytes in association with maturation, respectively. Epithelial cells of large intestine contained intense reactions for galectin-3 and galectin-4/6 but not for galectin-2. The stratified squamous epithelium of the forestomach was immunoreactive for galectin-3 and galectin-7. Outside the epithelium, Only galectin-1 was localized in the connective tissue, smooth muscles, and neuronal cell bodies. The subtype-specific localization of galectin suggests its important roles in host-pathogen interaction and epithelial homeostasis such as membrane polarization and trafficking in the gut (Nio-Kobayashi et al. 2009).

11.1.2 Galectin-4 Isoforms

Lactose-binding proteins with molecular masses of 14-, 17-, 18-, 28-, and 34-kDa were identified in extracts from porcine small intestinal mucosa. The most abundant of these proteins, was identified as porcine galectin-4. Galectin-4 is a member of the tandem-repeat subfamily of monomer divalent galectins. Galectin-4 (LGALS4) is encoded in humans by *LGALS4* gene. Galectin-4 was initially discovered as a soluble 17-kDa lectin in rat intestinal extracts by Leffler et al. (1989); later the cDNA cloning of galectin-4 revealed that it is a 36-kDa protein (Oda et al. 1993). The 323-amino acid galectin-4 protein contains two homologous, ~150-amino acid carbohydrate recognition domains and all amino acids typically conserved in galectins. Wooters et al. (2005b) identified two galectin-4 isoforms that differed in the length of their linker region. The larger isoform, galectin-4.1, is nine amino acids longer in its linker region than the smaller isoform, galectin-4.2. Based on nucleotide sequence similarities, the two isoforms are likely splice variants of galectin-4 pre-mRNA and not products of separate genes like murine galectins-4 and -6. The crystal of N-terminal domain, CRD1, of mGal-4 in complex with lactose belongs to tetragonal space group $P42_12$ with unit-cell parameters $a = 91.1$, $b = 91.16$, $c = 57.10$ A The initial electron-density maps of X-ray diffraction indicated the presence of one lactose molecule. The crystal structure of CRD1of mouse Gal-4 in complex

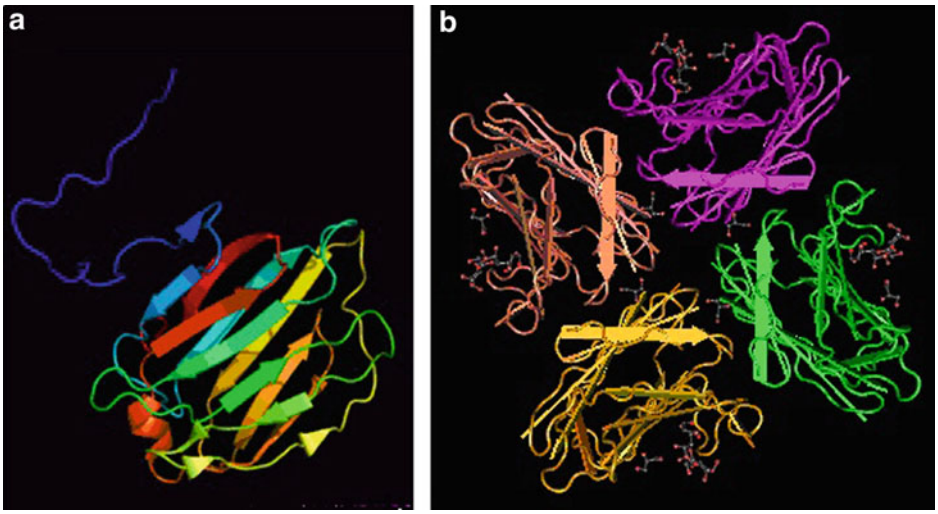

Fig. 11.1 (**a**) Solution structure of the C-terminal domain of galbinding lectin domain of human galectin-4 by NMR (PDB ID:1X50). The 323-amino acid LGALS4 protein contains two homologous, ~150-amino acid CRDs (Tomizawa et al. PDB ID: 1X50). (**b**) Structure of N-terminal CRD1of mouse Gal-4 in complex with lactose (PDB: ID 3I8T) (Krejčiříková et al. 2011)

with lactose was reported recently (Fig. 11.1b). Two lactose-binding sites were identified: a high-affinity site with a K_{D1} value of 600 ± 70 μM and a low-affinity site with K_{D2} = 28 ± 10 mM (Krejčiříková et al. 2008, 2011). Solution Structure of the C-terminal domain of Galactose-binding lectin domain of human Gal-4 by NMR (PDB ID:1X50 is shown in Fig. 11.1a.

Multiple members of galectin family are expressed in primary olfactory system. In particular, galectin-3 is expressed by ensheathing cells surrounding nerve fascicles in the submucosa and nerve fiber layer, where it may mediate cross-linking of axons. Galectin-4, -7, and -8 are expressed by the primary olfactory axons as they grow from the nasal cavity to the olfactory bulb. A putative role for nitric oxide donor compound-7 (NOC-7) and NOC-8 in axon fasciculation and the expression of multiple galectins in the developing olfactory nerve suggest that these molecules may be involved in the formation of this pathway, particularly in the sorting of axons as they converge towards their target (Storan et al. 2004). Cell-specific surface carbohydrates and carbohydrate-binding proteins within embryonic nervous system have raised the possibility that carbohydrate recognition contribute to the interactions of developing neurons. A cDNA from rat brain encodes a lectin, RL-14.5, which is homologous and identical in primary sequence to a lectin present in non-neural tissue. High levels of RL-14.5 mRNA are present in primary sensory neurons and motor neurons in the spinal cord and brain stem. The selective expression of oligosaccharide ligands for RL-14.5 on the same neurons are consistent with the idea that carbohydrate-mediated interactions contribute to the development of this subset of mammalian neurons (Hynes et al. 1990).

11.1.3 Gal-4 from Rodents and Other Animals

Gal-4 from Rat Intestine: Of the multiple soluble lactose-binding lectins in rat intestine, the major one, tentatively designated RI-H, was isolated as a polypeptide of M_r 17,000 Da. Surprisingly the cDNA encodes a protein of M_r 36,000, and this protein contains two homologous but distinct domains each with sequence elements that are conserved among all S-Lac lectins. The C-terminal domain, designated domain II, corresponds to lectin with M_r of 17,000 previously isolated from intestinal extracts and shown to have lactose binding activity. The new lectin, which was designated L-36, is highly expressed in full-length form in rat small and large intestine and stomach but was not detected in other tissues including lung, liver, kidney, and spleen. Each domain has approximately 35% sequence identity with the other domain and with the carbohydrate-binding domain of L-29, another S-Lac lectin, but only about 15% identity with other known S-Lac Lectins (Oda et al. 1993). Tardy et al. (1995) detected five β-galactoside binding lectins of 14–20 kDa in rat small intestinal mucosa and purified the prominent proteins of 17 and 19 kDa. Direct N-terminal sequencing of 17 kDa protein and intrachain sequencing of 19 kDa protein produced sequences which are part of N-terminal domain of L-36/galectin-4. The 17 and 19 kDa lectins were related to 36 kDa protein in human undifferentiated HT29 cells (Tardy et al. 1995).

Adherens Junction Lectin: A pig junction protein of M_r 37,000 was found in oral epithelium but not in epidermis, limited to suprabasal cells, and colocalizing with adherens junction proteins. Secondary structure predictions indicated that the 37% identical 16 to 17-kDa N- and C-terminal

domains from β-sheet-rich barrels are linked by a compact proline-rich segment. The protein is 72% identical in amino acid sequence and shares symmetrical two-domain structure with L-36, a lectin from rat intestine, indicating that the 37-kDa protein is the porcine form of L-36. The expressed protein binds a glycoprotein of 120 kDa from pig tongue epithelium and also inhibited by lactose. The lectin may be involved in the assembly of adherens junctions (Chiu et al. 1994).

Rabbit Bladder Galectin-4: The cDNA encoding rabbit bladder galectin-4 has been cloned and sequenced. The deduced 328 amino acid sequence predicts a multidomain structure consisting of an N-terminal peptide (19 residues) and two CRD (130 residues each) connected by a linker region (49 residues). Comparison of rabbit galectin-4 with related proteins reveals that two peptide motifs, M-A-F/Y-V-P-A-P-G-Y-Q-P-T-Y-N-P-T-L-P-Y in the N terminus and A-F-H-F-N-P-R-F-D-G-W-D-K-V-V-F in the first CRD are highly conserved in human, pig, rat, and mouse galectin-4 as well as in mouse galectin-6. The two peptide motifs were proposed as the signature sequences to identify new members of galectin-4 subfamily (Jiang et al. 1999). Comparison of rabbit galectin-4 sequence with those of human, pig, rat, and mouse revealed two invariant peptide motifs that are proposed as signature sequences for identifying related galectins (Bhavanandan et al. 2001).

Endogenous lactose binding lectin in retina has a subunit molecular weight of 16 kDa and a pI about 4.5 (Beyer et al. 1980) or C-16 (Sakakura et al. 1990) form of chicken endogenous soluble lactose-binding lectins. Retinal lectin might have a functional role during terminal differentiation of retinal cells (Castagna and Landa 1994). Two types of lectins have been isolated from extracts of axolotl (Ambystoma mexicanum) larvae: A thiol-independent lectin of subunit of 15 kDa and a thiol-dependent lectin of subunit of 18 kDa (Allen et al. 1992). A 16-kDa lactose-binding lectin comprises 5% or more of soluble protein in *Xenopus laevis* skin. This lectin is localized in the cytoplasm of granular gland cells and released onto skin surface by holocrine secretion in response to stress. Comparison of peptide sequences revealed expression of at least two isolectins, which differ in sequence at only two or three amino acids (Marschal et al. 1992).

11.1.4 Ligands for Galectin-4

Interaction with Linear Tetrasaccharides: The combining sites of mammalian galectins have overlapping carbohydrate specificities. Wu et al. (2002) analyzed CRD-I near the N-terminus of recombinant rat galectin-4 and suggested that among 35 glycans tested for lectin binding, galectin-4 reacted best with human blood group ABH precursor glycoproteins, and asialo porcine salivary glycoproteins, which contain high densities of blood group Ii determinants Galβ1-3GalNAc (the mucin-type sugar sequence on the human erythrocyte membrane) and/or GalNAcα1-Ser/Thr (Tn), whereas this lectin domain reacted weakly or not at all with most sialylated glycoproteins. Among oligosaccharides tested, Galβ1-3GlcNAcβ1-3Galβ1-4Glc was the best. Galectin-4 has a preference for the β-anomer of Gal at the non-reducing ends of oligosaccharides with a Galβ1-3 linkage, over Galβ1-4 and Galβ1-6. The fraction of Tn glycopeptide from asialo ovine submandibular glycoprotein was 8.3 times more active than Galβ1-3GlcNAc. The overall carbohydrate specificity of Galectin-4 can be defined as Galβ1-3GlcNAcβ1-3Galβ1-4Glc (lacto- N-tetraose) > Galβ1-4GlcNAcβ1-3Galβ1-4Glc (lacto- N -neo-tetraose) and Tn clusters > Galβ1-4Glc and GalNAcβ1-3Gal > Galβ1-3GalNAc > Galβ1-3GlcNAc > Galβ1-4GlcNAc > GalNAc > Gal. The definition of this binding profile provides the basis to detect differential binding properties relative to the other galectins with ensuing implications for functional analysis (Wu et al. 2002).

The core aspects of the carbohydrate specificity of domain-I of recombinant tandem-repeat-type galectin-4 from rat gastrointestinal tract (G4-N), especially its potent interaction with the linear tetrasaccharide Galβ1-3GlcNAcβ1-3Galβ1-4Glc (Iβ1-3L) have been defined. Analysis indicated that a high-density of polyvalent Galβ1-3/4GlcNAc (I/II), Galβ1-3GalNAc (T) and/or GalNAcα1-Ser/Thr (Tn) strongly favors G4-N/glycoform binding. These glycans were up to 2.3×10^6, 1.4×10^6, 8.8×10^5, and 1.4×10^5 more active than Gal, GalNAc, monomeric I/II and T, respectively. The distinct binding features of G4-N established the important concept of affinity enhancement by high density polyvalencies of glycotopes (vs. multiantennary I/II) and by introduction of an ABH key sugar to Galβ1-terminated core glycotopes (Wu et al. 2004).

Binding to Glycosphingolipids: Galectin-4 specifically binds to an $SO3^-$ —> 3Galβ1 —> 3GalNAc pyranoside with high affinity (Ideo et al. 2002). Galectin-4 binds to glycosphingolipids carrying 3-O-sulfated Gal residues, such as SB1a, SM3, SM4s, SB2, SM2a, and GM1, but not to glycosphingolipids with 3-O-sialylated Gal, such as sLc4Cer, snLc4Cer, GM3, GM2, and GM4. Galectin-4 was colocalized with SB1a, GM1, and carcinoembryonic antigen (CEA) in the patches on the cell surface of human colon adenocarcinoma cells. It appeared that SB1a and CEA in the patches on the cell surface of human colon adenocarcinoma cells could be biologically important ligands for galectin-4 (Ideo et al. 2005).

Interaction with Sulfatides: 1-benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (GalNAc α-O-bn), an inhibitor of glycosylation, perturbs apical biosynthetic trafficking

in polarized HT-29 cells suggesting the involvement of a lectin-based mechanism. Galectin-4 is one of the major components of detergent-resistant membranes (DRMs) isolated from HT-29 5M12 cells and also found in post-Golgi carrier vesicles. In galectin-4-depleted HT-29 5M12 cells apical membrane markers accumulated intracellularly. Galectin-4 depletion altered also the DRM association characteristics of apical proteins. Sulfatides with long chain-hydroxylated fatty acids, which were also enriched in DRMs, were identified as high-affinity ligands for galectin-4. It was proposed that interaction between galectin-4 and sulfatides plays a functional role in the clustering of lipid rafts for apical delivery (Delacour et al. 2005). Galectin-4 also binds to cholesterol 3-sulfate, which has no β-galactoside moiety. This characteristic of galectin-4 is unique within the galectin family. The site-directed mutated galectin-4-R45A suggested that Arg-45 of galectin-4 is indispensable for cholesterol 3-sulfate recognition. Results suggested that not only sulfated glycosphingolipids but also cholesterol 3-sulfate are endogenous ligands for galectin-4 in vivo (Ideo et al. 2007).

Murine Galectin-4: Murine (m) mGalectin-4 binds to α-GalNAc and α-Gal A and B type structures with or without fucose. While the CRD2 domain had a high specificity and affinity for A type-2 α-GalNAc structures, the CRD1 domain had a broader specificity in correlation to the total binding profile. Study suggested that CRD2 might be the dominant binding domain of mouse galectin-4. On mouse cryosections, all three forms (CRD1, CRD2 and mGalectin-4) bound to alveolar macrophages, macrophages of red pulp of the spleen and proximal tubuli of the kidney. However, mGalectin-4, but not CRD forms, bound to the suprabasal layer of squamous epithelium of the tongue, suggesting that the link region also plays an important role in ligand recognition (Markova et al. 2006).

11.1.5 Functions of Galectin-4

In absorptive cells, such as the small intestinal enterocyte and the kidney proximal tubule cell, the apical cell membrane is formed as a brush border, composed of regular, dense arrays of microvilli. Hydrolytic ectoenzymes make up the bulk of the microvillar membrane proteins, endowing the brush border with a huge digestive capacity. Several of the major enzymes are localized in lipid rafts, which are organized in a unique fashion, for enterocytes in particular. Glycolipids, rather than cholesterol, together with the divalent lectin galectin-4, define these rafts, which are stable and probably quite large. The architecture of these rafts supports a digestive/absorptive strategy for nutrient assimilation, but also serves as a portal for a large number of pathogens (Danielsen and Hansen 2006). Wasano and Hirakawa (1999) suggested that colorectal Gal-4 may be involved in crosslinking the lateral cell membranes of the surface-lining epithelial cells, thereby reinforcing epithelial integrity against mechanical stress exerted by the bowel lumen.

Role of Galectin-4 as Lipid Raft Stabilizer: The pig small intestinal brush border is a glycoprotein- and glycolipid-rich membrane that functions as a digestive/absorptive surface for dietary nutrients as well as a permeability barrier for pathogens. Galectins 3–4, intelectin, and lectin-like anti-glycosyl antibodies have been localized at brush border of pig small intestine. Together with the membrane glycolipids these lectins form stable lipid raft microdomains that also harbour several major digestive microvillar enzymes. Galectin-2 is enriched in microvillar detergent resistant fraction. Results suggest that galectins offer a maximal protection of the brush border against exposure to bile, pancreatic enzymes and pathogens (Hansen et al. 2005; Thomsen et al. 2009). Lipid rafts (glycosphingolipid/cholesterol-enriched membrane microdomains) have been isolated from many cell types. Superrafts are enriched in galectin-4 and harbor glycosylphosphatidylinositol-linked alkaline phosphatase and transmembrane aminopeptidase N. In microvillar membrane, galectin-4 functions as a core raft stabilizer/organizer for other, more loosely raft-associated proteins. The glycolipid rafts are stabilized by galectin-4 that cross-links galactosyl (and other carbohydrate) residues present on membrane lipids and several brush border proteins, including some of the major hydrolases. These supramolecular complexes are further stabilized by intelectin (Chap. 20) that also functions as an intestinal lactoferrin receptor. As a result, brush border hydrolases, otherwise sensitive to pancreatic proteinases, are protected from untimely release into the gut lumen. Finally, anti-glycosyl antibodies, synthesized by plasma cells locally in the gut, are deposited on the brush border glycolipid rafts, protecting epithelium from lumenal pathogens that exploit lipid rafts as portals for entry to the organism (Braccia et al. 2003; Danielsen and Hansen 2006).

Stechly et al. (2009) presented evidence of a lipid raft-based galectin-4-dependent mechanism of apical delivery of glycoproteins in these cells. First, galectin-4 recruits the apical glycoproteins in detergent-resistant membranes (DRMs) because these glycoproteins were depleted in DRMs isolated from galectin-4-knockdown (KD) HT-29 5M12 cells. DRM-associated glycoproteins were identified as ligands for galectin-4. Structural analysis showed that DRMs were markedly enriched in a series of complex

N-glycans in comparison to detergent-soluble membranes. Second, in galectin-4-KD cells, the apical glycoproteins still exit the Golgi but accumulated inside the cells, showing that their recruitment within lipid rafts and their apical trafficking required the delivery of galectin-4 at a post-Golgi level. This lectin that is synthesized on free cytoplasmic ribosomes is externalized from HT-29 cells mostly in the apical medium and follows an apical endocytic-recycling pathway that is required for the apical biosynthetic pathway. Together, data show that the pattern of N-glycosylation of glycoproteins serves as a recognition signal for endocytosed galectin-4, which drives the raft-dependent apical pathway of glycoproteins in enterocyte-like HT-29 cells.

Galectin-4 in Controlling of Intestinal Inflammation: Galectin-4 is selectively expressed and secreted by intestinal epithelial cells and binds potently to activated peripheral and mucosal lamina propria T-cells at CD3 epitope. The carbohydrate-dependent binding of galectin-4 at CD3 epitope is fully functional and inhibited T cell activation, cycling and expansion. Galectin-4 induced apoptosis of activated peripheral and mucosal lamina propria T cells via calpain-, but not caspase-dependent, pathways. Further, galectin-4 blockade by antisense oligonucleotides reduced TNF-α inhibitor induced T cell death and reduced pro-inflammatory cytokine secretion including IL-17 by T cells. In a model of experimental colitis, galectin-4 ameliorated mucosal inflammation, induced apoptosis of mucosal T-cells and decreased the secretion of pro-inflammatory cytokines. Thus, galectin-4 plays a unique role in the intestine and suggests a novel role of this protein in controlling intestinal inflammation by a selective induction of T cell apoptosis and cell cycle restriction. Thus, galectin-4 is a novel anti-inflammatory agent that could be therapeutically effective in diseases with a disturbed T cell expansion and apoptosis such as inflammatory bowel disease (Paclik et al. 2008b).

Galectins in Inflammatory Bowel Disease: Inflammatory bowel disease (IBD) is an immune-mediated intestinal inflammatory condition that is associated with an increase in autoantibodies, which bind to epithelial cells. The epithelial galectin-4 specifically stimulates IL-6 production by $CD4^+$ T cells. The galectin-4-mediated production of IL-6 is MHC class II independent and induced by PKCθ-associated pathway through the immunological synapse. The galectin-4-mediated stimulation of $CD4^+$ T cells is shown to exacerbate chronic colitis and delays the recovery from acute intestinal injury. These studies suggested the presence of an immunogenic, endogenous lectin in the intestine and indicated the biological role of lectin/$CD4^+$ T cell interactions under inflammatory conditions (Hokama et al. 2004).

Recent studies have identified immunoregulatory roles of galectins in intestinal inflammatory disorders. Unexpected roles of galectin-1 and galectin-4 and chi-lectin (chitinase 3-like-1) have been reported in intestinal inflammation. Galectin-1 and -2 contribute to the suppression of intestinal inflammation by the induction of effector T cell apoptosis. In contrast, galectin-4 is involved in the exacerbation of this inflammation by specifically stimulating intestinal $CD4^+$ T cells to produce IL-6 (Mizoguchi and Mizoguchi 2007). Hokama et al. (2008) reviewed how different members of galectins provide inhibitory or stimulatory signals to control intestinal immune response under intestinal inflammation. Inflammatory bowel diseases are characterized by various degrees of mucosal surface damage and subsequent impairment of intestinal barrier function. Resealing of the epithelial barrier requires intestinal cell migration and proliferation. Galectins are increasingly recognized as novel regulators of inflammation. Gal-2 and Gal-4 bind to epithelial cells at the E-cadherin/β-catenin complex. Both galectins significantly enhanced intestinal epithelial cell restitution in vitro. This enhancement of epithelial cell restitution was TGF-β-independent. In contrast, Gal-1 decreased epithelial cell migration TGF-β dependently. Gal-2 and Gal-4 were found to increase cyclin B1 expression and consequently cell cycle progression, while Gal-1 inhibited cell cycling. Studies on the influence of Gal-2 and Gal-4 on epithelial cell apoptosis showed no induction of apoptosis, whereas Gal-1 significantly induced apoptosis of epithelial cells caspase-independently. Thus, these galectins play a significant role in intestinal wound-healing processes and might exert beneficial effects in diseases characterized by epithelial barrier disruption like IBDs.

Galectin expression is related to the genetic background of control animals. In acute and chronic experimental colitis produced in C57BL/6 and BALB/c mice with acute dextran sodium sulphate colitis and in 129 Sv/Ev IL-10 knock-out ($IL\text{-}10^{-/-}$) mice, inflammation was associated with chronic colitis in $IL\text{-}10^{-/-}$ mice with increased galectin-4 expression. In contrast with two other models, no galectin-1 and -3 alterations were observed in $IL\text{-}10^{-/-}$ mice. Acute colitis in C57BL/6 and BALB/c mice showed increased galectin-3 expression in the lamina propria and the crypt epithelium, together with a decreased nuclear expression. These results suggest an involvement of galectins in the development and perpetuation of colonic inflammation and illustrate that the choice of the mouse strain for studying galectins might influence the outcome of the experiments (Mathieu et al. 2008).

Interaction of Galectin-4 with p27 in the yeast two-hybrid assay suggested that galectin-4 is involved in p27-mediated activation of myelin basic protein gene, possibly through

modulation of the glycosylation status of transcription factor Sp1 (Wei et al. 2007).

11.1.6 Galectin-4 in Cancer

Galectin-4 Expression in Carcinoid Tumors: Galectin-4 is localized in the enterochromaffin cells of the porcine and murine small intestine. A differential distribution of galectin-4 is observed in carcinoid tumors in different locations of the GI tract and the lungs. In primary and metastatic human ileal carcinoid tumors as well as in carcinoid tumors of the stomach, lung, and rectum, galectin-4 was most highly expressed in the ileal carcinoids and the levels of expression tended to be higher in primary ileal carcinoids compared to the metastatic tumors. Pancreatic neuroendocrine tumors were negative for Gal-1, Gal-3, and Gal-4. Gastric carcinoids also expressed Gal-4, but very few pulmonary or rectal carcinoids were positive for Gal-4. Lower levels of Gal-1 and Gal-3 expression were present in ileal carcinoids compared to primary pulmonary and rectal tumors (Rumilla et al. 2006).

Integrins and Galectins in Adamantinomatous Craniopharyngiomas: Lefranc et al. (2005) suggested that at least part of the adhesiveness of craniopharyngiomas to the surrounding tissue, such as optical chiasms and pituitary stalks, could be explained by the interactions between α2 β1 integrin expressed by craniopharyngiomas and collagens on the one hand, and vitronectin expressed by the surrounding tissue on the other. In addition, the levels of galectin-4 contribute significant information toward the delay in recurrence independently of surgical status (Lefranc et al. 2005).

Sinonasal adenocarcinomas are uncommon tumors which develop in ethmoid sinus after exposure to wood dust. Within microarray study in sinonasal adenocarcinoma Tripodi et al. (2009) identified LGALS4 and CLU, that were significantly differentially expressed in tumors compared to normal tissue. A further evaluation is necessary to evaluate the possibility of using them as diagnostic markers (Tripodi et al. 2009).

Gal-4 in Colorectal and Other Cancers: The Gal-4 mRNA is under expressed in colorectal cancer and restricted to small intestine, colon and rectum. In patients of colon, decreased galectin-4 mRNA expression may be an early event in colon carcinogenesis (Rechreche et al. 1997). Among five cell lines derived from colon carcinoma, only two (HT29 and LS174T) expressed galectin-4 mRNA (Rechreche et al. 1997). Huflejt et al. (1997) found two prominent proteins in human colon adenocarcinoma T84 cell line. Cloning of 36-kDa protein was human homolog of galectin-4, which was localized in the epithelial cells of rat and porcine alimentary tract. The other, a 29-kDa protein, was galectin-3, found in a number of different cell types including human intestinal epithelium. Despite the marked similarities in the CRDs of these two galectins, their cellular distribution patterns were strikingly different. In subconfluent T84 cells, each galectin is distributed to specific domains of lamellipodia, with galectin-4 concentrated in the leading edge and galectin-3 more proximally. The localization of galectin-4 suggests a role in cell adhesion which is also supported by the ability of immobilized recombinant galectin-4 to stimulate adhesion of T84 cells. Furthermore, galectin-4 is up-regulated in human hepatocellular carcinoma (Kondoh et al. 1999) and metastatic gastric cancer cells (Hippo et al. 2001). Galectin 4 (*LGALS4*) is highly and specifically expressed in Mucinous epithelial ovarian cancers (MOC), but expressed at lower levels in benign mucinous cysts and borderline (atypical proliferative) tumors, supporting a malignant progression model of MOC. *LGALS4* may have application as an early and differential diagnostic marker of MOC (Heinzelmann-Schwarz et al. 2006).

11.2 Galectin-6

In the course of studying mouse colon mRNA for galectin-4, Gitt et al. (1998a, b) detected a related mRNA of Galectin-6, which has two CRDs in a single peptide chain. The *Lgals4* gene encoding galectin-4 is distinct from *Lgals6*. The coding sequence of galectin-6 is specified by eight exons. The upstream region contains two putative promoters. Both *Lgals6* and the closely related *Lgals4* are clustered together about 3.2 centimorgans proximal to *apoE* gene on mouse chromosome 7. The syntenic human region is 19q13.1–13.3. The galectin-6 lacks a 24-amino acid stretch in the link region between the two CRDs that is present in galectin-4. Otherwise, these two galectins have 83% amino acid identity. Expression of both galectin-4 and galectin-6 is confined to the epithelial cells of the embryonic and adult GI tract. Expression of mouse galectin-4 and galectin-6 indicates that both are expressed in the small intestine, colon, liver, kidney, spleen and heart and P19X1 cells while only galectin-4 is expressed in BW-5147 and 3T3 cell lines. In situ hybridization confirmed the presence of galectin-4/-6 transcripts in the liver and small intestine.

11.3 Galectin-8

11.3.1 Galectin-8 Characteristics

Galectin-8 and galectin-9, which each consists of two carbohydrate recognition domains (CRDs) joined by a linker peptide, belong to the tandem-repeat-type subclass of the galectin family. Alternative splicing leads to the formation

of distinct splice variants (isoforms) of galectin-8 and galectin-9 with tandem-repeat-type structures. Galectin-8 consists of several isoforms, each made of two domains of approximately 140 amino-acids, both having a CRD. These domains are joined by a 'link peptide' of variable length. The isoforms share identical CRDs and differ only in linker region. Galectin-8 was discovered in prostate cancer cells and has been studied extensively in the last few years (Bidon et al. 2008). It is widely expressed in tumoral tissues and seems to be involved in integrin-like cell interactions. The human galectin-8 gene covers 33 kbp of genomic DNA. It is localized on chromosome 1 (1q42.11) and contains 11 exons. The gene produces by alternative splicing 14 different transcripts, altogether encoding 6 proteins. Galectin-8, like other galectins, is a secreted protein. Upon secretion galectin-8 acts as a physiological modulator of cell adhesion. Studies showed that the *LGALS8* gene encodes for almost seven mRNAs by alternative splicing pathways and various polyadenylation sites. These mRNAs could encode for six isoforms of galectin-8, of which three belong to the tandem-repeat galectin group (with two carbohydrate binding domains) and the three others to the prototype group (one carbohydrate binding domain). All these isoforms seem to be differentially expressed in various tumoral cells. This untypical galectin-8 subfamily seems to have a complex expression regulation that could be involved in cancer phenomena (Bidon et al. 2008; Bidon-Wagner and Pennec 2004).

A 35 kDa galectin was cloned from rat liver. Deduced amino acid sequence of galectin-8 contains two domains with conserved motifs that are implicated in carbohydrate binding of galectins. This protein was named galectin-8. In vitro, translation products of galectin-8 are biologically active and possess sugar binding and hemagglutination activity. The expected size (34 kDa) that binds to lactosyl-Sepharose is present in rat. Overall, galectin-8 is structurally related (34% identity) to galectin-4. Nonetheless, galectin-4 is confined to intestine and stomach where as galectin-8 is expressed in liver, kidney, cardiac muscle, lung, and brain. Unlike galectin-4, but similar to galectins-1 and -2, galectin-8 contains four Cys residues. The link peptide of galectin-8 is unique and bears no similarity to any known protein. The N-terminal carbohydrate-binding region of galectin-8 contains a unique WG-E-I motif instead of consensus WG-E-R/K motif implicated as playing an essential role in sugar-binding of all galectins (Hadari et al. 1995).

Linker Peptide in Functioning of Galectin-8 and Galectin-9 Galectin-8 has two covalently linked carbohydrate recognition domains (CRDs). The two CRDs are joined by a linker peptide. Ligation of integrins by galectin-8 induces a distinct cytoskeletal organization, associated with activation of the ERK and phosphatidylinositol 3-kinase signaling cascades. These properties of galectin-8 were mediated by the concerted action of its two CRDs and involved both protein-sugar and protein-protein interactions. Accordingly, the isolated N- or C-CRD domains of galectin-8 or galectin-8 mutated at selected residues implicated in sugar binding showed reduced sugar binding, resulting in severe impairment in the capacity of these mutants to promote the adhesive, spreading, and signaling functions of galectin-8. Deletion of the linker region similarly impaired the biological effects of galectin-8. These results provided evidence that cooperative interactions between the two CRDs and the "hinge" domain are required for proper functioning of galectin-8 (Levy et al. 2006; Sato et al. 2002).

Gal-8 and Gal-9 with Longest Linker Peptide are Susceptible to Thrombin Cleavage: The isoforms of galectin-8 and galectin-9 with the longest linker peptide, i.e. galectin-8L and galectin-9L (G8L and G9L), are highly susceptible to thrombin cleavage, whereas the predominant isoforms, galectin-8M and galectin-9M (G8M and G9M), and other members of human galectin family were resistant to thrombin. Amino acid sequence analysis of proteolytic fragments and site-directed mutagenesis showed that the thrombin cleavage sites (-IAPRT- and -PRPRG- for G8L and G9L, respectively) resided within the linker peptides. Although intact G8L stimulated neutrophil adhesion to substrate more efficiently than G8M, the activity of G8L but not of G8M decreased on thrombin digestion. Similarly, thrombin treatment almost completely abolished eosinophil chemoattractant (ECA) activity of G9L. Studies suggest that G8L and G9L play unique roles in relation to coagulation and inflammation (Nishi et al. 2006). Mutant forms lacking the entire linker peptide were highly stable against proteolysis and retained their biological activities. These mutant proteins need to be evaluated for the therapeutic potential (Nishi et al. 2005).

N-Glycans as Major Ligands: Binding of the galectins to the different CHO glycosylation mutants revealed that complex N-glycans are the major ligands for each galectin except the N-terminal CRD of galectins-8, and involve some fine differences in glycan recognition. Interestingly, increased binding of galectin-1 at 4°C correlated with increased propidium iodide (PI) uptake, whereas galectin-3 or -8 binding did not induce permeability to PI (Patnaik et al. 2006). Among galectins (N- and C-terminal domains) Galectin-4C and 8N were found to prefer the d-talopyranose configuration to the natural ligand d-galactopyranose configuration. Derivatization at talose O2 and/or O3 provided selective inhibitors for these two galectins (Oberg et al. 2008).

Gal-8 binding with β1 Integrins on Jurkat T Cells: Carcamo et al. (2006) studied the effects of immobilized Gal-8 on Jurkat T cells. Immobilized Gal-8 bound integrins α1β1, α3β1 and α5β1 but not α2β1 and α4β1 and adhered to these cells with similar kinetics to immobilized fibronectin (FN). The α5β1 was the main mediator of cell adhesion to galectin-8. Gal-8, but not FN, induced extensive cell spreading frequently leading to a polarized phenotype characterized by an asymmetric lamellipodial protrusion. Gal-8-induced Rac-1 activation and binding to α1 and α5 integrins are not known in any other cellular system. Strikingly, Gal-8 was also a strong stimulus on Jurkat cells in triggering ERK1/2 activation. Human galectin-8 induces reversible adhesion of peripheral blood neutrophils but not eosinophils, to a plastic surface in a lactose-sensitive manner and modulates neutrophil function related to transendothelial migration and microbial killing (Nishi et al. 2003). Galectin-8 induces neutrophil-adhesion through binding to integrin αM. Galectin-8, as well as tandem-repeat galectin-9, and several multivalent plant lectins, induced Jurkat T-cell adhesion to a culture plate, whereas single-CRD galectins-1 and -3 did not. Galectin-8 also induced the adhesion of peripheral blood leucocytes to human umbilical vein endothelial cells. It appeared that the di- or multivalent structure of galectin-8 is essential for the induction of cell adhesion and that this ability exhibits broad specificity for leucocytes (Yamamoto et al. 2008).

11.3.2 Functions of Galectin-8

Modulator of Cell Adhesion: When immobilized, it functions as a matrix protein equipotent to fibronectin in promoting cell adhesion by ligation and clustering of a selective subset of cell surface integrin receptors. Complex formation between galectin-8 and integrins involves sugar-protein interactions and triggers integrin-mediated signaling cascades such as Tyr phosphorylation of FAK and paxillin. In contrast, when present in excess as a soluble ligand, galectin-8 (like fibronectin) forms a complex with integrins that negatively regulates cell adhesion. Such a mechanism allows local signals emitted by secreted galectin-8 to specify territories available for cell adhesion and migration. Due to its dual effects on the adhesive properties of cells and its association with fibronectin, galectin-8 might be considered as a novel type of a matricellular protein (Zick et al. 2004).

Galectin-8 as a Modulator of Cell Growth: Galectin-8 functions as an extracellular matrix protein that forms high affinity interactions with integrins. Soluble galectin-8 inhibits cell cycle progression and induces growth arrest. These effects were not related to interference with cell adhesion but were attributed to an increase in the cellular content of the cyclin-dependent kinase inhibitor p21. The increase in p21 levels was preceded by an increase in JNK and protein kinase B (PKB) activities. This process involves activation of JNK, which enhances the synthesis of p21, along with the activation of PKB, which inhibits p21 degradation. These effects, due to protein-sugar interactions, were induced when galectin-8 was present as a soluble ligand or when it was overexpressed in cells (Arbel-Goren et al. 2005). Results indicated galectin-8 as a modulator of cell growth through up-regulation of p21.

11.3.3 Clinical Relevance of Gal-8

Galectin-8 Auto-Antibodies in SLE Patients: Patients with systemic lupus erythematosus (SLE) produce Gal-8-autoantibodies that impede both its binding to integrins and cell adhesion. These function-blocking autoantibodies reported for a galectin implied that Gal-8 constitutes an extracellular stimulus for T cells, able to bind specific β1 integrins and trigger signaling pathways conducive to cell spreading. Gal-8 could modulate a wide range of T cell-driven immune processes that eventually become altered in autoimmune disorders. During a search for antigens recognized by antibodies produced by a patient with SLE, Pardo et al. (2006) found reactivity against Gal-8, for which autoantibodies were not previously described. Higher frequency of autoantibodies against galectin-8 in patients with SLE suggests a pathogenic role. Further studies are needed to determine their clinical relevance (Pardo et al. 2006).

Galectin-8 in Primary Open Angle Glaucoma: Primary open angle glaucoma (POAG), a major blindness-causing disease, is characterized by elevated intraocular pressure due to an insufficient outflow of aqueous humor. The trabecular meshwork (TM) lining the aqueous outflow pathway modulates the aqueous outflow facility. TM cell adhesion, cell-matrix interactions, and factors that influence Rho signaling in TM cells are thought to play a pivotal role in the regulation of aqueous outflow. Galectin-8 modulates the adhesion and cytoskeletal arrangement of TM cells and that it does so through binding to β1 integrins and inducing Rho signaling. Thus studies that Gal8 modulates TM cell adhesion and spreading, at least in part, by interacting with α2-3-sialylated glycans on β1 integrins (Diskin et al. 2009).

11.3.4 Isoforms of Galectin-8 in Cancer

Studies showed that galectin-8 is widely expressed in tumor tissues as well as in normal tissues. The level of galectin-8 expression may correlate with the malignancy of human colon cancers and the degree of differentiation of lung

squamous cell carcinomas and neuro-endocrine tumors. The differences in galectin-8 expression levels between normal and tumor tissues have been used as a guide for the selection of strategies for the prevention and treatment of lung squamous cell carcinoma. Experiments suggest the potential of galectin-8 in understanding of, and possibly prevent, the process of neoplastic transformation (Bidon-Wagner and Pennec 2004). Galectin-8 levels of expression positively correlate with certain human neoplasms, prostate cancer being the best example studied thus far. The overexpressed lectin might give these neoplasms some growth and metastasis related advantages due to its ability to modulate cell adhesion and cellular growth. Hence, galectin-8 may modulate cell-matrix interactions and regulate cellular functions in a variety of physiological and pathological conditions (Zick et al. 2004).

11.4 Galectin-9

11.4.1 Characteristics

Galectin-9, a tandem-repeat type galectin, is a 40-kDa protein consisting of 353 amino acids. The sequence identity between the N- and C-terminal CRDs is 35%. The C-terminal CRD (CCRD) is highly homologous to rat galectin-5 CRD with an amino acid sequence identity of 70%, but the N-terminal CRD (NCRD) is only moderately homologous with known galectins. Among these, the galectin-9 NCRD shows the highest sequence identity (40%) with the galectin-3 CRD. Galectin-9 was first cloned from tumor cells from Hodgkin disease, a condition characterized by blood and tissue eosinophilia. Moreover, the recombinant galectin-9 causes thymocyte apoptosis in mouse cells, suggesting a possible role for galectin-9 in negative selection during T-cell development (Wada and Kanwar 1997; Wada et al. 1997). Interestingly, galectin-9 was shown to be related to a novel eosinophil chemoattractant produced by T lymphocytes, previously designated "ecalectin" (Matsumoto et al. 1998). Mutation studies showed that both the NCRD and CCRD of galectin-9 were required for the eosinophil chemoattraction activity (Matsushita et al. 2000). Additionally, galectin-9 interacts with Tim-3, which is specifically expressed on the surface of T helper type 1 (Th1) cell, through recognition of Tim-3 carbohydrates, and the Tim3-galectin9 pathway induces cell death in Th1cells. This suggests that galectin-9 plays a role in down-regulating the effector Th1 responses (Zhu et al. 2005). Galectin-9 interacts with carbohydrate(s) covalently attached to the surface of Tim-3, but the molecular and structural basis for this recognition is unknown. In vitro analyses showed that galectin-9 has a high affinity for a variety of oligosaccharides containing β-galactosides (Hirabayashi et al. 2002), and the NCRD and CCRD of galectin-9 have different oligosaccharide-binding affinities. The biological activities of galectin-9 may be related to the ligand binding specificity of each CRD and the multivalent binding conferred by two CRDs. To date, the structures of many CRDs from fungi to human have been solved, but there is no structural information about the structure of tandem-repeat type galectin CRDs. Such information should greatly clarify the mechanism of carbohydrate recognition by the CRDs and the multivalent properties that lead to multiple functions for a single protein.

A 36-kDa lectin, galectin-9 from mouse embryonic kidney, has the characteristic conserved sequence motif of galectins. Galectin-9 from liver and thymus, as well as recombinant galectin-9 exhibited specific binding for lactosyl group. It had two distinct N- and C-terminal CRDs connected by a link peptide, with no homology to any other protein. Galectin-9 had an alternate splicing isoform, exclusively expressed in small intestine with a 31-amino acid insertion between the N-terminal domain and link peptide. Sequence analysis revealed that C-terminal CRD of mouse galectin-9 showed an extensive similarity to that of monomeric rat galectin-5, which was demonstrated in mouse. Sequence comparison of rat galectin-5 and rat galectin-9 cDNA did not reveal identical nucleotide sequences in the overlapping C-terminal carbohydrate-binding domain, indicating that galectin-9 is not an alternative splicing isoform of galectin-5. However, galectin-9 had a sequence identical with that of its intestinal isoform in the overlapping regions in both species. Genomic analyses indicated the presence of a novel gene encoding galectin-9 in both mice and rats. In contrast to galectin-5, which is mainly expressed in erythrocytes, galectin-9 was found to be widely distributed, i.e. in liver, small intestine, thymus > kidney, spleen, lung, cardiac and skeletal muscle > reticulocyte, brain (Wada and Kanwar 1997).

11.4.2 Stimulation of Galectin-9 Expression by IFN-γ

Galectin-9 was detected in membrane and cytosolic fractions of human umbilical vein endothelial cells (HUVECs) after stimulation with IFN-γ, which also enhanced the adhesion of human eosinophilic leukemia-1 cells to endothelial monolayers. The polyinosinic-polycytidylic acid (poly IC), a double-stranded RNA (dsRNA), induces the expression of galectin-9 in HUVECs and involves TLR3, PI3K, and IFN regulatory factor 3 (IRF3) (Imaizumi et al. 2007). The expression of galectin-9 by endothelium from patients with inflammatory diseases indicates that IFN-γ-induced production of galectin-9 plays an important role in immune responses by regulating interactions between vascular wall

and eosinophils (Imaizumi et al. 2002). IFN-γ up-regulated the expression of galectin-9 in primary human dermal fibroblasts and surface expression of galectin-9 on human lung fibroblast cell line, HFL-1. It suggests that IFN-γ-induced galectin-9 expression in fibroblasts mediates eosinophil adhesion to the cells, suggesting a crucial role of galectin-9 in IFN-γ-stimulated fibroblasts at the inflammatory sites (Asakura et al. 2002).

11.4.3 Crystal Structure of Galectin-9

Nagae et al. (2006) reported the crystal structures of mouse galectin-9 N-terminal CRD (NCRD) in free and complexed form with four ligands. X-ray structure of galectin-9 NCRD showed it to be composed of six-stranded (S1–S6) and five stranded (F1–F5) β-sheets, which together form a β-sandwich arrangement (Fig. 11.2a). Based on this structure, the galectin-9 NCRD is not buried in membranes. The carbohydrate binding site is formed by the S4, S5, and S6 β-strands, and the carbohydrate recognition mechanism is similar to those of other galectins. All structures formed same dimmer (Fig. 11.2b), which was quite different from the canonical two-fold symmetric dimer seen for galectin-1 and -2. Thus, the β-galactoside recognition mechanism in the galectin-9 NCRD is highly conserved among other galectins. In the apo form structure, water molecules mimiced the ligand hydrogen-bond network. The galectin-9 NCRD could bind both N-acetyllactosamine (Galβ1-4GlcNAc) and T-antigen (Galβ1-3GalNAc) with the proper location of Arg-64. Moreover, the structure of the N-acetyllactosamine dimer (Galβ1-β1-3Galβ1-4GlcNAc) complex showed a unique binding mode of galectin-9. Surface plasmon resonance assay showed that the galectin-9 NCRD forms a homophilic dimer not only in the crystal but also in solution (Nagae et al. 2006).

Dimer formation—In asymmetric unit of apo form1, two molecules, referred as chains A and B, are related by a two-fold non-crystallographic axis perpendicular to the β-sheets. They form a continuous 12-stranded antiparallel β-sheet through interactions between the β strands of chain-A S6 and chain-B S6 (Fig. 11.2b). On the dimer interface, the main chain oxygen and nitrogen atoms of Arg-86 form hydrogen bonds with the corresponding main-chain atoms of Arg-86 of the other monomer (Fig. 11.2c). The N and C termini of each monomer are positioned at the opposite side of the dimer interface (Fig. 11.2a) (Nagae et al. 2006).

Nagae et al. (2008, 2009) also reported the crystal structures of human galectin-9 NCRD in presence of lactose and Forssman pentasaccharide. Human galectin-9 NCRD exists as a monomer in crystals, despite a high sequence identity to the mouse homologue. Comparative frontal affinity chromatography analysis of the mouse and human galectin-9 NCRDs revealed different carbohydrate binding specificities, with disparate affinities for complex glycoconjugates. Human galectin-9 NCRD exhibited a high affinity for Forssman pentasaccharide; the association constant for which was 100-fold more than for mouse galectin-9 NCRD. The combination of structural data with mutational studies demonstrated that non-conserved amino acid residues on the concave surface were important for determination of target specificities. The human galectin-9 NCRD exhibited greater inhibition of cell proliferation than the mouse NCRD. The biochemical and structural differences have been reported between highly homologous proteins from different species (Nagae et al. 2008).

11.4.4 Galectin-9 Recognizes *L. major* Poly-β-galactosyl Epitopes

The glycan epitopes on Leishmania parasites are involved in the pathogenesis of Leishmaniasis. Established species-specific glycan structures is the poly-β-galactosyl epitope (Galβ1-3)n found on *L. major*, which can develop cutaneous infection with strong inflammatory responses. The host galectin-3 can distinguish *L. major* from other species through its binding to poly-β-galactosyl epitope, proposing a role for galectin-3 as an immunomodulator that could influence the *L. major*-specific immune responses in Leishmaniasis. In addition, Galectin-9 can also recognize *L. major* by binding to *L. major*-specific polygalactosyl epitope. The galectin-9 affinity for polygalactose was enhanced in proportion to the number of Galβ1-3 units present. Although both Galectin-3 and Galectin-9 have comparable affinities toward the poly-galactosyl epitopes, Pelletier et al. (2003) suggested that only galectin-9 can promote the interaction between *L. major* and macrophages in the development of leishmaniasis in the host. Compared with galectin-9 C-terminal CRD, the N-terminal CRD shows striking affinities for complex glycoconjugates such as Forssman pentasaccharide and polymerized *N*-acetyllactosamine (Hirabayashi et al. 2002; Walser et al. 2004). The specific interactions of galectin-9 NCRD with the carbohydrates is thought to be the clue for understanding the physiological mechanism of galectin-9. Nagae et al. (2006) reported the crystal structures of mouse galectin-9 N-terminal CRD in absence and in presence of carbohydrate ligands. These structures suggest a potential mechanism for the specificity of carbohydrate recognition and binding.

11.4.5 Functions of Galectin-9

Galectin-9 as a Ligand for T-Cell Immunoglobulin Mucin-3: T cell immunoglobulin and mucin domain

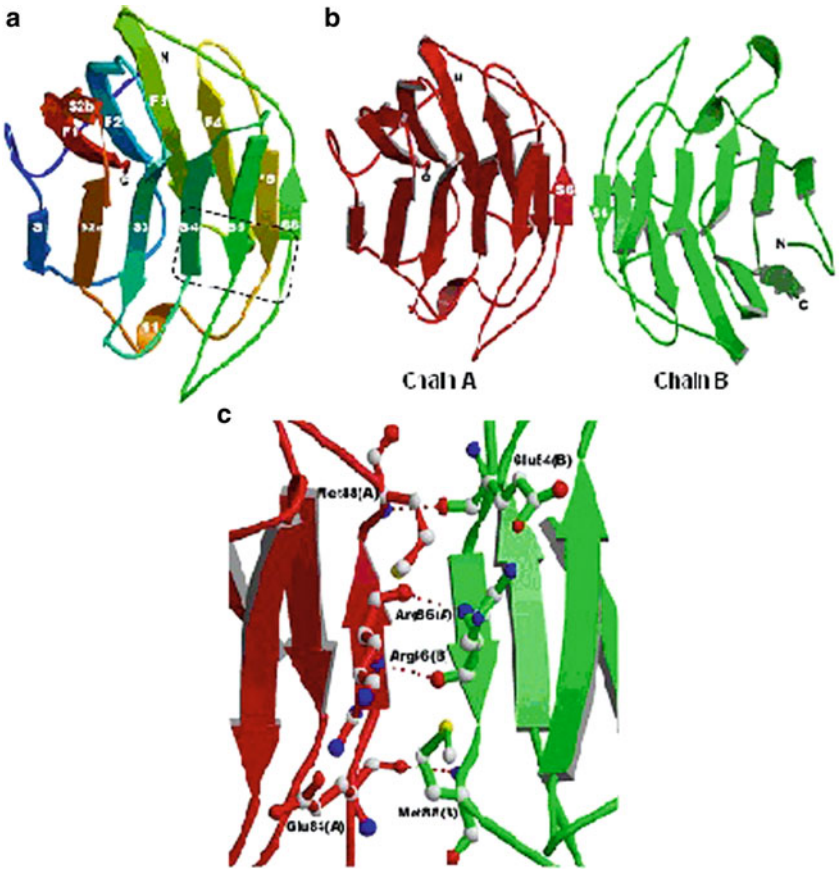

Fig. 11.2 Crystal structure of the mouse galectin-9 *N*-terminal CRD (NCRD). (**a**) Ribbon diagram of monomeric structure of apo form 1 of galectin-9 NCRD is shown. The five-stranded (*F1–F5*) and six-stranded (S1–S6) β-sheets and one short helix (H1) are indicated by the *number code*. The carbohydrate binding site is shown by a *dotted box*. (**b**) Dimeric structure of galectin-9 NCRD is shown. Two monomers in an asymmetric unit in apo form1 crystal are shown for chain-A (in *red*) and chain-B (*green*). (**c**) Close up view of the dimer interface. The amino acid residues involved in the dimer formation are shown in *ball-and-stick* model. The carbon, oxygen, nitrogen, and sulfur atoms are shown in *white*, *red*, *blue*, and *yellow spheres*, respectively. Hydrogen bonds are depicted by *dotted lines* (Adapted by permission from Nagae et al. 2006 © American Society for Biochemistry and Molecular Biology)

(TIM)-3 is a member of the TIM family of proteins (T-cell immunoglobulin mucin) involved in the regulation of $CD4^+$ T-cells. TIM-3 is a Th1-specific type 1 membrane protein that regulates Th1 proliferation and the development of tolerance. TIM-3 is a molecule expressed on terminally differentiated murine Th1 cells but not on Th2 cells. TIM-3 seems to regulate Th1 responses and the induction of peripheral tolerance. Zhu et al. (2005) showed that galectin-9 is the TIM-3 ligand. Galectin-9-induced intracellular calcium flux, aggregation and death of Th1 cells were TIM-3-dependent in vitro, and administration of galectin-9 in vivo resulted in selective loss of IFN-γ-producing cells and suppression of Th1 autoimmunity. These data suggested that the TIM-3-galectin-9 pathway may have evolved to ensure effective termination of effector Th1 cells (Zhu et al. 2005). Stimulation of TIM-3 by its ligand galectin-9 results in increased phosphorylation of Y265, suggesting that this tyrosine residue plays an important role in downstream signalling events regulating T-cell fate. Given the role of TIM proteins in autoimmunity and cancer, the conserved SH2 binding domain surrounding Y265 could represent a possible target site for pharmacological intervention (van de Weyer et al. 2006). The immunoregulatory TIM-3 in TGF-β-stimulated mast cells and melanoma cells may support the survival of this tumor type (Wiener et al. 2007).

Identification of galectin-9 as a ligand for TIM-3 has now firmly established the TIM-3-galectin-9 pathway as an important regulator of Th1 immunity and tolerance induction. TIM-3 is similarly expressed on human Th1 cells and not on Th2 cells, which suggests that TIM-3 might also contribute to Th1 regulation in humans. Binding of galectin-9 to the extracellular domain of TIM-3 resulted

in apoptosis of Th1 cells, though the intracellular pathways involved in the regulatory function of TIM-3 remained unknown. In addition, genetic data associate the TIM locus and specific TIM-3 polymorphisms with various immune-mediated diseases. Most importantly, recent data suggest a novel paradigm in which dysregulation of the TIM-3-galectin-9 pathway could underlie chronic autoimmune disease states, such as multiple sclerosis (Anderson and Anderson 2006). Unlike TIM-1, which is expressed in renal epithelia and cancer, TIM-3 has been described in neuronal besides T-cells. TIM-3 has been found in malignant and non-malignant epithelial tissues. Lensch et al. (2006) indicated distinct functions for galectin-5 and -9 especially in erythropoiesis and unknown mechanisms to compensate its absence from galectin network in other mammals.

Maturation of Human Monocyte-Derived Dendritic Cells: Dai et al. (2005) assessed the role of galectin-9 in DC maturation. Culture of immature DCs with exogenous Gal-9 markedly increased the surface expression of CD40, CD54, CD80, CD83, CD86, and HLA-DR in a dose-dependent manner, although Gal-9 had no or little effect on differentiation of human monocytes into immature DCs. Gal-9-treated DCs secreted IL-12 but not IL-10, and elicited the production of Th1 cytokines (IFN-γ and IL-2) but not that of the Th2 cytokines (IL-4 and IL-5) by allogeneic CD4+ T cells. Gal-9 induced phosphorylation of the MAPK p38 and ERK1/2 in DCs, and an inhibitor of p38 signaling, but not inhibitors of signaling by either ERK1/2 or PI3K, blocked Gal-9-induced up-regulation of costimulatory molecule expression and IL-12 production. These findings suggest that Gal-9 plays a role not only in innate immunity but also in acquired immunity by inducing DC maturation and promoting Th1 immune responses (Dai et al. 2005).

Endometrial Epithelial Marker: Galectin-9 mRNA is expressed in the human endometrium, specifically in the human endometrial epithelial cells but not in stromal or immune cells. It is expressed at very low concentrations during the proliferative phase and the early-secretory phase and shows a sharp and significant increase in the mid- and late-secretory phases, the window of implantation, as well as in the decidua. Galectin-9 protein is also exclusively increased in human endometrial epithelial cells during the mid- and late-secretory phases and in the decidua, however, not in endometrial stromal cells or decidualized cells in vivo or in vitro. A regulation in vitro by estradiol, progesterone, epidermal growth factor, and IFN-γ could not be detected. Based on the functional studies of other galectins, galectin-9 was suggested as a novel endometrial marker for the mid- and late-secretory and decidual phases (Popovici et al. 2005).

Gal-9 and Gal-1 Determine Distinct T Cell Death Pathways: Galectin-9 and galectin-1 both kill thymocytes, peripheral T cells, and T cell lines; however, galectin-9 and galectin-1 require different glycan ligands and glycoprotein receptors to trigger T cell death. The two galectins also utilize different intracellular death pathways, as galectin-9, but not galectin-1, T cell death was blocked by intracellular Bcl-2, whereas galectin-1, but not galectin-9, T cell death was blocked by intracellular galectin-3. To define structural features responsible for distinct activities of the tandem repeat galectin-9 and dimeric galectin-1, Shuguang et al. (2008) created a series of bivalent constructs with galectin- 9 and galectin-1 CRDs connected by different peptide linkers and suggested that the N-terminal CRD and linker peptide contributed to the potency of these constructs. However, the C-terminal CRD was the primary determinant of receptor recognition, death pathway signaling, and target cell susceptibility. Thus, CRD specificity, presentation, and valency make distinct contributions to the specific effects of different galectins in initiating T cell death (Shuguang et al. 2008).

11.4.6 Galectin-9 in Clinical Disorders

Galectin-9 is known as a versatile immunomodulator that is involved in various aspects of immune regulations. It induces various biological reactions such as chemotaxis of eosinophils and apoptosis of T cells. A 36-kDa protein with characteristics of S-type lectins was detected in Hodgkin's disease. While the N-terminal lectin domain showed merely moderate homologies with known galectins, the C-terminal lectin domain was highly homologous to rat galectin-5 with an amino acid sequence identity of 70%. In accordance, the galactoside binding protein was designated as galectin-9 (Türeci et al. 1997). Drug-induced liver injury, often caused by an allergic mechanism, is occasionally accompanied by eosinophilic infiltration. High expression of Gal-9 is suggested to be a specific finding of drug-induced liver injury. However, tissue eosinophilia in drug-induced liver injury cannot be explained by the augmentation of Gal-9 expression (Takahashi et al. 2006).

The mechanisms of leucocyte traffic across the vascular endothelium induced by dsRNA involved the expression of galectin-9 as one of key molecules in the regulation of the interaction between vascular wall and white blood cells. In HUVEC in culture upregulation of galectin-9 by poly-IC in the

vascular endothelium may explain part of the mechanism for leucocyte traffic through the vascular wall (Ishikawa et al. 2004).

Involvement of Gal-9 in Dermal Eosinophilia of Th1-Polarized Skin Inflammation: Skin eosinophilia is a common feature of allergic skin diseases, but it is not known how epidermal and dermal eosinophil infiltration is controlled. It appears that among eosinophil-specific chemoattractants in dermal fibroblasts and epidermal keratinocytes, eotaxin-3 contributes to dermal and epidermal eosinophil infiltration in Th2-polarized skin inflammation in which IL-4 is produced. In contrast, IFN-γ-dominated inflammation appears to mediate eosinophil extravasation into the dermis and eosinophil adhesion to dermal fibroblasts via galectin-9 in association with decreased chemoattractant activity of epidermal galectin-9. A mechanism of dermal eosinophilia in IFN-γ-mediated skin inflammation reflects concerted chemoattractant production involving dermal and/or epidermal eosinophilia during changes in the local cytokine profile (Igawa et al. 2006). Yamamoto et al. (2007) investigated the role of Gal-9 in asthma model in guinea pigs. In guinea pig, Gal-9 exists in three isoforms that differ only in the length of their linker peptides. Guinea pig Gal-9 was chemoattractant for eosinophils and promoted induction of apoptosis in sensitized but not non-sensitized T lymphocytes. Results provide evidence that Gal-9 is not involved in airway hypersensitivity, but is partly involved in prolonged eosinophil accumulation in the lung. Galectin-9 is expressed in human melanoma cells. Among these, MM-BP proliferated with colony formation, but MM-RU failed. High galectin-9 expression was inversely correlated with the progression of this disease, suggesting that high galectin-9 expression in primary melanoma links to better prognosis (Kageshita et al. 2002).

Galectin-9 as Eosinophil Chemoattractant in Nasal Polyps: Ecalectin (galectin-9) is an eosinophil chemoattractant from T lymphocytes. Ecalectin, is produced in the mucosa of nasal polyps and, seems to play an important role in the accumulation and activation of eosinophils in nasal polyps, regardless of the presence or absence of atopic predisposition. The role of ecalectin in nasal polyp tissues associated with various nasal and paranasal diseases and in the pathogenesis of eosinophilia in patients having chronic sinusitis with nasal polyposis has been clarified by the presence of ecalectin-positive cells in the subepithelial layer, where many EG2-positive cells were present. This was substantiated by the presence of many ecalectin mRNA-positive cells in nasal polyps with an accumulation of EG2-positive cells (Iino et al. 2006).

Gal-9 is a High Affinity IgE-Binding Lectin with Anti-allergic Effect: *Gal* attenuated asthmatic reaction in guinea pigs and suppressed passive-cutaneous anaphylaxis in mice and stabilizing effect on mast cells. In vitro studies of mast cell demonstrated that Gal-9 suppressed degranulation from the cells stimulated by IgE plus antigen and that the inhibitory effect was completely abrogated in presence of lactose thus indicating that lectin activity of Gal-9 is critical. It was found that Gal-9 strongly and specifically bound IgE, which is a heavily glycosylated Ig, and that the interaction prevented IgE-antigen complex formation, clarifying the mode of action of the anti-degranulation effect. The fact that immunological stimuli of MC/9 cells augmented Gal-9 secretion from cells implies that Gal-9 is an autocrine regulator of mast cell function to suppress excessive degranulation. Findings suggest a beneficial role of Gal-9 for the treatment of allergic disorders including asthma (Niki et al. 2009).

Periodontal Ligament Cells (PDL): Considerable evidence suggests that periodontal disease not only is caused by bacterial infection but also is associated with host susceptibility. Lipopolysaccharide (LPS) extracted from *Porphylomonas gingivalis (P. gingivalis* LPS) enhances the expression level of galectin-9 mRNA and protein in a time-dependent manner together with interleukin-8. In addition, strong immunoreaction for galectin-9 was detected in the PDL consisting of the periodontal pocket of a patient with severe periodontal disease. Furthermore, significant up-regulation of galectin-9 mRNA expression was detected in the mRNA from PDLs of patients with periodontal disease when compared with healthy donors. These results suggest that galectin-9 expression is associated with inflammatory reactions in the PDL (Kasamatsu et al. 2005).

The mechanisms of leucocyte traffic across the vascular endothelium induced by dsRNA involved the expression of galectin-9 as one of key molecules in the regulation of the interaction between vascular wall and white blood cells. In HUVEC in culture upregulation of galectin-9 by poly IC in the vascular endothelium may explain part of the mechanism for leucocyte traffic through the vascular wall (Ishikawa et al. 2004).

11.4.7 Galectin-9 in Cancer

In Management of T-Cell Leukemia: Galectin-9 is known as an apoptosis inducer of activated T lymphocytes. Caspase-dependent and-independent death pathways exist in Jurkat cells, and the main pathway might vary with the

T-cell type (Lu et al. 2007). ATL is a fatal malignancy of T lymphocytes caused by human T-cell leukemia virus type I (HTLV-I) and remains incurable. The protease-resistant galectin-9 by modification of its linker peptide, hG9NC (null), prevents cell growth of HTLV-I-infected T-cell lines and primary ATL cells. The suppression of cell growth was inhibited by lactose, but not by sucrose, indicating that β-galactoside binding is essential for hG9NC(null)-induced cell growth suppression. The hG9NC(null) induced cell cycle arrest by reducing the expression of cyclin D1, cyclin D2, cyclin B1, Cdk1, Cdk4, Cdk6, Cdc25C and c-Myc, and apoptosis by reducing the expression of XIAP, c-IAP2 and survivin. Hence, hG9NC(null) can be a suitable agent for the management of ATL(Okudaira et al. 2007).

Antimetastatic Potential in Breast Cancer: Galectin-9 induces aggregation of certain cell types. Irie et al. (2005), who assessed the contribution of galectin-9 to the aggregation of breast cancer cells as well as the relation between galectin-9 expression in tumor tissue and distant metastasis in patients with breast cancer, showed that MCF-7 subclones with a high level of galectin-9 expression formed tight clusters during proliferation in vitro, whereas a subclone (K10) with the lowest level of galectin-9 expression did not. However, K10 cells stably transfected with a galectin-9 expression vector aggregated in culture and in nude mice. Galectin-9 is a possible prognostic factor with antimetastatic potential in breast cancer (Irie et al. 2005). Yamauchi et al. (2006) suggested a relationship between galectin-9 expression in tumor tissue and distant metastasis exists in breast cancer. Tumors in 42 of the 84 patients were galectin-9-positive, and tumors in 19 of the 21 patients with distant metastasis were galectin-9-negative (Yamauchi et al. 2006).

Oral Squamous Cell Carcinomas: Oral squamous cell carcinomas (OSCCs) are the most common neoplasms of the head and neck. Galectin-9 is correlated with cellular adhesion and aggregation in melanoma cells. Galectin-9 mRNA and protein were commonly down-regulated in OSCC cell lines (Ca9-22, HSC-2, and HSC-3) and normal oral keratinocytes (NOKs). An adhesion assay revealed an increased cellular adherence ratio in overexpressed galectin-9 cells compared with nontransfected cells. Results supported that galectin-9 is correlated with oral cancer cell-matrix interactions and may therefore play an important role in the metastasis of OSCCs (Kasamatsu et al. 2005).

Nasopharyngeal Carcinomas: Nasopharyngeal carcinomas (NPC) are etiologically related to the Epstein-Barr virus (EBV), and malignant NPC cells have consistent although heterogeneous expression of the EBV latent membrane protein 1 (LMP1). LMP1 trafficking and signaling require its incorporation into membrane rafts. Conversely, raft environment is likely to modulate LMP1 activity. Galectin 9 was identified as a novel LMP1 partner. Galectin 9 is abundant in NPC biopsies as well as in LCLs, whereas it is absent in Burkitt lymphoma cells (Pioche-Durieu et al. 2005). Study provides the proof of concept that NPC cells can release HLA class-II positive exosomes containing galectin 9 and/or LMP1. It confirms that the EBV-encoded LMP1 has intrinsic T-cell inhibitory activity (Keryer-Bibens et al. 2006).

Galectin-9 Isoforms Influence Adhesion Between CCCs and HUVEC In Vitro: Galectin-9 plays multiple roles in a variety of cellular functions, including cell adhesion, aggregation, and apoptosis. Galectin-9 has three isoforms (named galectin-9L, galectin-9M, and galectin-9S), which differ in their functions. Transient expression of galectin-9L decreased E-selectin levels, while transient expression of galectin-9M or galectin-9S increased E-selectin levels in LoVo cells, which do not express endogenous galectin-9. Over-expression of three galectin-9 isoforms led to increased attachment of LoVo cells to extracellular matrix proteins respectively, while over-expression of galectin-9M or galectin-9S increased the adhesion of LoVo cells to HUVEC in vitro. Findings indicate that different isoforms of galectin-9 exhibit distinct biological functions (Zhang et al. 2009).

11.5 Galectin-12

Galectin-12 consists of conserved CRDs and is preferentially expressed in peripheral blood leukocytes and adipocytes. It is a major regulator of adipose tissue development and has been shown to be a predominantly adipocyte-expressed protein. Galectin-12 from human adipose tissue contained two potential CRDs with the second CRD being less conserved compared with other galectins (Hotta et al. 2001). In vitro translated galectin-12 bound to a lactosyl-agarose column far less efficiently than galectin-8. Galectin-12 mRNA was predominantly expressed in adipose tissue of human and mouse and in differentiated 3T3-L1 adipocytes. Caloric restriction and treatment of obese animals with troglitazone increased galectin-12 mRNA levels and decreased the average size of the cells in adipose tissue. The induction of galectin-12 expression by the thiazolidinedione, troglitazone, was paralleled by an increase in the number of apoptotic cells in adipose tissue. Galectin-12 was localized in the nucleus of adipocytes, and transfection with galectin-12 cDNA induced apoptosis of COS-1 cells. Hotta et al. (2001) suggested that galectin-12, an adipose-expressed galectin-like molecule, may participate in the apoptosis of adipocytes.

Galectin-12 is induced by cell cycle block at G_1 phase and causes G_1 arrest when overexpressed (Yang et al. 2001). The galectin-12 gene is expressed in mouse preadipocytes and is up-regulated when preadipocytes undergo cell cycle arrest, concomitant with acquisition of the competence to undergo differentiation in response to adipogenic hormone stimulation. Down-regulation of endogenous galectin-12 expression by RNA interference greatly reduced the expression of the adipogenic transcription factors CCAAT/enhancer-binding protein-β and -α and peroxisome proliferator-activated receptor-γ and severely suppressed adipocyte differentiation as a result of defective adipogenic signaling. It was suggested that galectin-12 is required for signal transduction that conveys hormone stimulation to the induction of adipogenic factors essential for adipocyte differentiation (Yang et al. 2004). Treatment of 3T3-L1 cells with isoproterenol, insulin, TNFα, and dexamethasone reduced galectin-12 gene expression between 47% and 85%; the inhibitory effect of isoproterenol could be reversed by pretreatment with the β-adrenergic antagonist propranolol and mimicked by stimulation of G(S)-proteins with cholera toxin or by activation of adenylyl cyclase with forskolin and dibutyryl-cAMP. Findings imply a role for galectin-12 in the pathogenesis of insulin resistance (Fasshauer et al. 2002).

The transcription factors CCAAT enhancer-binding protein α, β, and δ, and PPAR-γ are known to be crucial to the differentiation of adipocytes and are expressed in sebaceous gland cells. Galectin-12, resistin, SREBP-1, and stearoyl-CoA desaturase mRNAs are expressed in SZ95 sebocytes. Evidence suggests that pathways of differentiation in adipocytes and sebocytes could be similar and therefore further understanding of sebaceous gland differentiation and lipogenesis and potential therapies for sebaceous gland disorders may be obtained from our knowledge of adipocyte differentiation (Harrison et al. 2007).

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Galectin-3: Forms, Functions, and Clinical Manifestations

12

Anita Gupta

12.1 General Characteristics

Galectins-3 (Gal-3) (formerly known as CBP35, Mac2, L-29, L-34, IgEBP, and LBP) is widely spread among different types of cells and tissues, found intracellularly in nucleus and cytoplasm or secreted via non-classical pathway out of cell, thus being found on the cell surface or in the extracellular space. Through specific interactions with a variety of intra- and extracellular proteins Gal-3 affects numerous biological processes and seems to be involved in different physiological and pathophysiological conditions, such as development, immune reactions, and neoplastic transformation and metastasis. Most members of the galectin family including Gal-1 possess apoptotic activities, whereas Gal-3 possesses anti-apoptotic activity as well. Information on structural, biochemical and intriguing functional properties of Gal-3 have been reviewed extensively (Dumic et al. 2006).

12.1.1 Galectin-3 Structure

Galectin-3 is structurally unique member of galectin family. It is a 31-kDa chimeric galectin characterized by a single C-terminal carbohydrate recognition domain (CRD) for carbohydrate binding and an N-terminal aggregating domain that interacts with a noncarbohydrate ligand and allows the formation of oligomers. Gal-3 has an extra long and flexible N-terminal domain consisting of 100–150 amino acid residues, according to species of origin, made up of repetitive sequence of nine amino acid residues rich in proline, glycine, tyrosine and glutamine and lacking charged or large side-chain hydrophobic residues (Hughes et al. 1994; Birdsall et al. 2001) (Fig. 12.1a). The N-terminal domain functions to cross-link both carbohydrate and noncarbohydrate ligands (Hughes 1994; Birdsall et al. 2001; Brewer et al. 2002) and contains sites for phosphorylation (Ser^{6}, Ser^{12}) (Huflejt et al. 1993; Mazurek et al. 2000) and other determinants important for the secretion of the lectin by a novel, nonclassical mechanism (Menon and Hughes 1999). The CRD consists of about 135 amino acid residues and displays an identical topology and very similar three-dimensional structure to that reported for CRD of homodimeric Gal-1 and -2 (Liao et al. 1994; Lobsanov et al. 1993) with which it shares 20–25% sequence identity. Like Gal-1 and -2, it is arranged in 12β strands (F1-F5 and S1-S6a/6b) (Seetharaman et al. 1998).

Of multiple soluble lactose-binding lectins in rat intestine, the major one, tentatively designated RI-H, was isolated as a polypeptide of M_r 17,000. Surprisingly the cDNA encodes a protein of M_r 36,000, and this protein contains two homologous but distinct domains each with sequence elements that are conserved among all S-Lac lectins. The C-terminal domain, designated domain II, corresponds to lectin with M_r of 17,000 isolated from intestinal extracts and shown to have lactose binding activity. The new lectin, which was designated L-36, is highly expressed in full-length form in rat small and large intestine and stomach but was not detected in other tissues including lung, liver, kidney, and spleen. Each domain has approximately 35% sequence identity with the other domain and with the carbohydrate-binding domain of L-29, another S-Lac lectin, but only about 15% identity with other known S-Lac lectins (Oda et al. 1993).

Sugar Binding Subsites in Galectins: A carbohydrate-binding subsite has been suggested in hamster Gal-3 involving Arg^{139}, Glu^{230}, and Ser^{232} for NeuNAc-[α]2,3-; Arg^{139} and Glu^{160} for fucose-[α]1,2-; and Arg^{139} and Ile^{141} for GalNAc-[α]1,3- substituents on the primary galactose. Each of these positions is variable within the whole galectin family. Two of these residues, Arg^{139} and Ser^{232}, were probed for their importance in this putative subsite. Mutagenesis studies indicated that residue 139 adopts main-chain dihedral angles characteristic of an isolated bridge structural feature, while residue 232 is C-terminal residue of [β]-strand-11, and is

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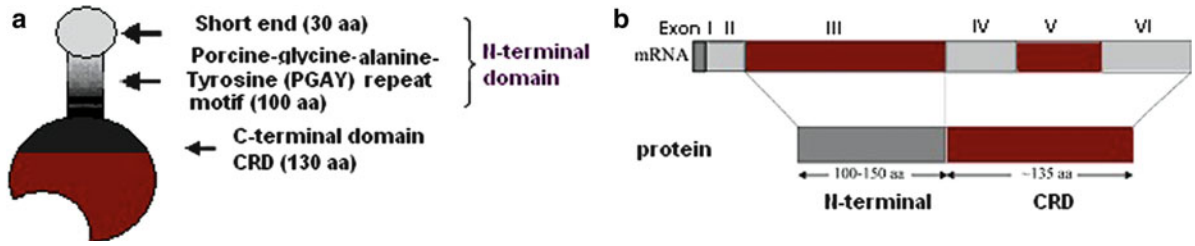

Fig. 12.1 The structure (**a**) and gene organization (**b**) of galectin-3

followed immediately by an inverse [γ]-turn. Arg^{139} was suggested to recognize the extended sialylated ligand (Henrick et al. 1998).

The murine Gal-3 is a monomer in absence and presence of N-acetyllactosamine (LacNAc), a monovalent sugar. However, Gal-3 precipitates as a pentamer with a series of divalent pentasaccharides with terminal LacNAc residues. Although the majority of Gal-3 in solution is a monomer, a rapid equilibrium exists between the monomer and a small percentage of pentamer. The latter, in turn, precipitates with divalent oligosaccharides, resulting in rapid conversion of monomer to pentamer by mass action equilibria. It forms heterogenous, disorganized cross-linking complexes with the multivalent carbohydrates. This contrasts with Gal-1 and many plant lectins (Ahmad et al. 2004a).

12.1.2 Galectin-3 Gene

The structure of Gal-3 gene is consistent with the multi-domain organization of the protein. The gene for Gal-3 is composed of six exons and five introns (human locus 14q21-22). Exon I encodes the major part of 5′untranslated sequence mRNA. Exon II contains the remaining part of 5′untranslated sequence, the protein translation initiation site and the first six amino acids including the initial methionine. Galectin-3 is coded by a single gene in the human genome. The gene is composed of six exons and five introns, spanning a total of approximately 17 kb. Based on primer extension and ribonuclease protection analyses, there are two transcription initiation sites located 52 and 50 nt upstream of the exon I-intron 1 border, and defined as +1a and +1b, respectively. The translation start site is in exon II. The ribonucleoprotein-like N-terminal domain, containing the proline-glycine-alanine-tyrosine (PGAY) repeat motif, is found entirely within exon III. Genomic fragments encompassing −836 to +141 nt (relative to +1a) have significant promoter activity. Because galectin-3 is an immediate-early gene whose expression is dependent on the proliferative state of the cell, results provide the basis for determining the molecular mechanisms of transcriptional regulation in neoplasia or cellular senescence. Exons IV, V and VI code the C-terminal half of the protein. The carbohydrate recognition sequence is found entirely within exon V (Fig. 12.1b) (Hughes et al. 1994, 1997; Kadrofske et al. 1998).

Murine Galectin-3 (Mac-2) Gene: The murine Mac-2 gene is composed of six exons dispersed over 10.5 kb. S1 nuclease mapping showed multiple transcription initiation sites, clustered within a 30-bp region. Sequence analysis revealed that a consensus initiator sequence is located in this area which lacks a TATA motif. The untranslated first exon contains an alternative splice donor site, confirming the existence of two cDNA species with the potential to encode proteins differing at their NH2 termini. In vitro expression and translocation experiments demonstrated that both the alternatively spliced variants of Mac-2 encode proteins which lack a functional signal peptide. Most of the Mac-2 protein is present in the cytosol. Results support the view that Mac-2 is exported from the cell by an unusual mechanism which does not depend on the presence of a signal peptide (Rosenberg et al. 1993).

12.1.3 Tissue and Cellular Distribution

Gal-3 has been detected in many immunocompetent/inflammatory cells, the epithelium of gastrointestinal and respiratory tracts, the kidneys and some sensory neurons (Hughes et al. 1997, 1999). In immunocompetent/inflammatory cells, Gal-3 is expressed in monocytes, dendritic cells (DCs), macrophages, eosinophils, mast cells, NK cells, and activated T and B cells (Chen et al. 2005; Dumic et al. 2006; Sato and Nieminen 2004). Moreover, Gal-3 displays pathological expression in many tumors, e.g., human pancreas, colon and thyroid carcinomas (see Chap. 13). It is mainly found in the cytoplasm, although, depending on cell type and proliferative state, a significant amount of this lectin can also be detected in the nucleus, on cell surface or in extracellular environment. In mouse fibroblasts, the nuclear versus cytoplasmic distribution of Gal-3 depends on the proliferation state of analysed cells. In quiescent cultures of fibroblasts, phosphorylated

derivative of Gal-3 (pI ~8.2) was predominantly cytoplasmic; however, proliferating cultures of same cells showed intense nuclear staining for nonphosphorylated native polypeptide (pI ~8.7). The intracellular location of Gal-3 is connected with its role in the regulation of nuclear pre-mRNA splicing and protection against apoptosis. On the other hand, its extracellular location on the cell surface and in the extracellular milieu indicates its participation in cell-cell and cell-matrix adhesion. A distinct expression profile of Gal-3 was determined in various murine organs when set into relation to homodimeric galectins-1 and -7. Lohr et al. (2008) demonstrated cell-type specificity and cycle-associated regulation for Gal-3 with increased presence in atretic preantral follicles and in late stages of luteolysis. Wang et al. (2004) reviewed lectins, which have been localized in the cytoplasm and nucleus of the cells. Gal-1 and Gal-3 were identified as pre-mRNA splicing factors in the nucleus, in conjunction with their interacting ligand, Gemin4. Gal-1 and Gal-3 are directly associated with splicing complexes throughout the splicing pathway in a mutually exclusive manner and they bind a common splicing partner through weak protein-protein interactions (Wang et al. 2006).

Transport of Gal-3 Between the Nucleus and Cytoplasm: Gal-3 shuttles between nucleus and the cytoplasm. Gal-3 compartmentalization in nucleus versus cytoplasm affects the malignant phenotype of various cancers. The mechanism by which Gal-3 translocates into nucleus remains debatable. In mouse fibroblasts, the Gal-3 (residues 1–263) fusion protein was localized predominantly in the nucleus. Mutants of this construct, containing truncations of the Gal-3 polypeptide from amino terminus suggested that the amino-terminal half was dispensable for nuclear import. Mutants of the same construct, containing truncations from the carboxyl terminus, showed loss of nuclear localization. Site-directed mutagenesis of the sequence ITLT (residues 253–256) suggested that nuclear import was dependent on the IXLT type of nuclear localization sequence, first shown in the *Drosophila* protein Dsh (dishevelled). In Gal-3 polypeptide, the activity of this nuclear localization sequence is modulated by a neighboring leucine-rich nuclear export signal (Davidson et al. 2006). It appeared that the export of Gal-3 from the nucleus may be mediated by the CRM1 receptor. The nuclear export signal fitting the consensus sequence recognized by CRM1 can be found between residues 240 and 255 of the murine Gal-3 sequence. Results indicated that residues 240–255 of the Gal-3 polypeptide contain a leucine-rich nuclear export signal that overlaps with the region (residues 252–258) identified as important for nuclear localization (Li et al. 2006).

Nakahara et al. (2006b) suggested that Gal-3 nuclear translocation is governed by dual pathways, whereas the cytoplasmic/nuclear distribution may be regulated by multiple processes, including cytoplasmic anchorage, nuclear retention, and or nuclear export. Gal-3 can be imported into the nucleus through at least two pathways; via passive diffusion and/or active transport (Nakahara et al. 2006a). The process mediated by the active nuclear transport of Gal-3 involves a nuclear localization signal (NLS)-like motif in its protein sequence, ^{223}HRVKKL^{228}, that resembles p53 and c-Myc NLSs [^{378}SRHKKL^{383}, ^{322}AKRVKL^{327}], respectively. It was suggested that Gal-3, in part, is translocated to the nucleus via the importin-α/β route and that Arg-224 residue of human Gal-3 is essential for its active nuclear translocation and molecular stability (Nakahara et al. 2006a).

Secretion: Gal-3 is secreted/externalized from cells through a nonclassical and a less understood transport pathway called ectocytosis, which is independent of classical secretory pathway through ER and Golgi system (Dumic et al. 2006; Henderson et al. 2006; Krześlak and Lipińska 2004; Nickel 2003; Rabinovich et al. 2002). Gal-3 is synthesized on free ribosomes in cytoplasm and lacks any signal sequence for translocation into ER (Hughes et al. 1999). As an extracellular protein, it interacts with glycoproteins within extracellular matrix to form a glycoprotein lattice or act as a soluble ligand to crosslink with carbohydrates of surface proteins by N-terminal oligomerization, thus evoking signal transduction and cell functions. Although it does not traverse endoplasmic reticulum/Golgi network, there is abundant evidence for Gal-3 also having an extracellular location. The N-terminus of Gal-3 has been proposed to contain targeting information for nonclassical secretion (Menon and Hughes 1999). A hamster Gal-3 CRD fragment lacking N-terminal domains, when expressed in transfected Cos cells, is not secreted, whereas addition of N-terminal segment to a normal cytosolic protein such as CAT-fusion protein efficiently exported it from transfected Cos cells. A short segment of Gal-3 N-terminal sequence comprising residues 89–96 (Tyr-Pro-Ser-Ala-Pro-Gly-Ala-Tyr) plays a critical role in Gal-3 secretion. However, this sequence is not sufficient on its own to cause direct secretion of CAT fusion protein, indicating that it is operative in association with a large part of N-terminal sequence of Gal-3 (Menon and Hughes, 1999). Studies indicate that the first step in Gal-3 secretion is its accumulation at cytoplasmic side of the plasma membrane. The acylation of Lck was an essential requirement for the retention and functioning of these proteins at the cytoplasmic side of plasma membranes (Zlatkine et al. 1997). In next step of secretion, the evaginating membrane domain(s) is/are pinched of and extracellular vesicles in which Gal-3 is protected against

proteolysis is released. Isolated vesicles were more stable, and indicated that rapid breakdown of vesicles requires some factor(s) released by cells. Hughes (1999) suggested that a PLA2 may catalyse the hydrolysis of an sn-2 fatty acyl bond of phospholipids and liberate free fatty acids and lysophospholipids (Krześlak and Lipińska 2004).

12.2 Ligands for Galectin-3: Binding Interactions

12.2.1 Extracellular Matrix and Membrane Proteins

Potential ligands for Gal-3 are the lysosomal associated membrane proteins 1 and 2 (Lamp 1 and 2) (Sarafian et al. 1998). Lamps are lysosomal membrane proteins, and are rarely found on the plasma membrane of normal cells. But Lamp 1 and 2 increase on cell surface of tumor cells, especially in highly metastatic ones, where they are major carriers for poly-N acetyllactosamines. Recombinant Gal-3 binds to Lamp-expressing metastasing melanoma cells, which indicates that Lamps could be ligands for cell adhesion molecules and participate in the process of tumor invasion and metastasis. MP20, the lens membrane integral protein and a member of the tetraspanin superfamily, also interacts with Gal-3 (Gonen et al. 2001; Serafian et al. 1998). It is not known exactly what role the MP20/Gal-3 complex could play in the lens. It is conceivable that Gal-3 plays an essential role in modulating the ability of MP20 to form adhesive junctions at this critical stage of development (Gonen et al. 2001). Because of its affinity for polylactosamine glycans, Gal-3 binds to glycosylated extracellular matrix components, including laminin, fibronectin, tenascin and Mac-2 binding protein (Woo et al. 1990; Rosenberg et al. 1991; Koths et al. 1993; Sato and Hughes 1992; Probstmeier et al. 1995; Ochieng and Warfield 1995) (Table 12.1). Some cell- surface adhesion molecules, for instance integrins, are also ligands for Gal-3. Evidence suggests that galectins, through binding to extracellular domains of one or both subunits of an integrin, may modulate integrin activation, and affect the binding with extracellular ligands (Hughes 2001). A major ligand for Gal-3 on mouse macrophage is α-subunit of the integrin αMβ2, also known as CD11b/18 (Hughes 2001; Dong and Hughes 1997). Moreover, Gal-3 also interacts with integrin α1β1 via its CRD domain in a lactose dependent manner (Ochieng et al. 1998). Gal-3 also seems to be an endogenous cross-linker of CD98 antigen, leading to the activation of integrin mediated adhesion (Hughes 2001). While gal-1 specifically recognizes CD2, CD4, CD7, CD43, and CD45, Gal-3 binds to IgE, Fc receptors, CD66, and CD98 (Pace et al. 1999; Walzel et al. 2000; Hughes 2001). The two CRDs of Gal-4 have different preferences for carbohydrate ligands, suggesting that bivalent galectins may cross-link different ligands (Oda et al. 1993). Recognition of unique glycan ligands probably allows different galectins to exert distinct biological effects in various tissues.

12.2.2 Intracellular Ligands

All above listed ligands for Gal-3 are extracellular matrix or membrane proteins. However, Gal-3 is also known to have an intracellular location and to interact with several proteins inside the cell, i.e., cytokeratins (Goletz et al. 1997), CBP70 (Sève et al. 1993), Chrp (Menon et al. 2000), Gemin4 (Park et al. 2001), Alix/AIP-1 (Liu et al. 2002), and Bcl-2 (Akahani et al. 1997). Nuclear Gal-3 interacts with Gemin-4, a component of a complex containing ~15 polypeptides, including SMN (survival of motor neuron) protein, Gemin-2, Gemin-3, some of the Sm core proteins of snRNPs and others. The identification of Gemin-4 as an interacting partner of Gal-3 provides evidence that Gal-3 can play a role in splicesome assembly in vivo (Liu et al. 2002). The precise mechanism by which Gal-3 and other cytosolic proteins that lack signal peptides are secreted is yet to be elucidated. It is worth noting that almost all of the mentioned intracellular ligands interact with Gal-3 via protein-protein rather than lectin-glycoconjugate interactions. However, Goletz et al. (1997) have shown that cytokeratins of some human cells carry a posttranslational modification, a glycan with a terminal α linked GalNAc, which are recognized in vitro by Gal-3. A carbohydrate binding protein of 70 kDa (CBP70), which is a nuclear and cytoplasmic lectin glycosylated by the addition of N- and O-linked oligosaccharide chains (Rousseau et al. 2000), interacts with Gal-3 via a protein-protein interaction mediated by the addition of lactose, probably resulting in modification of the Gal-3 conformational structure. Menon et al. (2000) reported a cytoplasmic cysteine-histidine rich protein – called Chrp that assists intracellular trafficking of Gal-3. Chrp recognizes the CRD domain of Gal-3 and not the N-terminal repeat sequence (Bawumia et al. 2003). The functional significance of Chrp-Gal-3 interaction remains to be elucidated.

Another Gal-3 binding protein is a human homologue of ALG-2 linked protein X (Alix) or ALG-2 interacting protein-1 (AIP-1) (Liu et al. 2002). This protein interacts with ALG-2, a calcium binding protein necessary for cell death induced by different stimuli. AIP-1 cooperates with ALG-2 in executing the calcium dependent requirements along the cell death pathway (Vito et al. 1999). Vlassara et al. (1995) suggested that Gal-3 is one of the AGE receptors, which might be present in macrophages, astrocytes and umbilical vein endothelial cells (Pricci et al. 2000). Results

Table 12.1 Ligands for galectin-3: Binding interactions (Iacobini et al. 2003)

	Ligand	Source/cell	Reference
Extracellular matrix proteins	IgE	Eosinophils and neutrophils	Liu 2005
	Laminin	EHS, macrophage, neutrophil, placenta	Woo et al. 1990; Ochieng and Warfield 1995; Kuwabara et al. 1996
	Fibronectin	Foetal	Sato and Hughes 1992
	Tenascin	Brain	Probstmeier et al. 1995
	M2BP	Brain	Rosenberg et al. 1991; Koths et al. 1993
	Collagen I/IV	Madin-Darby canine kidney cells	Friedrichs et al. 2008
	gp90kDa	Colorectal cancer	Iacovazzi et al. 2010
	Elastin	Breast carcinoma	Ochieng et al. 1999
	AGE	Ubiquitous	Vlassara et al. 1995; Pricci et al. 2000; Zhu et al. 2001
Membrane proteins	IgE receptor	Mast cell (and basophil)	Pricci et al. 2000
	Integrins:		
	αM/β2(CD11b/18)	Macrophage	Dong and Hughes 1997
	α1/β1	Adenocarcinoma	Ochieng et al. 1998
	N-CAM	Mouse brain	Probstmeier et al. 1995
	L1	Mouse brain	Probstmeier et al. 1995
	MAG	Mouse brain	Probstmeier et al. 1995
	LAMP-1,2	Ubiquitous	Dong and Hughes 1997; Sarafian et al. 1998; Ohannesian et al. 1995
	MP20	Rat lens	Gonen et al. 2001
	CD98	Human T lymphoma Jurkat cells	Yang et al. 1996
	Mucin	Colon cancer	Bresalier et al. 1998
	CD66	Human neutrophils	Feuk-Lagerstedt et al. 1999
	Bacterial LPS	*Klebsiella pneumoniae*	Mey et al. 1996
Intracellular proteins	Cytokeratins	HeLa, MCF-7	Goletz, et al. 1997
	Chrp	Murine 3T3 fibroblasts	Menon, et al. 2000; Bawumia et al. 2003
	CBP70	HL60	Sève, et al. 1993
	Alix/AIP-1	Human T lymphoma Jurkat cells	Liu et al. 2002
	Bcl-2	Human T lymphoma Jurkat cells	Yang et al. 1996
	β-Catenin	Breast cancer cells; colon cancer cells	Shimura et al. 2004
	Gemin-4	HeLa	Park et al. 2001
	CEA and other glycoconjugates	Colon carcinoma cells	Ohannesian et al. 1995
	98 kDa (Mac-2-BP) and 70 kDa species	Human melanoma cells	Inohara and Raz 1994

showed that AGE binds to Gal-3 CHO cells, followed by endocytosis and subsequent lysosomal degradation. Further studies revealed that acetylated and oxidized low density lipoproteins also undergo receptor-mediated endocytosis by these cells. GGal-3 is likely to play an important role in the formation of atherosclerotic lesions in vivo by the modification of the endocytic uptake of AGE and by modified low density lipoproteins (Zhu et al. 2001; Krześlak and Lipińska 2004).

It is found that Gal-3 can interact directly with membrane lipids in solid phase binding assays and has its own capacity to traverse the lipid bilayer. The interaction of Gal-3 with the plasma membrane may involve cholesterol-rich membrane domains where Gal-3 can be concentrated and form multimers or interact covalently with other proteins (Lukyanov et al. 2005).

12.2.3 Carbohydrate Binding

Gal-3 has an affinity for lactose (Lac) and N-acetyllactosamine (LacNAc) and acts as a receptor for ligands containing poly-N-acetyllactosamine sequences, which consist of many disaccharide units: Gal α1,4GlcNAc binds to each other by α1,3 linkage. The binding site of Lac/LacNAc is located to β-strands S4-S6a/S6b. In recent years, it has been demonstrated that Gal-3 binds to multimeric LN (LacNAc) and LDN (LacdiNAc) forming GalNAcß1-4GlcNAc motifs (van den Berg et al. 2004) as well as to other carbohydrate structures on glycoproteins and glycolipids (including LPS) from many pathogens such as (myco)bacteria, protozoan parasites, and yeast (Ochieng et al. 2004). Gal-3 appears to have an increased affinity for complex oligosaccharides (Hughes 1999; Hirabayashi et al. 2002). Gal-1, -2, and -3 exhibit differential

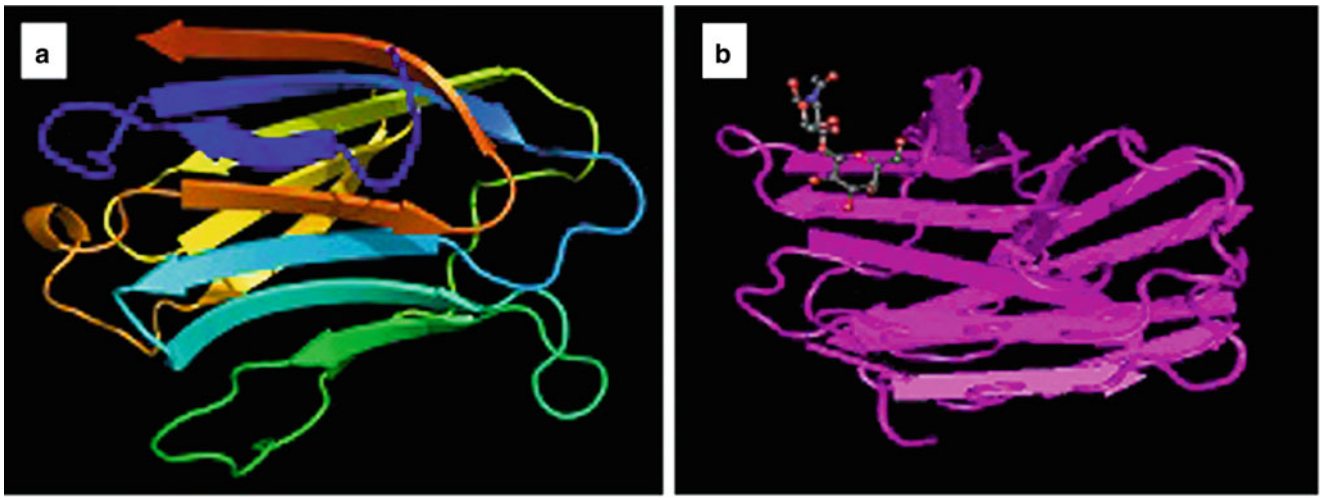

Fig. 12.2 Structure of human galectin-3 CRD (**a**) (Seetharaman et al. 1998) at 2.1 Å (PDB ID: 1A3K) and (**b**) bound to lactose at 1.35 Å (PDB ID: 2NN8) (Collins et al. 2007)

recognition of sialylated glycans and blood group antigens (Stowell et al. 2008). Complex N-glycans are the major ligands for Gal-1, -3, and -8 on Chinese hamster ovary cells (Patnaik et al. 2006). Extension at the nonreducing end of disaccharide units with NeuNAcα2, 3 or with GalNAcα1,3 and Fuc α1,2 substituents greatly enhances affinity for Gal-3 (Hughes 1999, 2001). Structural and mutagenic studies helped in identification of contact residues in Gal-3 CRD responsible for recognition of complex carbohydrates (Seetharaman et al. 1998). Studies revealed that Arg-144 in human Gal-3 is well positioned to interact with GlcNAc moiety or other saccharide residues linked to O-3 of terminal galactose (Seetharaman et al. 1998) (Figs. 12.2, 12.3). The C-4 hydroxyl group of galactose moiety (Gal) plays a central role in binding, probably accepting hydrogen bonds from the highly conserved residues His-158 and Arg-162, and at the same time donating hydrogen bonds to Asn-160 and a water molecule (W1) (Seetharaman et al. 1998). The galactose C-6 hydroxyl group also displays this cooperative hydrogen bonding pattern, interacting with Glu-184, Asn-174 and W3.

In case of N-acetylglucosamine (GlcNAc), only its C-3 hydroxyl group makes direct hydrogen bonds with Glu-184 and Arg-162 of protein. The only other contacts involving GlcNAc moiety are mediated through its N-acetyl group; the amide proton is hydrogen bonded through W2 to Glu-165 and the methyl group makes a van der Waals contact with guanidine head group of Arg-186. The van der Waals interaction and the strength of hydrogen bond involving two position of Glc/GlcNAc moiety represent the only significant differences between Lac and LacNAc complexes, and presumably account for the ~5-fold higher binding affinity for N-acetyllactosamine over lactose shown by human Gal-3 (Seetharaman et al. 1998) (Fig. 12.3). It seems that the N-terminal non-CRD domain is responsible for enhanced affinity of Gal-3 for extended structures of basic recognition units such as Lac or LacNAc (Hirabayashi et al. 2002). Affinity chromatography revealed that intact Gal-3 has 3.8 times higher affinity for oligosaccharides terminating in fucose or sialic acid residues than its deletion product in which N-terminal domain was removed by collagenase digestion.

Binding of Bivalent Oligosaccharides to Gal-3: Binding properties of recombinant murine Gal-3 to synthetic analogs containing two LacNAc residues separated by a varying number of methylene groups, as well as biantennary analogs possessing two LacNAc residues and binding of multivalent carbohydrates to C-terminal CRD domain of Gal-3 showed that each bivalent analog is bound by both LacNAc residues to two galectins. However, Gal-1 showed a lack of enhanced affinity for bivalent straight chain and branched-chain analogs, although Gal-3 showed enhanced affinity for only lacto-N-hexaose, a natural branched chain carbohydrate (Ahmad et al. 2004b).

Human Monocytes Recognize Porcine Endothelium via Interaction of Galectin 3: Gal-3 is mainly expressed in human monocytes, not lymphocytes and plays a key role in CD13-mediated homotypic aggregation of key inflammatory monocytic cells (Mina-Osorio et al. 2007). Monocytes, recruited to xenografts, play an important role in delayed xenograft rejection and have the ability to bind to major xenoantigen [Gal-α(1,3)Gal-β(1,4) GlcNAc-R]. Evidences show that Gal-3 is the receptor that recognizes β-galactosides (Gal-β(1-3/4)GlcNAc) and plays diverse roles in many physiological and pathological events. Human monocyte binding is strikingly

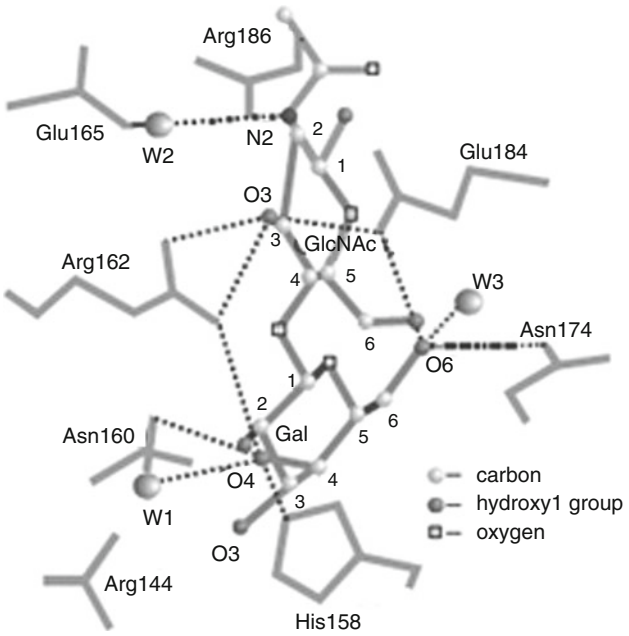

Fig. 12.3 The interaction of human Gal-3 with the N-acetyllactosamine moiety (for details, see text). The water molecules are labeled W1–W3. Potential hydrogen bonds are shown as *dotted lines*. The positions of the carbon atoms and the main hydroxyl groups are numbered (Adapted by permission from Seetharaman et al. 1998 © The American Society for Biochemistry and Molecular Biology)

increased on porcine aortic endothelial cells (PAEC), which express high levels of [Gal-α(1,3)Gal-β(1,4) GlcNAc-R], compared with human aortic endothelial cells. Human monocytes from healthy donors and purified Gal-3 bind to Gal-α(1,3)Gal-β(1,4)GlcNAc-R at variable intensities. Soluble Gal-3 binds preferentially to PAEC vs human aortic endothelial cells, and this binding can be inhibited by lactose, indicating dependence on CRD of Gal-3. Thus, Gal-3 expressed in human monocytes is a receptor for the major xenoantigen [Gal-α(1,3)Gal-β(1,4) GlcNAc-R], expressed on porcine endothelial cells (Jin et al. 2006).

12.2.4 Carbohydrate-Independent Binding

In addition to carbohydrate-dependent extracellular functions, Gal-3 participates in carbohydrate-independent intracellular signaling pathways, including apoptosis, via protein-protein interactions, some of which engage the carbohydrate-binding groove. When ligands bind within this site, conformational rearrangements are induced. The information on unliganded Gal-3 is therefore valuable for structure-based drug design. Removal of cocrystallized lactose from human Gal-3 CRD was achieved via crystal soaking, but took weeks despite low affinity (Collins et al. 2007).

The composition of mycolic acids (MA), the major constituents of *Mycobacterium tuberculosis* (Mtb) cell envelope, and other cell wall-associated lipids contribute to the virulence of a given strain. Gal-3, present mainly in cytoplasm of inflammatory cells and also on cell surface, can recognize mycobacterial MA. The MA can inhibit the lectin self-association but not its carbohydrate-binding abilities and can selectively interfere in the interaction of lectin with its receptors on temperature-sensitive DC line. This suggested that Gal-3 could be involved in the recognition of trafficking mycolic acids and can participate in their interaction with host cells (Barboni et al. 2005).

12.3 Functions

12.3.1 Galectin-3 is a Multifunctional Protein

Gal-3 is a multifunctional protein implicated in a variety of biological functions, including tumor cell adhesion, proliferation, differentiation, angiogenesis, cancer progression and metastasis. Multiple functions of Gal-3 depend on its location in the cell. Gal-3 is the only family member that is composed of a glycine/proline rich N-terminal repeated sequence and a C-terminal carbohydrate-binding domain. Although galectins lack a classical signal sequence, they are present in extracellular fluid and on the cell surface and are also located inside the cells (Hughes 1999; Liu et al. 2002). Extracellular Gal-3 can bind to cell surface through glycosylated proteins and thereby trigger or modulate cellular responses such as mediator release or apoptosis. Intracellular Gal-3 has been reported to inhibit apoptosis, regulation of tumor proliferation and angiogenesis, regulation of cell cycle, and participate in the nuclear splicing of pre-mRNA. Studies implicate Gal-3 in both innate and adaptive immune responses, where it participates in the activation or differentiation of immune cells. It is the only chimera-type member of galectin family of endogenous lectins, which share specificity with β-galactosides and have a jelly-roll-like folding pattern. Intracellular Gal-3 exhibits the activity to suppress drug induced apoptosis and anoikis (apoptosis induced by the loss of cell anchorage) that contribute to cell survival. Resistance to apoptosis is essential for cancer cell survival and plays a role in tumor progression. Conversely, it was shown that tumor cells secreted Gal-3 induces T-cells' apoptosis, thus playing a role in the immune escape mechanism during tumor progression through induction of apoptosis of cancer-infiltrating T-cells. Nakahara et al. (2005) summarized evidences on the role of Gal-3 as an anti-apoptotic and/or pro-apoptotic factor in various cell types and discussed the understanding of the molecular mechanisms of Gal-3 in apoptosis (Nakahara et al. 2005).

Gal-3 has been implicated in many facets of inflammatory response including neutrophil adhesion and activation (Kuwabara and Liu 1996), chemoattraction of monocytes/macrophages (Sano et al. 2000), and activation of mast cells and lymphocytes. Secreted Gal-3 can cross-link surface glycoproteins and activate pathways involved in several innate immune responses such as the oxidative burst in neutrophils (Janeway and Medzhitov 2002; Nieminen et al. 2005; Liu and Robinovich 2005) and degranulation in mast cells (Frigeri et al. 1993). Gal-3 also contributes to chemotaxis by mediating cell-cell and cell-substratum adhesion (Dumic et al. 2006). Mice lacking Gal-3 develop a dominant Th1 phenotype and exhibit abnormalities in several inflammatory disease models including asthma and diabetes, suggesting that Gal-3 may be involved in the regulation of inflammatory and Th1 responses (Pugliese et al. 2001; Zuberi et al. 2004; Bernardes et al. 2006; Nomoto et al. 2006). Gal-3 regulates peritoneal B1-cell differentiation into plasma cells (Oliveira et al. 2009).

12.3.2 Role in Cell Adhesion

Through its ability to bind polylactosamine structures [such as $(Galß1\text{-}4GlcNAc)_n$] on endogenous ligands (Hirabayashi et al. 2002), Gal-3 intervenes in many cellular processes in vitro. Despite the specific binding of galectin to glycoconjugates, there are still controversies as to whether galectins facilitate or inhibit cell adhesion. Gal-3 was shown to reduce the adhesion and spreading of baby hamster kidney epithelial cells on laminin 1 coated wells (Sato and Hughes 1992; Sato et al 1993). On the other hand, purified Gal-3 was shown to promote the adhesion of human neutrophils to laminin (Kuwabara and Liu 1996). This binary action of Gal-3 may be related to its concentration as well as to the expression level and glycosylation of its cell surface and matrix ligands (Hughes 2001). In particular, it favors cell-cell and cell-matrix glycoprotein adhesion (Ochieng et al. 1998), exerts chemotactic effects (Sano et al. 2000), controls cell proliferation (Demetriou et al. 2001; Yang et al. 1996), and promotes phagocytosis by macrophages (Sano et al. 2003; van den Berg et al. 2004).

12.3.3 Gal-3 at the Interface of Innate and Adaptive Immunity

Chen et al. (2005) reviewed the role of Gal-3 in the immune system and discussed the possible underlying mechanisms. Bernardes et al. (2006) studied Gal-3-deficient ($gal3^{-/-}$) mice after their response to *Toxoplasma gondii* infection, which is characterized by inflammation in affected organs, Th-1-polarized immune response, and accumulation of cysts in the CNS. In wild ($gal3^{+/+}$) orally infected mice, Gal-3 was highly expressed in the leukocytes infiltrating the intestines, liver, lungs, and brain. Compared with $gal3^{+/+}$, infected $gal3^{-/-}$ mice developed reduced inflammatory response in all of these organs but the lungs. Brain of $gal3^{-/-}$ mice displayed a significantly reduced number of infiltrating monocytes/macrophages and $CD8^+$ cells and a higher parasite burden. It shows that Gal-3 exerts an important role in innate immunity, including not only a pro-inflammatory effect but also a regulatory role on dendritic cells, capable of interfering in the adaptive immune response.

Gal-3 exerts cytokine-like regulatory actions in rat and mouse brain-resident immune cells. Both the expression of Gal-3 and its secretion into the extracellular compartment were significantly enhanced in glia under IFN-γ-stimulated, inflamed conditions. After exposure to Gal-3, glial cells produced high levels of proinflammatory mediators and exhibited activated properties. Notably, within minutes after exposure to Gal-3, JAK2 and STAT1, STAT3, and STAT5 showed considerable enhancement of tyrosine phosphorylation; thereafter, downstream events of STAT signaling were also significantly enhanced. Thus, Gal-3 acts as an endogenous danger signal under pathological conditions in the brain, providing a potential explanation for the molecular basis of Gal-3-associated pathological events (Jeon et al. 2010).

Gal-3 as an Opsonin: Galectins recognizes saccharide ligands on a variety of microbial pathogens, including viruses, bacteria, and parasites. It has been suggested that Gal-3 may serve as a pathogen pattern recognition receptor to visualize PAMPs from bacteria (Mandrell et al. 1994; Gupta et al. 1997), parasites (Pelletier and Sato 2002; van den Berg et al. 2004), and fungi (Kohatsu et al. 2006; Fradin et al. 2000). Gal-3, a galectin expressed by macrophages, dendritic cells, and epithelial cells, binds bacterial and parasitic pathogens including *Leishmania major*, *Trypanosoma cruzi*, and *Neisseria gonorrhoeae*. Gal-3 bound only to *Candida albicans* species that bear α-1,2-linked oligomannans on the cell surface, but did not bind *Saccharomyces cerevisiae* that lacks α-1,2-linked oligomannans. Binding induced death of *Candida* species containing specific α-1,2-linked oligomannosides by directly interaction (Kohatsu et al. 2006).

Gal-3 has been demonstrated to have antimicrobial activity toward the pathogenic fungus *C. albicans* (Kohatsu et al. 2006). After pneumococcal infection of lungs, Gal-3 accumulates in the alveolar space, and this correlates with the onset of neutrophil extravasation. However, although neutrophils were actively recruited into *E. coli* pneumonia-infected lungs, there was no increase in Gal-3 expression. Furthermore Gal-3 was released by alveolar macrophages on incubation with *S. pneumoniae* membrane fraction (Sato et al. 2002). In addition, LPS expressed on *E. coli* has been

shown to down-regulate Gal-3 expression (Sato et al. 2002). The antimicrobial activity may also be relevant for other pathogens, thus revealing an interesting therapeutic use of this galectin. However, Gal-3 plays a role in leukocyte recruitment in a murine model of lung infection by *S. pneumoniae* (Nieminen et al. 2008). Gal-3$^{-/-}$ mice develop more severe pneumonia after infection with *S. pneumoniae*, as demonstrated by increased septicemia and lung damage compared to WT mice. Neutrophil recruitment to the alveolar space was reduced in Gal-3$^{-/-}$ mice; however, myeloperoxidase activity in lung homogenates was not reduced in these mice compared to WT. This would suggest that neutrophils accumulate in the interstitial lung tissue during pneumonia in Gal-3$^{-/-}$ mice but are hindered from transmigrating into the alveolar space in the absence of Gal-3 (Farnworth et al. 2008).

Although mouse neutrophils express very low levels of endogenous Gal-3 (Sato et al. 2002), they can be activated by extracellular Gal-3, which is up-regulated in the surrounding tissue environment after infection. However, in Gal-3-null mice this Gal-3 up-regulation does not occur, resulting in reduced neutrophil recruitment into the alveolar spaces, activation, and phagocytosis. Gal-3 expression is up-regulated after pneumococcal infection, and macrophages are capable of secreting large amounts of Gal-3 (Sato et al. 2002). Longevity of human neutrophils is increased after incubation with exogenous Gal-3, thus delaying spontaneous apoptosis in vitro. Furthermore, Gal-3$^{-/-}$ macrophages displayed reduced phagocytosis of apoptotic human neutrophils compared to WT. The resultant accumulation of apoptotic neutrophils in the lungs of Gal-3$^{-/-}$ mice after infection would cause considerable damage to lung tissue, thus allowing bacteria to traverse the lung epithelia and enter the blood stream resulting in septicemia.

During *S. pneumoniae* infection the Gal-3$^{-/-}$ mouse mounts a greater Th1 response as demonstrated by increased IL-6 and TNF-α cytokine levels compared to WT mice or Gal-3$^{-/-}$ mice treated with recombinant Gal-3. This increased Th1 response may contribute to lung damage and subsequent septicemia. Gal-3$^{-/-}$ macrophages demonstrated a deficit in their ability to adopt an anti-inflammatory alternative (M2) phenotype (MacKinnon et al. 2008). It was proposed that, in addition to reduced neutrophil activation and apoptotic neutrophil clearance by macrophages in the Gal-3$^{-/-}$ mice, these mice were less able to dampen down the excessive inflammation and destructive potential of pneumococcal infection.

Gal-3 can directly activate both human and mouse neutrophils and potentiate the effect of fMLP. Whole blood neutrophils can be directly activated by concentrations of Gal-3 lower than those that directly activate isolated neutrophils. This suggests that in whole blood there may be factors present in the serum (eg, GM-CSF) functioning to prime circulating neutrophils. The Gram-positive *Streptococcus pneumoniae* is the leading cause of community-acquired pneumonia worldwide, resulting in high mortality. In vivo studies show that Gal-3$^{-/-}$ mice develop more severe pneumonia after infection with *S. pneumoniae*, as demonstrated by increased bacteremia and lung damage compared to wild-type mice and that Gal-3 reduces the severity of pneumococcal pneumonia in part by augmenting neutrophil function. Specifically, (1) Gal-3 directly acts as a neutrophil-activating agent and potentiates the effect of fMLP, (2) exogenous Gal-3 augments neutrophil phagocytosis of bacteria and delays neutrophil apoptosis, (3) phagocytosis of apoptotic neutrophils by Gal-3$^{-/-}$ macrophages is less efficient compared to wild type, and (4) Gal-3 demonstrates bacteriostatic properties against *S. pneumoniae* in vitro. Furthermore, recombinant Gal-3 in vivo protects Gal-3-deficient mice from developing severe pneumonia. Studies demonstrate that Gal-3 is a key molecule in the host defense against pneumococcal infection. Strategies designed to augment Gal-3 expression in the lung may result in the development of novel treatments for pneumococcal pneumonia (Farnworth et al. 2008).

Modulation of Neutrophil Activation: Exogenously added Gal-3 increased the uptake of apoptotic neutrophils by monocyte-derived macrophages (MDM). The effect was lactose-inhibitable and required Gal-3 affinity for N-acetyllactosamine, a saccharide typically found on cell surface glycoproteins. Perhaps, Gal-3 functions as a bridging molecule between phagocyte and apoptotic prey, acting as an opsonin. Results imply that the increased levels of Gal-3 often found at inflammatory sites could potently affect apoptosis (Karlsson et al. 2009).

Human neutrophils are activated by Gal-3, provided cells are primed by in vivo extravasation or by in vitro preactivation with, for example, LPS. Removal of terminal sialic acid can change neutrophil functionality and responsiveness due to exposure of underlying glycoconjugate receptors or change in surface charge. Such alteration of cell surface carbohydrate composition can alter the responsiveness of the cells to Gal-3. Earlier studies had shown that priming of the neutrophil response to Gal-3 with LPS was paralleled by degranulation of intracellular vesicles and granules and upregulation of potential Gal-3 receptors (Almkvist et al. 2004).

While investigating the effects of Gal-3 on central effector functions of human neutrophils, Fernandez et al. (2005) supported the notion that Gal-3 and soluble fibrinogen are two physiological mediators present at inflammatory sites that activate different components of the MAPK pathway and could be acting in concert to modulate the

functionality and life span of neutrophils. Whereas those activities are likely to be associated with ligand cross-linking by this lectin, Gal-3, unlike other members of the galectin family, exists as a monomer. Consequently, it was proposed that oligomerization of the N-terminal domains of Gal-3, after ligand binding by the C-terminal domain, is responsible for this cross-linking. The oligomerization status of Gal-3 could, thus, control the majority of its extracellular activities. Data of Nieminen et al. (2007) suggested that Gal-3 lattices are robust and could be involved in the restriction of receptor clustering. However, little is known about the actual mode of action through which Gal-3 exerts its function.

Annexin 1 (ANXA1), Gal-1 and Gal-3 regulate leukocyte migration. The expression of these proteins was studied in human neutrophils and endothelial cells (ECs) during a transmigration process induced by IL-8. ANXA1 and Gal-3 changed in their content and localization when neutrophils adhered to endothelia, suggesting a process of sensitive-balance between two endogenous anti- and pro-inflammatory mediators (Gil et al. 2006). Chronic morphine treatment in an *S. pneumoniae* infection model suppresses NF-kB gene transcription in lung resident cells, which, in turn, modulates the transcriptional regulation of MIP-2 and inflammatory cytokines. The decreased synthesis of MIP-2 and inflammatory cytokines coupled with the decreased release of Gal-3 result in reduced migration of neutrophils to the site of infection, thereby increasing susceptibility to *S. pneumoniae* infection after morphine treatment (Wang et al. 2005).

12.3.4 Regulation of T-Cell Functions

The rapid expansion of the field of galectin research has positioned Gal-3 as a key regulator of T-cell functions (Hsu et al. 2009). Gal-3 is absent in resting $CD4^+$ and $CD8^+$ T cells but is inducible by various stimuli. These include viral transactivating factors, T-cell receptor (TCR) ligation, and calcium ionophores. In addition, Gal-3 is constitutively expressed in human regulatory T cells and $CD4^+$ memory T cells. It exerts extracellular functions because of its lectin activity and recognition of cell surface and extracellular matrix glycans. Formation of lattices can result in restriction of receptor mobility and cause attenuation of receptor functions. Because of Gal-3 presence in intracellular locations, several functions have been described for Gal-3 inside T cells. These include inhibition of apoptosis, promotion of cell growth, and regulation of TCR signal transduction. Gal-3 takes part in control of T-cell and monocyte survival and activation. For instance, extracellular Gal-3, by associating with *N*-glycans on TCR has been shown to down-modulate TCR responsiveness and to regulate the production of Th1 and Th2 cytokines by differentiated T cells (Demetriou et al. 2001; Joo et al. 2001; Morgan et al. 2004). On the other hand, intracellular Gal-3 can positively or negatively impact intracellular signaling pathways by regulating the activities of various kinases, including protein kinases C, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (Chen et al. 2005). Studies of cell surface glycosylation have led to convergence of glycobiology and galectin biology and provided new clues on how Gal-3 may participate in the regulation of cell surface receptor activities (Hsu et al. 2009).

Ocklenburg et al. (2006) reported the identification of the ubiquitin-like gene, diubiquitin (UBD) as a downstream element of FOXP3 in human activated regulatory $CD4^+CD25^{hi}$ T cells (T_{reg}). Ocklenburg et al. (2006) suggested that UBD contributes to the anergic phenotype of human regulatory T cells and acts downstream in FOXP3 induced regulatory signaling pathways, including regulation of *LGALS3* expression. High level of *LGALS3* expression represents a FOXP3-signature of human antigen-stimulated $CD4^+CD25^{hi}$ derived regulatory T cells (Ocklenburg et al. 2006).

12.3.5 Pro-apoptotic and Anti-apoptotic Effects

Extracellular Gal-3 induces T-cell apoptosis (Fukumori et al. 2003) whereas intracellular Gal-3 results in an inhibition of apoptosis (Yang et al. 1996). Furthermore, peritoneal macrophages taken from Gal-$3^{-/-}$ mice are more prone to undergo apoptosis than wild-type (WT) macrophages (Hsu et al. 2000). Clearance of apoptotic neutrophils by macrophages is a key step in the resolution of inflammation. Without this step, apoptotic neutrophils will undergo secondary necrosis resulting in the release of damaging toxic products. Removal of potentially toxic apoptotic neutrophils results in the release of anti-inflammatory and preparative cytokines such as TGF-β1. These clearance and resolution phases help to limit the degree of tissue injury. Intracellular Gal-3 suppresses drug induced apoptosis and anoikis (apoptosis induced by the loss of cell anchorage) that contribute to cell survival. Resistance to apoptosis is essential for cancer cell survival and plays a role in tumor progression. Conversely, tumor cells' secreted Gal-3 induces T-cells' apoptosis, thus playing a role in immune escape mechanism during tumor progression through induction of apoptosis of cancer-infiltrating T-cells. Cells with over-expresed Gal-3 display increased resistance to the apoptotic stimuli induced by the anti-Fas antibody, staurosporine, TNF, radiation and nitric oxide (Akahani et al. 1997; Matarrese et al. 2000; Moon et al. 2001; Yang et al. 1996). Gal-3 has significant sequence similarity with Bcl-2 protein, a suppressor of apoptosis. The four amino acid motif, Asn-Trp-Gly-Arg (NWGR) of lectin is a highly conserved within BH1 domain

of the Bcl-2 family proteins and is crucial for Bcl-2 protein function in the inhibition of programmed cell death (Akahani et al. 1997; Yang et al. 1996). An amino acid substitution of Gly to Ala at position 182 in this motif of Gal-3 prevents its anti-apoptotic activity (Akahani et al. 1997). Yang et al. (1996) demonstrated that Gal-3 can interact with Bcl-2 in a lactose-inhibitable manner, which is surprising since Bcl-2 is not a glycoprotein. Yang et al. (1996) suggested that the Asn-Trp-Gly-Arg motif is present within CRD of Gal-3, and is closely involved in interaction with Bcl-2. Lactose binding to Gal-3 may induce a conformational change in the critical region of this protein, which prevents its interaction with Bcl-2. Though the mechanism by which Gal-3 regulates apoptosis induced by different agents remains to be elucidated, it is possible that Gal-3 can replace or mimic Bcl-2 protein. Bcl-2 is located on the outer membranes of mitochondria and regulates apoptosis by blocking the release of cytochrome C from the mitochondria (Nagata 2000; Zörnig et al. 2001). Moon et al. (2001) showed that Gal-3 inhibition of nitrogen free radical-mediated apoptosis in breast carcinoma BT549 cells involved the protection of mitochondrial integrity, the inhibition of cytochrome C release and the activation of caspase. Thus, Gal-3 appears to be a mitochondrial-associated apoptotic regulator in addition to Bcl-2 (Matarrese et al. 2000; Moon et al. 2001). Studies have demonstrated that Gal-3 translocates into the mitochondrial membrane following a variety of apoptotic stimuli (Yu et al. 2002). Moreover, Gal-3 prevents mitochondrial damage and cytochrome C release. Such a location of Gal-3 is regulated by Gal-3 interacting proteins such as a synexin, a 51 kDa member of annexin family of proteins, which can bind to phospholipid membranes. Gal-3 regulates the time course of the apoptotic process in pancreatic acinar cells (Gebhardt et al. 2004).

Anti-apoptotic Function of Gal-3 and Cell Cycle Arrest: Gal-3 can protect against apoptosis induced by loss of cell anchorage (anoikis). Although BT549 cells (human breast epithelial cells) undergo anoikis, Gal-3-overexpressing BT549 cells respond to the loss of cell adhesion by inducing G1 arrest without detectable cell death. Gal-3-mediated G1 arrest involves down-regulation of G1-S cyclin levels (cyclin E and cyclin A) and up-regulation of their inhibitory protein levels (p21(WAF1/CIP1) and p27KIP1). After the loss of cell anchorage, Rb protein becomes hypophosphorylated in Gal-3-overexpressing cells. Interestingly, Gal-3 induces cyclin D1 expression (an early G1 cyclin) and its associated kinase activity in the absence of cell anchorage. Kim et al. (1999) proposed that Gal-3 inhibition of anoikis involves cell cycle arrest at an anoikis-insensitive point (late G1) through modulation of gene expression and activities of cell cycle regulators. The study suggests that Gal-3 may be a critical determinant for anchorage-independent cell survival of disseminating cancer cells in the circulation during metastasis. Studies have revealed that Gal-3 suppresses apoptosis and anoikis that contribute to cell survival during metastatic cascades. Human Gal-3 undergoes post-translational signaling modification of Ser-6 phosphorylation that acts as an "on/off" switch for its sugar-binding activity. Nakahara et al. (2005) summarized evidences on the role of Gal-3 as an anti-apoptotic and/or pro-apoptotic factor in various cell types and discussed the role of Gal-3 in apoptosis.

Apart from cell cycle arrest at late G1 in response to loss of cell adhesion, Gal-3 influences G2/M arrest of BT 549 cells following genistein treatment (Lin et al. 2000). Lin et al. (2000) showed that genistein effectively induces apoptosis without detectable cell cycle arrest in BT 549, which does not express Gal-3 at a detectable level. It is also likely that nuclear Gal-3 may directly modulate gene expression through regulation of transcription and/or mRNA splicing (Lin et al. 2002). Lin et al. (2002) showed that Gal-3 induces cyclin D1 promoter activity in BT 549 cells through multiple cis-elements, including SP1 and CREB binding sites. The Gal-3 induction of cyclin D1 promoter activity may result from stabilization of nuclear protein-DNA complex formation at the CRE site of cyclin D1 promoter.

Studies suggest that Gal-3 phosphorylation is required for its anti-apoptotic activity and anti-anoikis activity (Yoshii et al. 2002). Human Gal-3 is phosphorylated at Ser 6 by casein kinase I. Ser 6 phosphorylation of human Gal-3 significantly reduces its binding to ligands, e.g. laminin and asialomucin, while dephosphorylation fully restores the sugar binding activity (Mazurek et al. 2000). Yoshii et al. (2002) demonstrated that Gal-3 phosphorylation can also influence its other biological activities. Ser 6 mutation resulted in a relative decline in the level of Gal-3's ability to protect cells against *cis*platin-induced cell death and poly(ADP-ribose)polymerase from degradation compared with WT Gal-3.

Gal-3 and Gal-1 Bind Distinct Cell Surface Receptors to Induce T Cell Apoptosis: Extracellular Gal-1 directly induces death of T cells and thymocytes, while intracellular Gal-3 blocks T cell death. In contrast to the antiapoptotic function of intracellular Gal-3, extracellular Gal-3 directly induces death of human thymocytes and T cells. However, events in Gal-3- and Gal-1-induced cell death differ in a number of ways. Thymocyte subsets demonstrate different susceptibility to the two galectins: whereas Gal-1 kills double-negative and double-positive human thymocytes with equal efficiency, Gal-3 preferentially kills double-negative thymocytes. Gal-3 binds to a complement of T cell surface glycoprotein receptors distinct from that recognized by Gal-1. Of these glycoprotein receptors, CD45 and CD71, but not CD29 and CD43, appear to be

involved in Gal-3-induced T cell death. In addition, CD7 that is required for Gal-1-induced death is not required for death triggered by Gal-3. Following Gal-3 binding, CD45 remains uniformly distributed on the cell surface, in contrast to the CD45 clustering induced by Gal-1. Thus, extracellular Gal-3 and Gal-1 induce death of T cells through distinct cell surface events. However, as Gal-3 and Gal-1 cell death are neither additive nor synergistic, the two death pathways may converge inside the cell (Stillman et al. 2006).

Gal-3 as a CD95-Binding Partner in Selecting Apoptotic Signaling Pathways: Studies on CD95 (APO-1/Fas), a member of death receptor family, have revealed that it is involved in two primary CD95 apoptotic signaling pathways, one regulated by the large amount of active caspase-8 (type I) formed at the death-inducing signaling complex and the other by the apoptogenic activity of mitochondria (type II). It is still unclear which pathway has to be activated in response to an apoptotic insult. Fukumori et al. (2004) demonstrated that the Gal-3, which contains the four amino acid-anti-death-motif (NWGR) conserved in the BH1 domain of the Bcl-2 member proteins, is expressed only in type I cells. In addition, Gal-3 is complexed with CD95 in vivo identifying Gal-3 as a novel CD95-binding partner that determines which of the CD95 apoptotic signaling pathways the cell will select (Fukumori et al. 2004).

Repression of Gal-3 by HIPK2-Activated p53: A New Apoptotic Pathway: It has been demonstrated that p53-induced apoptosis is associated with transcriptional repression of Gal-3. Phosphorylation of p53 at Ser46 is important for transcription of proapoptotic genes; induction of apoptosis and the homeodomain-interacting protein kinase 2 (HIPK2) is specifically involved in these functions. Cecchinelli et al. (2006) showed that HIPK2 cooperates with p53 in Gal-3 repression and this cooperation required HIPK2 kinase activity. Gene-specific RNA interference demonstrated that HIPK2 is essential for repression of Gal-3 upon induction of p53-dependent apoptosis. Furthermore, the expression of a nonrepressible Gal-3 prevents HIPK2- and p53-induced apoptosis. These results revealed a new apoptotic pathway induced by HIPK2-activated p53 requiring repression of the antiapoptotic factor Gal-3.

Nucling Mediates Apoptosis by Inhibiting Expression of Gal-3: Nucling is involved in cytochrome c/Apaf 1/caspase-9 apoptosome induction following pro-apoptotic stress. It is able to interact with Gal-3, which participates in apoptotic cell death. Nucling was found to down-regulate the expression level of Gal-3 mRNA/protein. Nucling-deficient cells, in which Gal-3 expression is up-regulated, appeared to be resistant to some forms of pro-apoptotic stress as compared with wild-type cells. In addition, the preputial gland from Nucling-deficient mice expressed a significant level of Gal-3 and exhibited a high incidence of inflammatory lesions, indicating that Nucling plays a crucial role in the homoeostasis of this gland by interacting with the Gal-3 and regulating the expression of Gal-3. Up regulation of Gal-3 was also observed in the heart, kidney, lung, testis and ovary of the Nucling-deficient mice. Nucling was shown to interfere with NF-kB activation via the nuclear translocation process of NF-kB/p65, thus inhibiting the expression of Gal-3. It was proposed that Nucling mediates apoptosis by interacting and inhibiting expression of Gal-3 (Liu et al. 2004).

Gal-3 Gene Encodes a Mitochondrial Protein that Promotes Cytochrome C Release: A Gal-3 internal gene (*Galig*) as an internal gene transcribed from the second intron of the human Gal-3 gene is implicated in cell growth, cell differentiation and cancer development. *Galig* expression causes morphological alterations in human cells, such as cell shrinkage, cytoplasm vacuolization, nuclei condensation, and ultimately cell death. *Galig* encodes a mitochondrial-targeted protein named mitogaligin. Structure studies revealed that the mitochondrial mitogaligin relies on an internal sequence that is required for the release of cytochrome c and cell death upon cell transfection. Moreover, incubation of isolated mitochondria with peptides derived from mitogaligin induces cytochrome c release. Thus, *Galig* is a cell death gene that encodes mitogaligin, a protein promoting cytochrome c release upon direct interaction with the mitochondria (Duneau et al. 2005).

12.3.6 Role in Inflammation

The role of Gal-3 in promotion and control of inflammation and in the regulation of the immune response has been evaluated in different models. Gal-3 has been proposed to be a powerful proinflammatory signal in vitro (Hsu et al. 2000) and in Gal-3-deficient mice (Zuberi et al. 2004). For instance, the lack of Gal-3 protects mice against asthma reactions, an effect associated with an enhanced Th1 response in challenged animals (Zuberi et al. 2004). On the other hand, administration of Gal-3 (by means of gene therapy) inhibits asthmatic reactions (Lopez et al. 2006). These studies reveal opposite effects of the endogenous and exogenous Gal-3 and suggest that, according to its location (extracellular versus intracellular), Gal-3 differentially controls immune/inflammatory cell survival, migration, and cytokine release. Along with its role in inflammation and immune responses in noninfectious conditions, Gal-3 can also sense certain microorganisms through multimeric LacNAc and LacdiNAc forming GalNAcß1-4GlcNAc motifs (van den Berg et al. 2004) as well as to other carbohydrate structures on glycoproteins

and glycolipids (including LPS) from many pathogens such as (myco)bacteria, protozoan parasites, and yeast (Ochieng et al. 2004). Through this activity, Gal-3 participates in the phagocytosis of some microorganisms by macrophages and triggers host responses to pathogens, at least in vitro (Jouault et al. 2006; Sano et al. 2003). Whether it also contributes to signaling events in accessory cells during in vivo infection is unclear. Likewise, its role in signaling pathways triggered by Toll-like receptors (TLRs), which represent key sensors in innate cells (Janeway and Medzhitov 2002), is still elusive. The in vivo role of Gal-3 during infection has not been extensively studied. It appears to clear late *Mycobacterium tuberculosis* infection (Beatty et al. 2002), to contribute to neutrophil recruitment at the site of *Streptococcal pneumonia* infection (Sato et al. 2002), and to exert an important role in innate immunity during *Toxoplasma gondii* infection (Bernardes et al. 2006).

Gal-3 is a Negative Regulator of LPS-Mediated Inflammation: LPS, a major pathogen-associated molecular pattern (PAMP) from the outer membrane of Gram-negative bacteria, is a potent immune activator closely associated with many infectious and inflammatory diseases. LPS consists of hydrophobic lipid A, the 0-polysaccharide chain, and a core oligosaccharide that may be recognized differently by the host immune system. The visualization of LPS requires the TLR4 complex and triggers MyD88-dependent and independent signaling pathways. This leads to the activation of NF-κB and kinases, including MAPK, ERK1/2, p38, and JNK, which subsequently turn on the expression of many inflammatory genes including NADPH oxidase and inducible NO synthase. Since LPS is a powerful immune activator and may be fatal, the response to LPS must be tightly regulated to maintain the immune response at an appropriate level (Takeda et al. 2003; Miller et al. 2005; Liew et al. 2005; Li et al. 2008). Gal-3 interacts with LPS via both N′ and C′ terminals (Mey et al. 1996).

Li et al. (2008) demonstrated that Gal-3 is a negative regulator for LPS function and negatively regulates TCR-mediated CD4+ T-cell activation at the immunological synapse (Chen et al. 2009). Macrophages spontaneously express Gal-3, which specifically binds to LPS. Macrophages from Gal-3-deficient mice have elevated inflammatory cytokine production in response to LPS and lipid A compared with wild-type cells. This is accompanied by an increased phosphorylation of JNK, p38, ERK, and NF-κBp65. The increased inflammatory cytokine production by Gal-3 knockout cells could be normalized by recombinant Gal-3 protein. In vivo, mice lacking Gal-3 excessively produced inflammatory cytokines and NO and were more susceptible to LPS shock. On the other hand, such mice were more resistant to *Salmonella* infection due to the skewing of a Th1 response and increasing the levels of NO and hydrogen peroxide. Thus, Gal-3 is a natural negative regulator for LPS function, which protects against endotoxic shock but may be detrimental by helping in early *Salmonella* infection.

Modulation of T Cell Activity in the Inflamed Intestinal Epithelium: Gal-3, although mostly described as proinflammatory, can also act as an immunomodulator by inducing apoptosis in T cells. In inflamed intestinal mucosa Gal-3 is expressed at comparable levels as in controls and inflammatory bowel disease (IBD) patients in remission. In the normal mucosa, Gal-3 protein was mainly observed in differentiated enterocytes, preferentially at the basolateral side. However, Gal-3 was significantly downregulated in inflamed biopsies from IBD patients. Study suggested that down-regulation of epithelial Gal-3 in the inflamed mucosa reflects a normal immunological consequence, whereas under noninflammatory conditions, its constitutive expression may help to prevent inappropriate immune responses against commensal bacteria or food compounds. Hence, Gal-3 may be a useful parameter for manipulating disease activity (Muller et al. 2006).

Gal-3 in intestine binds to specific *C. albicans* glycans and is involved in inflammation. Inflammation strongly promoted *C. albicans* colonization. Conversely, *C. albicans* augmented inflammation induced by dextran sulfate sodium (DSS). The absence of Gal-3 reduced DSS inflammation and abolished the response of TLR-2 and TNF-α to *C. albicans* colonization. DSS-induced colitis provides a model for establishing *C. albicans* colonization in mice. This model reveals that *C. albicans* augments inflammation and confirms the role of Gal-3 in both inflammation and the control of host responses to *C. albicans* (Jawhara et al. 2008).

Gal-3 Regulates JNK Gene in Mast Cells: Gal-3 plays an important role in mast cell biology. To determine the role of Gal-3 in the function of mast cells, Chen et al. (2006) studied bone marrow-derived mast cells (BMMC) from wild-type ($gal3^{+/+}$) and Gal-3-deficient ($gal3^{-/-}$) mice. Results indicated that there is a defect in the response of mast cells in $gal3^{-/-}$ mice. Unexpectedly, $gal3^{-/-}$ BMMC showed a dramatically low level of JNK1 protein compared with $gal3^{+/+}$ BMMC, which was probably responsible for the lower IL-4 production. The decreased JNK1 level in $gal3^{-/-}$ BMMC was accompanied by a lower JNK1 mRNA level, suggesting that Gal-3 regulates the transcription of the JNK gene or processing of its RNA (Chen et al. 2006).

12.3.7 Gal-3 in Wnt Signaling

Gal-3, a pleiotrophic protein, is an important regulator of tumor metastasis, which, like, β-catenin shuttles between the nucleus and the cytosol in a phosphorylation-dependent manner. Reports show that β-catenin stimulation of cyclin D1 and c-myc expression is Gal-3 dependent. Gal-3 binds to β-catenin/Tcf complex, colocalizes with β-catenin in the nucleus, and induces the transcriptional activity of Tcf-4. The β-catenin- Gal-3-binding sequences which are in the NH2 and COOH termini of the proteins encompassing amino acid residues 1–131 and 143–250 respectively have been identified. Report indicated that Gal-3 is a binding partner for β-catenin involved in the regulation of Wnt/β-catenin signaling pathway (Shimura et al. 2004). The human Gal-3 sequence revealed a structural similarity to β-catenin as it also contains the consensus sequence (S92XXXS96) for glycogen synthase kinase-3β (GSK-3β) phosphorylation and can serve as its substrate. In addition, Axin, a regulator protein of Wnt that complexes with β-catenin, also binds Gal-3 using the same sequence motif identified by a deletion mutant analysis. Shimura et al. (2005) gave credence to the suggestion that Gal-3 is a key regulator in the Wnt/β-catenin signaling pathway and highlighted the functional similarities between Gal-3 and β-catenin.

12.3.8 In Urinary System of Adult Mice

Gal-3 is expressed in murine urinary system, starting from kidney to distal end of urethra; renal cortex expresses Gal-3 more intensely than medulla. An EM study demonstrated diffuse cytoplasmic localization of Gal-3 in principal cells of the collecting ducts and in the bladder epithelial cells. Urethral Gal-3 expression at pars spongiosa decreased in intensity near external urethral orifice, where predominant subtype of galectin was substituted by galectin-7. Observations indicated that adult urinary system shows intense and selective expression of Gal-3 in epithelia of the uretic bud- and cloaca-derivatives (Nio et al. 2006). Gal-3 modulates collecting duct growth/differentiation in vitro, and is expressed in human autosomal recessive polycystic kidney disease in cyst epithelia, almost all of which arise from collecting ducts. Moreover, exogenous Gal-3 restricts growth of cysts generated by Madin-Darby canine kidney collecting duct-derived cells in three-dimensional culture in collagen. The results suggest that Gal-3 may act as a natural brake on cystogenesis in cpk mice, perhaps via ciliary roles (Chiu et al. 2006). Bichara et al. (2006) Showed that Gal $3^{-/-}$ mice have mild renal chloride loss, which causes chronic ECF volume contraction and reduced blood pressure levels.

The adaptation of cortical collecting duct (CCD) to metabolic acidosis requires the polymerization and deposition in the extracellular matrix of the protein hensin. $HCO3^{-}$-secreting β-intercalated cells remove apical Cl^{-} : $HCO3^{-}$ exchangers and may reverse functional polarity to secrete protons. In intercalated cells in culture, Gal-3 facilitated hensin polymerization thereby causing their differentiation into the H^{+}-secreting cell phenotype. It appeared that Gal-3 may play important roles in the CCD, including mediating the adaptation of β-intercalated cells during metabolic acidosis (Schwaderer et al. 2006).

12.3.9 Gal-3 in Reproductive Tissues

Gal-3 expression has been identified in human, rat and porcine testes where it is under hormonal control. Gal-3 is present in Sertoli cells and appears to be absent in human and in rat germ cells. Expression of 31 kDa Gal-3 in cultured porcine Sertoli cells was under positive control of FSH as well as of cytokines EGF and TNF-α. Gal-3 expression in Sertoli cells is also under the control of mature germ cells since an increased expression was observed in adult rat testes depleted in spermatocytes or spermatids (Deschildre et al. 2007). In human testes, Gal-3 is specifically expressed in mature Sertoli cells and Leydig cells, and is absent from fetal and pre-pubertal testes, suggesting a hormone-dependence of this gene (Devouassoux-Shisheboran et al. 2006). Presence of Gal-3 in the connective tissues in the male reproductive organs suggests its role in extracellular matrix (Kim et al. 2006). Gal-1 and Gal-3 are predominantly expressed in the mouse ovary, where they mediate progesterone production and metabolism in luteal cells via different mechanisms (Nio and Iwanaga 2007). In human ovaries, Gal-3 is absent from granulosa cells, as well as from granulosa cell and Sertoli-Leydig cell tumors, and is not a useful marker in Sertoli-Leydig cell tumors (Devouassoux-Shisheboran et al. 2006). Conceptus tissue expresses potential receptors for endometrial HT1 antigen. Carbohydrate-lectin interactions may facilitate attachment of the apical surfaces of uterine epithelial cells and trophectoderm during the early stages of placentation (Woldesenbet et al. 2004).

12.3.10 Gal-3 on Chondrocytes

Endochondral bone formation is characterized by the progressive replacement of cartilage anlagen by bone at the growth plate with a tight balance between the rates of chondrocyte proliferation, differentiation, and cell death. Gal-3 is

detected in mature and early hypertrophic chondrocytes. Notochord-specific expression of a prototype galectin has been characterized during early embryogenesis in zebrafish (Danio rerio) (Ahmed et al. 2004). Gal-3 may play a part in osteoarthritis (OA), possibly related to the interaction of chondrocytes and the cartilage matrix (Guevremont et al. 2004). Presence of Gal-3 at the surface of chondrocytes shows a strong correlation with integrin-β1. Deficiency of matrix metalloproteinase-9 (MMP-9) leads to an accumulation of late hypertrophic chondrocytes. It was found that Gal-3, the in vitro substrate of MMP-9, accumulates in the late hypertrophic chondrocytes and their surrounding ECM in the expanded hypertrophic cartilage zone. These results indicated that extracellular Gal-3 could be an endogenous substrate of MMP-9 that acts downstream to regulate hypertrophic chondrocyte death and osteoclast recruitment during endochondral bone formation. Thus, the disruption of growth plate homeostasis in *Mmp*-9 null mice links Gal-3 and MMP-9 in the regulation of the clearance of late chondrocytes through regulation of their terminal differentiation (Ortega et al. 2005; Li et al. 2009). Because of its ubiquitous expression, Gal-3 cannot be used as a marker of notochordal cells in the postnatal rat disc (Oguz et al. 2007).

12.3.11 Role of Gal-3 in Endothelial Cell Motility and Angiogenesis

During cross-talk between pericytes and endothelial cells (EC), binding of soluble NG2 proteoglycan to the EC surface induces cell motility and multicellular network formation in vitro and stimulated corneal angiogenesis in vivo. The process seems to involve both Gal-3 and α3β1 integrin in the EC response to NG2 and the formation of a complex on cell surface involving NG2, Gal-3, and α3β1. It appears that Gal-3-dependent oligomerization may potentiate NG2-mediated activation of α3β1. In conjunction with earlier studies, this study suggested that pericyte-derived NG2 is an important factor in promoting EC migration and morphogenesis during the early stages of neovascularization (Fukushi et al. 2004).

In two different models of corneal wound healing, re-epithelialization of wounds was significantly slower in Gal-3-deficient ($gal3^{-/-}$) mice compared with wild-type ($gal3^{+/+}$) mice. Exogenous Gal-3 accelerated re-epithelialization of wounds in $gal3^{+/+}$ mice but, surprisingly, not in the $gal3^{-/-}$ mice. In corresponding experiments, recombinant Gal-1 did not stimulate the corneal epithelial wound closure rate. The extent of acceleration of re-epithelialization of wounds with both Gal-3 and galectin-7 was greater than that observed in most of the published studies using growth factors. These findings have broad implications for developing novel therapeutic strategies for treating nonhealing wounds (Cao et al. 2002a, b).

12.3.12 Role in CNS

Microglia is the major cell type expressing Gal-3 in CNS. Ablation of Gal-3 did not affect PrP(Sc)-deposition and development of gliosis. However, $Gal\text{-}3^{-/-}$-mice showed prolonged survival times upon intracerebral and peripheral scrapie infections. Gal-3 plays a detrimental role in prion infections of the CNS, and the endo-/lysosomal dysfunction in combination with reduced autophagy may contribute to disease development (Mok et al. 2007). Schwann cells are considered to be closely involved in the success of peripheral nerve regeneration. The absence of Gal-3 allowed faster regeneration, which may be associated with increased growth of Schwann cells and expression of beta-catenin. This would favor neuron survival, followed by faster myelination, culminating in a better morphological and functional outcome (Narciso et al. 2009). Depletion of Gal-3 from MDCK cells results in missorting of non-raft-dependent apical membrane proteins to the basolateral cell pole. This suggests a direct role of Gal-3 in apical sorting as a sorting receptor (Delacour et al. 2006).

12.4 Clinical Manifestations of Gal-3

12.4.1 Advanced Glycation End Products (AGES)

The accumulation of irreversible advanced glycation end products (AGEs) on long-lived proteins, and the interaction of AGEs with cellular receptors such as AGE-R3/Gal-3 and receptors for AGE (RAGE) are considered the key events in the development of long-term complications of diabetes mellitus, Alzheimer's disease, uremia and ageing.

The expression and sub-cellular distribution of Gal-3, as well as its possible modulation by AGEs, has been investigated in MC3T3E1 mouse calvaria-derived osteoblasts and in UMR 106 rat osteosarcoma cells, both cell lines express 30 kDa (monomeric) Gal-3. Dimeric (70 kDa) Gal-3 was also observed in the UMR106 cells. Exposure to AGEs-BSA increased the cellular content of 30 kDa Gal-3 and decreased Gal-3 in culture media. These results confirmed the expression of Gal-3 in osteoblastic cells. Osteoblastic exposure to AGEs alters the expression of Gal-3, which may have significant consequences on osteoblast metabolism and thus on bone turnover (Mercer et al. 2004).

Corneal endothelial cell loss is a change that occurs with age. Interaction between AGE and its receptors is implicated in the corneal endothelial cell loss with age. AGEs

accumulate with ageing and may have a significant impact on age related dysfunction of the retinal pigment epithelium (RPE). Expression of RAGE and Gal-3 was detected in bovine corneal endothelial cells. Gal-3 was important in the internalization of AGE. In contrast, RAGE was important in the generation of reactive oxygen species and induction of apoptosis. Based on these data, the interaction of AGE in aqueous humor and AGE receptors expressed on the corneal endothelial cells was speculated to have a role in the corneal endothelial cell loss with age (Kaji 2005). Stitt et al. (2005) reported a significant suppression of angiogenesis by the retinal microvasculature during diabetes and implicated AGEs and AGE-receptor interactions in its causation (Stitt et al. 2005). The role of the Gal-3 receptor component was examined by transfection and overexpression using the D407 cell line. Primary cultures of bovine RPE cells and also a human cell line showed a pathological response to AGE exposure, an effect which appears to be modulated by the Gal-3 component of the receptor complex (McFarlane et al. 2005).

Mice lacking Gal-3/AGE-receptor 3 develop accelerated diabetic glomerulopathy. Gal-3 ablation is associated with increased susceptibility to diabetes- and AGE-induced glomerulopathy, thus indicating a protective role of Gal-3 as an AGE receptor (Iacobini et al. 2004, 2005). Gal-3 in chronic kidney disease induced by unilateral ureteral obstruction (UUO) showed significantly increased expression compared with basal levels. The degree of renal damage was more extensive in Gal-3-deficient mice at days 14 and 21. Therefore, Gal-3 not only protects renal tubules from chronic injury by limiting apoptosis but it may lead to enhanced matrix remodeling and fibrosis attenuation (Okamura et al. 2010)

12.4.2 GAL-3 and Protein Kinase C in Cholesteatoma

Cholesteatoma is a benign disease characterized by the presence of an unrestrained growth and the accumulation of keratin in the middle ear cavity. Ghanooni et al. (2006) studied the expression of protein kinase C-α, -δ, -γ, -η, and -ζ in epithelial tissues of human cholesteatomas and their correlations with distributions of p53, Gal-3, retinoic acid receptor-β and macrophage migration inhibitory factor (MIF). The patterns of PKC-α, and -δ expression, but not of PKC-γ, -η and -ζ correlated significantly and positively with Gal-3 expression. In addition, the correlation levels between the expression of PKC-α, and -δ and that of Gal-3 varied depending on the infection and recurrence status. Thus, modifications occurring at the level of keratinocyte differentiation in human cholesteatomas involve the activation of PKC-α, -δ, -γ, -η and -ζ (Ghanooni et al. 2006).

12.4.3 Gal-3 and Cardiac Dysfunction

Inflammatory mechanisms have been proposed to be important in heart failure (HF), and cytokines have been implicated to enhance the progression of HF. In a comprehensive study, Gal-3 has emerged as the most robustly overexpressed gene in failing *versus* functionally compensated hearts from homozygous transgenic TGRmRen2-27 (Ren-2) rats. Myocardial biopsies obtained at an early stage of hypertrophy before apparent HF showed that expression of Gal-3 was increased specifically in the rats that later rapidly developed HF. Gal-3 colocalized with activated myocardial macrophages. Gal-3-binding sites were found in cardiac fibroblasts and the extracellular matrix. Sharma et al. (2004) showed that an early increase in Gal-3 expression identifies failure-prone hypertrophied hearts. Gal-3, a macrophage-derived mediator, induces cardiac fibroblast proliferation, collagen deposition, and ventricular dysfunction. This implies that HF therapy needs to antagonize multiple inflammatory mediators, including Gal-3. Nonetheless, the amino-terminal pro-brain natriuretic peptide (NT-proBNP) is considered superior to either apelin or Gal-3 for diagnosis of acute HF, although Gal-3 levels were significantly higher in subjects with HF compared with those without. However, recent data showed potential utility of Gal-3 as a useful marker for evaluation of patients with suspected or proven acute HF, whereas apelin measurement was not useful for these indications. Moreover, the combination of Gal-3 with NT-proBNP was the best predictor for prognosis in subjects with acute HF (van Kimmenade et al. 2006).

In the ApoE-deficient mouse model of atherosclerosis Nachtigal et al. (2008) showed an age-related increase in the incidence of aorta atheromatous plaques and periaortic vascular channels in ApoE-deficient mice. By contrast ApoE/Gal-3 double-knockout mice did not show an increase in pathological changes with age. The effect of Gal-3 deficiency on atherogenesis decrease could be related to its function in macrophage chemotaxis, angiogenesis, lipid loading, and inflammation. Atherosclerosis and renal disease are related conditions, sharing several risk factors. This includes hyperlipidaemia, which may result in enhanced lipoprotein accumulation and chemical modification, particularly oxidation, with formation of advanced lipoxidation end products (ALEs). Mice knocked out for $Gal3^{-/-}$ have shown that Gal-3 exerts a significant role in the uptake and effective removal of modified lipoproteins with diversion of these products from RAGE-dependent pro-inflammatory pathways associated with downregulation of RAGE expression. Iacobini et al. (2009a, b)

suggest a unique protective role for Gal-3 in the uptake and effective removal of modified lipoproteins, with concurrent down-regulation of proinflammatory pathways responsible for atherosclerosis initiation and progression.

12.4.4 Gal-3 and Obesity

In streptozotocin induced diabetic C57BL/6 Gal-$3^{+/+}$ and Gal-$3^{-/-}$ mice, Gal-3 is involved in immune mediated β cell damage and is required for diabetogenesis in this mouse model (Mensah-Brown et al. 2009). Gal-3 might be associated with the pathophysiology of obesity in obesity-prone C57BL/6 J mice (Kiwaki et al. 2007). Adipocytes synthesize Gal-3 whose deficiency protects from inflammation associated with metabolic diseases. Circulating Gal-3 was elevated in type 2 diabetes (T2D) and obesity compared with normal-weight individuals. In T2D patients, Gal-3 was increased in serum of patients with elevated C-reactive protein and negatively correlated with glycated hemoglobin. It was suggested that systemic Gal-3 is elevated in obesity and negatively correlates with glycated hemoglobin in T2D patients, pointing to a modifying function of Gal-3 in human metabolic diseases (Weigert et al. 2010). There remains a need for robust mouse models of diabetic nephropathy (DN) that mimic key features of advanced human DN. The recently developed mouse strain BTBR with the ob/ob leptin-deficiency mutation develops severe type 2 diabetes, hypercholesterolemia, elevated triglycerides, and insulin resistance. The abnormalities closely resemble advanced human DN more rapidly than most other murine models, making this strain particularly attractive for testing therapeutic interventions (Hudkins et al. 2010).

12.4.5 Autoimmune Diseases

Gal-3 in Induction of Type 1 Diabetes: Pro-apoptotic cytokines have been associated with the pathogenesis of Type 1 diabetes (T1D). Rat islets identified Gal-3 as the most up-regulated protein. Combined proteome-transcriptome-genome and functional analyses identified gal-3 as a candidate gene/protein in T1D susceptibility that may prove valuable in future intervention/prevention strategies (Karlsen et al. 2006). Macrophages are potent immune regulators in the development of autoimmune diabetes and are important effector cells during diabetogenesis. The role of macrophages in autoimmune diabetes has been described with particular emphasis on the role of Gal-3 and T1/ST2, an IL-1 receptor-like protein, both of which play significant roles in the immunomodulatory functions of macrophages in BALB/c mice. It appeared that functional capacity of macrophages influences their participation in Th1-mediated autoimmunity and the development of autoimmune diabetogenesis (Mensah-Brown et al. 2006).

Gal-3 in Rheumatoid Arthritis: Galectin 3 is present in the inflamed synovium in patients with rheumatoid arthritis suggesting that the protein is associated with the pathogenesis of this disease. In murine model, Gal-3 plays a pathogenic role in the development and progression of antigen-induced arthritis (AIA) and the disease severity is accompanied by alterations of antigen-specific IgG levels, systemic levels of TNFα and IL-6, and frequency of IL-17-producing T cells. Rheumatoid arthritis (RA) is a chronic debilitating autoimmune disease that results in joint destruction and subsequent loss of function. Gal-3 is expressed in synovial tissue of patients with RA, particularly at sites of joint destruction. Gal-3 is induced either by proinflammatory cytokines or by adhesion to cartilage components. Results suggest four times more RA synovial fibroblasts (SF) than osteoarthritis SF adhered to COMP (cartilage oligomeric matrix protein) coated plates. The adhesion of RA SF to COMP was found to increase the intracellular level of Gal-3. In contrast, intracellular Gal-3 decreased after exposure to TNF-α. It was concluded that the increase of Gal-3 occurs after adhesion to COMP, and the αVβ3 receptor (CD51/CD61) has a pivotal role in this process (Neidhart et al. 2005). To better understand the pathogenesis of RA, Shou et al. (2006) profiled the rat model of collagen-induced arthritis (CIA) to discover and characterize blood biomarkers for RA. Genes known to be involved in autoimmune response and arthritis, such as those encoding Gal-3, Versican, and Socs3, were identified and validated. Analysis confirmed that Gal-3 was secreted over time in plasma as well as in supernatant of cultured tissue synoviocytes of the arthritic rats, which is consistent with disease progression (Shou et al. 2006; Forsman et al. 2011). Gal-3 also plays an important disease-exacerbating role in EAE through its multifunctional roles in preventing cell apoptosis and increasing IL-17 and IFN-γ synthesis, but decreasing IL-10 production (Jiang et al. 2009).

Gal-3 in Psoriatic Skin: Gal-3 is critical for development of allergic inflammatory response in a mouse model of atopic dermatitis (Saegusa et al. 2009). Contrary to normal epidermis, the psoriatic epithelium does not express Gal-3 and glycoligands for Gal-1. Strong expression of Gal-3/Gal-3-reactive glycoligands in capillaries of psoriatic dermis represents one of the most important findings demonstrating the activation of endothelium in the course of the disease. The altered galectin expression and binding pattern in psoriatic skin indicates the modified process of keratinocyte maturation in hyperactivated psoriatic epithelium. The enhanced

expression of Gal-3/Gal-3-reactive glycoligands in dermal capillaries of psoriatic skin can be important for rearrangement of the capillary network and migration of inflammatory cells to psoriatic skin (Lacina et al. 2006). Keratinocytes undergo apoptosis in a variety of physiological and pathological conditions. Gal-3 mRNA was transiently upregulated in ultraviolet-B (UVB)-irradiated wild-type keratinocytes. Gal-$3^{-/-}$ keratinocytes were significantly more sensitive to apoptosis induced by UVB as well as various other stimuli, both in vitro and in vivo, than wild-type cells. It suggests that endogenous Gal-3 is an anti-apoptotic molecule in keratinocytes functioning by suppressing ERK activation and enhancing AKT activation and may play a role in the development of apoptosis-related skin diseases (Saegusa et al. 2008).

12.4.6 Myofibroblast Activation and Hepatic Fibrosis

Gal-3 expression is up-regulated in human fibrotic liver disease and is temporarily and spatially related to the induction and resolution of experimental hepatic fibrosis. Disruption of the Gal-3 gene blocks myofibroblast activation and procollagen (I) expression in vitro and in vivo, markedly attenuating liver fibrosis. The reduction in hepatic fibrosis was observed in the gal$3^{-/-}$ mouse despite equivalent liver injury and inflammation, and similar tissue expression of TGF-β. TGF-β failed to transactivate gal$3^{-/-}$ hepatic stellate cells, in contrast with WT (gal$3^{+/+}$) hepatic stellate cells. It indicates that Gal-3 is required for TGF-β mediated myofibroblast activation and matrix production. Further, in vivo siRNA knockdown of Gal-3 inhibited myofibroblast activation after hepatic injury and may therefore provide an alternative therapeutic approach to the prevention and treatment of liver fibrosis (Henderson et al. 2006).

Gal-3 may play an important role in inflammatory responses. Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as a liver condition that may progress to end-stage liver disease. Based on the known functions of Gal-3, it was hypothesized that Gal-3 might play a role in the development of NAFLD. Study in Gal-3 knockout gal$3^{-/-}$ mice suggested that the absence of Gal-3 can cause clinicopathological features in male mice similar to those of NAFLD (Nomoto et al. 2006).

Colonic lamina propria fibroblasts (CLPFs) play an important role in the pathogenesis of fibrosis and strictures in Crohn's disease. Soluble Gal-3 was identified as a strong activator of CLPFs produced by CEC. Gal-3 induced NF-kB activation and IL-8 secretion in these cells may be a target for future therapeutic approaches to reduce or avoid stricture formation (Lippert et al. 2007).

12.5 Gal-3 as a Pattern Recognition Receptor

The galectin family of lectins recognizes saccharide ligands on a variety of microbial pathogens, including viruses, bacteria, yeasts, and parasites. Gal-3, expressed by macrophages, dendritic cells, and epithelial cells, binds bacterial and parasitic pathogens including *Leishmania major*, *Trypanosoma cruzi*, and *Neisseria gonorrhoeae*. Galectins may have direct effects on microbial viability. Kohatsu et al. (2006) showed that Gal-3 binds only to those *C. albicans* species that bear β-1,2-linked oligomannans on the cell surface, but did not bind *S. cerevisiae* that lacks β-1,2-linked oligomannans. Surprisingly, binding directly induced death of *Candida* species containing specific β-1,2-linked oligomannosides. Thus, Gal-3 can act as a pattern recognition receptor that recognizes a unique pathogen-specific oligosaccharide sequence (Kohatsu et al. 2006).

12.5.1 Gal-3 Binds to *Helicobacter pylori*

Helicobacter pylori causes gastritis and some infections result in peptic ulceration, gastric adenocarcinoma or gastric lymphoma. The role for the lipopolysaccharide O-antigen side-chain in this process has been identified. Evidence indicates that the receptor recognized by the O-antigen side-chain is Gal-3. Expression of Gal-3, is upregulated by gastric epithelial cells following adhesion of *H. pylori,* suggesting that in addition to colonization this protein also plays a role in the host response to infection. Upregulation of Gal-3 is inhibited by treating gastric epithelial cells with MAPK inhibitors and does not occur in cells infected with either *H. pylori* cagE or cagA isogenic mutants. This implies that *H. pylori*-mediated expression of Gal-3 is dependent on delivery of CagA into the host cell cytosol and the subsequent stimulation of MAPK signalling. A further consequence of *H. pylori* adhesion is that it elicits a rapid release of Gal-3 from infected cells (Fowler et al. 2006).

12.5.2 Recognition of *Candida albicans* by Macrophages Requires Gal-3

β-1,2-linked oligomannoside residues are associated with mannan and a glycolipid, the phospholipomannan, at the *Candida albicans* cell wall surface. β-1,2-linked oligomannoside residues act as adhesins for macrophages and stimulate these cells to undergo cytokine production. The macrophage receptor involved in the recognition of *C. albicans* β-1,2-oligomannoside repeatedly led to detection of a 32-kDa

macrophage protein. The purified peptides from the 32-kDa tryptic digest showed complete homology to Gal-3, an endogenous lectin which is expressed in a wide variety of cell types with which *C. albicans* interacts as a saprophyte or a parasite (Fradin et al. 2000). Glycans present in both *C. albicans* and *S. cerevisiae* cell walls have been shown to act as ligands for different receptors leading to different stimulating pathways, some of which need receptor co-involvement. However, among these ligand-receptor couples, none has been shown to discriminate the pathogenic yeast *C. albicans*. Jouault et al. (2006) explored the role of Gal-3, which binds *C. albicans* β-1,2 mannosides and suggested that macrophages differently sense *C. albicans* and *S. cerevisiae* through a mechanism involving TLR2 and Gal-3, which probably associate for binding of ligands expressing β-1,2 mannosides specific to the *C. albicans* cell wall surface.

12.5.3 Gal-3 is Involved in Murine Intestinal Nematode and Schistosoma Infection

Conflicting studies have addressed the role of endogenous Gal-3 during *Schistosoma* infection (Bickle and Helmby 2007; Oliveira et al. 2007). Gal-3 recognizes the GalNAcβ1-4GlcNAc (LDN) epitope present on many helminth antigens, including those of the schistosome eggs. However, Gal-3 is not a critical component in the development of Th2 responses during helminth infection in vivo, nor it is essential for schistosome egg granuloma formation (Bickle and Helmby 2007). The N-terminal lectin domain (Nh) of the tandem repeat-type nematode galectin LEC-1 has a lower affinity for sugars than the C-terminal lectin domain. LEC-1 forms a complex with N-acetyllactosamine-containing glycoproteins. The formation of a crosslinked product with the Q38C mutant suggested the low-affinity interaction of Nh with the glycoprotein (Arata et al. 2006). van den Berg et al. demonstrated that LDN motifs, which are expressed by *Schistosoma mansoni* eggs (Khoo and Dell 2001; Srivatsan et al. 1992), bind Gal-3 and suggested that this interaction may play an important role in Th2-mediated inflammatory response that occurs in the liver (van den Berg et al. 2004). Oliveira et al. (2007) and Breuilh et al. (2007) showed that, relative to wild-type (WT) mice, *S. mansoni*-infected Gal-3-deficient mice develop reduced granuloma formation and have a dramatically decreased number of total lymphocytes in spleen. Although Gal-3 deficiency in DCs does not impact their differentiation and maturation processes, it greatly influences the strength (but not the nature) of adaptive immune response that they trigger, suggesting that Gal-3 deficiency in some other cell types may be important during murine schistosomiasis. As a whole, Gal-3 is a modulator of immune/inflammatory responses during helminthic infection and that Gal-3 expression in DCs is pivotal to control the magnitude of T-lymphocyte priming (Breuilh et al. 2007).

12.5.4 Up-Regulation of Gal-3 and Its Ligands by *Trypanosoma cruzi* Infection

Human Gal-3 binds to the surface of *Trypanosoma cruzi trypomastigotes* and human coronary artery smooth muscle (CASM) cells. CASM cells express Gal-3 on their surface and secrete it. Exogenous Gal-3 increased the binding of *T. cruzi* to CASM cells. Trypanosome binding to CASM cells was enhanced when either *T. cruzi* or CASM cells were preincubated with Gal-3. Thus, host Gal-3 expression is required for *T. cruzi* adhesion to human cells and exogenous Gal-3 enhances this process, leading to parasite entry (Kleshchenko et al. 2004). The expression of Gal-3 and its ligands on splenic DCs (sDCs) from *T. cruzi* infected mice are markedly up-regulated and adhesiveness is increased with Gal-3-coated substratum. Results documented that a parasitic infection can modulate both in vivo and in vitro the expression of Gal-3 and of ligands for this lectin in DCs with functional consequences on their capacities of adhesion and migration (Vray et al. 2004). During acute infection with *T. cruzi*, the thymus undergoes intense atrophy followed by a premature escape of $CD4^+CD8^+$ immature cortical thymocytes. Studies provide evidence of a role for Gal-3 in the regulation of thymus physiology and in identifying a potential mechanism based on protein-glycan interactions in thymic atrophy associated with acute *T. cruzi* infection (Silva-Monteiro et al. 2007).

12.6 Gal-3 as a Therapeutic Target

12.6.1 Gal-3: A Target for Anti-inflammatory/Anticancer Drugs

Dabelic et al. (2006) investigated the effects of non-steroidal anti-inflammatory drugs (aspirin and indomethacin) and glucocorticoids (hydrocortisone and dexamethasone) on macrophage Gal-3, which in general acts as a strong pro-inflammatory signal. All immunomodulatory drugs in clinically relevant doses affect both the gene (LGALS3) and protein expression level of Gal-3. Study revealed Gal-3 as a new target molecule of immunomodulatory drugs, thus suggesting an additional pathway of their action on immune response (Dabelic et al. 2006). Targeting efficacy of anticancer drug (doxorubicin) was improved when the drug was conjugated to N-(2-hydroxypropyl) methacrylamide (HPMA)-based copolymers with bio-recognizable groups, such as simple carbohydrates. HPMA-based copolymers

are efficient carriers for anticancer drugs because of their good biocompatibility. Thus, the binding of the glycoside-bearing HPMA copolymer-DOX conjugates to the cells was mediated not only by Gal-3, but HPMA Copolymer conjugates bearing multivalent galactoside residues can improve their cytotoxicity (David et al. 2004).

Galectins-3 is implicated in asthma. In a murine model of ovalbumin (OVA)-induced asthma, (1) peribronchial inflammatory cells express large amounts of Gal-3; (2) bronchoalveolar lavage fluid from OVA-challenged mice contained higher levels of Gal-3 compared to control mice; and (3) macrophages in bronchoalveolar lavage fluid were the major cell type that contained Gal-3. Further investigations revealed that OVA-sensitized $gal3^{-/-}$ mice developed fewer eosinophils and lower goblet cell metaplasia, after airway OVA challenge compared to similarly treated $gal3^{+/+}$ mice. In addition, the OVA-sensitized $gal3^{-/-}$ mice developed significantly less airway hyperresponsiveness and developed a lower Th2 response, but a higher Th1 response, suggesting that Gal-3 regulates the Th1/Th2 response after airway OVA challenge. Thus, Gal-3 might play an important role in the pathogenesis of asthma and inhibitors of this lectin could prove useful for treatment of this disease (Zuberi et al. 2004). The inhibitory effects of Gal-3 on eosinophilic inflammation in guinea pig asthma models provided evidence for an eosinophil recruitment from bone marrow to circulation blood to lung in asthmatic response, in which overexpression of IL-5, Eotaxin, and CCR-3 could be involved. Gal-3, a selective inhibitor of IL-5 mRNA transcription, might potentially suppress eosinophilc inflammation and be a compromising specific anti-asthma reagent (Li et al. 2005). The role of galectins in chronic obstructive pulmonary disease (COPD), characterized by epithelial changes and neutrophil infiltration remains unknown. Studies support the hypothesis that distal airways represent an important site for detecting changes in COPD: viz., in patients with severe disease increased Gal-3 expression and neutrophil accumulation in the small airway epithelium, correlating with epithelial proliferation and airway obstruction (Pilette et al. 2007). In chronic asthmatic mice, treatment with Gal-3 gene led to an improvement in the eosinophil count and the normalization of hyperresponsiveness to methacholine. Concomitantly, this treatment resulted in an improvement in mucus secretion and sub-epithelial fibrosis in the chronically asthmatic mice, with a measured reduction in lung collagen, a prominent feature of airway remodeling (Lopez et al. 2006).

Studies have revealed that Gal-3 demonstrates anti-apoptotic effects which contribute to cell survival in several types of cancer cells. Intracellular Gal-3 in particular, which contains the NWGR anti-death motif of the Bcl-2 family, inhibits cell apoptosis induced by chemotherapeutic agent such as cisplatin and etoposide in some types of cancer cells. The nuclear export of phosphorylated Gal-3 regulates its anti-apoptotic activity in response to chemotherapeutic drugs. It is suggested that targeting Gal-3 could improve the efficacy of anticancer drug chemotherapy in several types of cancer. Gal-3, which plays an important role in the biology of angiosarcoma (ASA) in humans and hemangiosarcoma (HSA) in dogs is identified as a potential therapeutic target in tumors arising from malignant endothelial cells (Fukumori et al. 2007; Johnson et al. 2007) (See Chap, 13).

12.7 Xenopus-Cortical Granule Lectin: A Human Homolog of Gal-3

Cortical granule lectin (xCGL) is a candidate target glycoprotein of Xenopus galectin-VIIa (xgalectin-VIIa) in Xenopus embryos. In addition, another member of the xCGL family is xCGL2. Expression of the mRNAs of xCGL and xCGL2, as well as that of xgalectin-VIIa occurs throughout early embryogenesis. Two and three potential N-glycosylation sites were deduced from the amino acid sequences of xCGL and xCGL2, respectively. The xgalectin-VIIa recognizes N-glycans linked to a common site in xCGL and xCGL2 in addition to N-glycans linked to a site specific to xCGL2. However, interaction between xgalectin-Ia and xCGLs was not detectable. The oligosaccharide specificity pattern of xgalectin-VIIa was similar to that of human homolog Gal-3. The N-acetyllactosamine type, biantennary N-glycans exhibit high affinity for xgalectin-VIIa ($K_D = 11$ μM) families (Shoji et al. 2005).

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Galectin-3: A Cancer Marker with Therapeutic Applications

13

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13.1 Galectin-3: A Prognostic Marker of Cancer

Galectin-3 (Gal-3) is a pleiotropic carbohydrate-binding protein involved in a variety of normal and pathological biological processes. Its carbohydrate-binding properties constitute the basis for cell-cell and cell-matrix interactions (Chap. 12) and cancer progression. Gal-3 is known to be expressed in various neoplasms including thyroid tumors. Gal-3 is widely spread among different types of cells and tissues, found intracellularly in nucleus and cytoplasm or secreted via non-classical pathway outside the cell, thus being found on the cell surface or in the extracellular space. Gal-3 is involved in RNA processing and cell cycle regulation through activation of transcription factors when translocated to the nucleus. It affects numerous biological processes and seems to be involved in different physiological and pathophysiological conditions. Most members of the galectin family including galectin-1 possess apoptotic activities, whereas Gal-3 possesses anti-apoptotic activity (Dumic et al. 2006). Studies lead to the recognition of Gal-3 as a diagnostic/prognostic marker for specific cancer types, such as thyroid and prostate (Califice et al. 2004b). Though the immune system recognizes diverse cancer antigens, tumors can evade the immune response, therefore growing and progressing. However, there is strong evidence indicating that the regulation of galectins function in the human tumor microenvironment is a complex process that is influenced by diverse biological circumstances.

13.2 Discriminating Malignant Tumors from Benign Nodules of Thyroid

Galectin-3 has been regarded as a useful tool for discriminating malignant tumors from benign nodules of the thyroid, including the distinction between follicular carcinoma and adenoma. Gal-3 is over-expressed in neoplastic human thyroid, while its expression in normal tissue and adenomas was absent or weak (Cvejic et al. 2005a, c; Kawachi et al. 2000; Yoshii et al. 2001; Coli et al. 2002; Faggiano et al. 2002; Herrmann et al. 2002; Nucera et al. 2005; Oestreicher-Kedem et al. 2004; Pisani et al. 2004; Takenaka et al. 2003). Gal-3 may potentially serve as a marker in difficult differential diagnosis cases involving Hürthle cell adenomas and Hürthle cell carcinomas (Nascimento et al. 2001). In addition, Gal-3 mRNA was observed in all malignant thyroid lesions, while in normal and non-malignant tissues, it was not detectable (Inohara et al. 1999). Gal-3 was predominantly found in the cytoplasm of follicular and parafollicular cells; a nuclear location was also sometimes observed. Gal-3 was significantly higher in papillary carcinomas with metastases than those without metastases (Kawachi et al. 2000). However, expression of Gal-3 in lymphatic metastases of papillary carcinoma and in lymph node metastases of medullary thyroid carcinoma appeared to be lower than in primary lesions (Faggiano et al. 2002; Kawachi et al. 2000). The antisense inhibition of Gal-3 expression in thyroid papillary carcinoma resulted in a marked reduction of malignant phenotype (Yoshii et al. 2001). Takenaka et al. (2003) gave evidence, which suggested that over-expression of Gal-3 in transfected Gal-3 cDNA normal thyroid follicular cells leads to the acquisition of malignant phenotype. The genes with increased expression include: retinoblastoma (RB), proliferating cell nuclear antigen (PCNA) and replication factor C (RCF), all of which are involved in the G1-S transition of cell cycle. Therefore, the possibility of Gal-3 involvement in the cell cycle regulation exists. From a biochemical point of view, the evaluation of cytoplasmic Gal-3 expression in epithelial cells isolated from FNAB should serve to make a differential presurgical diagnosis between benign follicular adenomas and differentiated carcinomas (i.e., papillary and widely and minimally invasive follicular carcinomas). Serum levels of galectins-1 and -3 are relatively high in patients with thyroid malignancy but there is

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considerable overlap in serum Gal-3 concentrations between those with benign and malignant nodular thyroid disease and, to a lesser extent, between those with and without nodular thyroid disease (Saussez et al. 2008c).

However, there are follicular tumors with unclear vascular or capsular invasion, which makes diagnosis more difficult. To find a relationship between Gal-3 expression and the degree of vascular or capsular invasion of follicular tumors, Ito et al. (2005) suggested that Gal-3 plays a role in the transformation of follicular tumors from benign to malignant. Moreover, in follicular tumors, the presence of this protein should not be required for diagnosing malignant transformation in all cases. Hence, Gal-3 should only be considered as an adjuvant marker for follicular carcinoma (Ito et al. 2005). Analyses of 202 specimens of papillary thyroid carcinoma (PTC) in relation to histomorphologic subtypes and clinicopathologic data showed that Gal-3 itself is not an indicator of local metastatic spread or extrathyroid invasion of PTC (Cvejic et al. 2005c). Another study indicated that Gal-3 gene is expressed at the protein level in most papillary microcarcinomas, a variant of PTC, although with slightly lower frequency than that reported for clinically evident PTC. The presence of Gal-3 in clinically silent microcarcinomas may indicate that Gal-3 is not related to growth or aggressiveness of papillary thyroid microcarcinomas but rather plays some other role in thyroid tumor biology. Results on the relationship between Gal-3 and proliferating cell nuclear antigen (PCNA) suggested that overexpression of Gal-3 is not clearly related to proliferative activity of PTC cells as assessed by PCNA immunostaining (Cvejic et al. 2005a, b; Pisani et al. 2004). In addition to 12/15 papillary carcinomas, among the malignant thyroid lesions, Gal-3 was also detected in 3/4 Hurthle cell carcinomas, 4/6 follicular carcinomas and 0/3 anaplastic carcinomas. Conversely, Gal-3 expression was absent in NT and in all benign thyroid lesions, but 1/15 Hashimoto's thyroiditis (HT) and 10/22 follicular adenomas (FA). Gal-3 cytoplasmic-perinuclear immunolocalization was observed in the majority of thyroid carcinomas and in more than half of the FA, theoretically suggesting an involvement of this protein in thyroid tumorigenesis throughout an antiapoptotic activity. Moreover, Gal-3 expression in FA might anticipate the likelihood of evolution of these benign lesions towards malignancy (Nucera et al. 2005; Oestreicher-Kedem et al. 2004). Diagnostic problems may arise in the presence of Hurthle cell proliferation or minimally invasive follicular carcinoma.

13.2.1 Large-Needle Aspiration Biopsy

Gasbarri et al. (2004a) illustrated the clinical impact of a new diagnostic test-method, named "Gal-3 thyrotest", which is based on the immunodetection of Gal-3 in specimens derived from thyroid nodular lesions. This diagnostic test method, which consistently improves the accuracy of conventional cytology, has been validated in a large multicenter study and is going to impact hardly the clinical management of patients bearing thyroid nodular diseases. The rationale of this diagnostic approach, the possibility to improve its performance in selected cases by using large needle aspiration biopsy (LNAB) together with technical details have been presented and discussed (Gasbarri et al. 2004a). Among 85 thyroid specimens, 42 Gal-3-positive cases were discovered preoperatively (11 thyroid cancers and 3 adenomas confirmed at the final histology), whereas Gal-3-negative cases were 71 (1 carcinoma and 70 benign proliferations at the final histology). Sensitivity, specificity and diagnostic accuracy of this integrated morphologic and phenotypic diagnostic approach were 91.6%, 97.2% and 95.3%, respectively. In conclusion, LNAB plus Gal-3 expression analysis when applied preoperatively to selected thyroid nodules candidate to surgery can potentially reduce unnecessary thyroid resections (Carpi et al. 2006). The routine correct use of Gal-3 can lead to a sensitive reduction of useless thyroid surgeries.

13.2.2 Fine-Needle Aspiration Biopsy

Among diagnostic modalities, fine-needle aspiration biopsy (FNAB) of clinically suspicious thyroid nodules is becoming increasingly popular. Assays using tumor-specific markers may improve the sensitivity and accuracy of FNA and so may be expected to reduce the frequency of open surgical procedures by identifying those patients with demonstrably benign lesions who do not require definitive surgical excision of their lesions for diagnosis. At the same time, thyroid-specific mRNA assays (especially thyroglobulin mRNA testing) have been used by investigators in the postoperative follow-up of patients with thyroid cancer as a potential means of detecting tumor recurrence in the peripheral blood (Rodrigo et al. 2006). Mills et al. (2005) suggested that Gal-3 does not appear to be a useful adjunct to diagnosis in thyroid FNA as it does not reliably distinguish malignant and benign lesions. Many thyroid aspirates are of low cellularity and are not suitable for cell block immunohistochemistry (Mills et al. 2005). Moreover, Gal-3 in the tissue of follicular adenomas with grave dysplasia and follicular carcinoma is an unfavourable prognostic sign and not a reliable immunohistochemical marker to distinguish benign from malignant thyroid follicular lesions (Mehrotra et al. 2004).

Although FNAB is the most reliable preoperative diagnostic procedure, it shows inherent limitations in differentiating adenoma from follicular carcinoma and, sometimes, follicular variants of papillary carcinoma. Gal-3 cytoplasmic neoexpression has been proposed as a peculiar

feature of thyroid malignant cells, easily detectable in cytological and histological samples. Among 39 follicular carcinomas, 26 papillary carcinomas (PTC), and 105 adenomas in both cell-block samples and their histological counterparts, all papillary carcinoma samples showed high levels of Gal-3 immunoreactivity. Thirty-four follicular carcinomas were positive, whereas five were negative in cell-blocks but positive in their histological counterparts (Saggiorato et al. 2004). In Indian population, Gal-3 positivity was seen in 80% of papillary carcinomas, 13.5% of follicular neoplasms and in 60% of benign nodules. Study showed that Gal-3 was strongly expressed in smears of papillary carcinoma. However, since it is also expressed in a variety of benign lesions, its role as a pre-surgical markerfor differentiating benign from malignant thyroid nodules is limited (Aron et al. 2006).

From a total of 426 follicular lesions from patients who had undergone thyroidectomy for either benign or malignant nodules, Gal-3 could be used as a useful supplementary marker for cytologic diagnosis, although it was not an absolute marker in determining whether a lesion was benign or malignant (Bartolazzi and Bussolati 2006; Kim et al. 2006). Collet et al. (2005) suggested that Gal-3 constitutes a useful marker in the diagnosis of thyroid lesions classified as undeterminate by conventional cytology. Torres-Cabala et al. (2006) concluded that gal-3, gal-1 and S100C can be used to help in discriminating benign and malignant thyroid lesions.

Papillary carcinomas showed a strong, but cytoplasmic pattern of staining. Gal-3 was strongly positive in papillary carcinomas, and negative in benign lesions, confirming its value in differential diagnosis. The S100C, highly expressed in papillary carcinomas, is expressed in the nuclei of normal tissue, hyperplastic nodules, and follicular adenomas and carcinomas. It is helpful in pathological study of thyroid lesions, especially in cases in which follicular variants of papillary carcinoma and follicular carcinoma are considered in differential diagnosis (Torres-Cabala et al. 2004). Quantification of the TFF3/Gal-3 mRNA ratio (T/G ratio) may be a useful tool for the distinction between follicular adenomas and carcinomas, which is most difficult in thyroid pathology (Takano et al. 2005).

13.2.3 Combination of Markers

Gal-3 and HBME-1 Markers: Oncocytic cell tumors (OCTs) of the thyroid include oncocytic cell adenomas (OCAs) and oncocytic cell carcinomas (OCCs). Oncocytic variant of papillary carcinoma (OVPC) has also been described. These tumors may present similar diagnostic problems as their non-oncocytic counterparts, in both conventional histology and FNAB. Several markers can distinguish benign from malignant thyroid follicular tumors, Gal-3 and HBME-1 being the most promising ones. Though controversial data have been reported on their discriminatory potential in the small series of OCTs, Volante et al. (2004) assessed the role of Gal-3 and HBME-1 in a large series of 152 OCTs (including 50 OCAs, 70 OCCs and 32 OVPCs). Using a biotin-free detection system, the sensitivity of Gal-3 was 95.1%, while that for HBME-1 was nearly 53%. The combination of Gal-3 and HBME-1 increased the sensitivity up to 99%. However, for both markers, the specificity was 88%, lower than that reported for non-oncocytic follicular tumors. The relationship between the markers investigated and the nuclear changes suggest that the tumors containing them are pathogenetically linked to papillary carcinomas (Papotti et al. 2005). Immunochemical stains of cytokeratin (CK19), Gal-3 and HBME-1, especially when used in combination, can be an important adjunct to the histopathological diagnoses of thyroid lesions (Teng et al. 2004; Rossi et al. 2006). According to de Matos et al. (2005), from a panel of thyroid malignancy markers including HBME-1, CK19 and Gal-3, HBME-1 is the most sensitive marker though the three markers may be useful in specific cases (de Matos et al. 2005; Prasad et al. 2005).

The combined panel of antibodies against RET, HBME-1, and Gal-3 and the nuclear pleomorphism of follicular cells were effective in distinguishing between thyroid nodules requiring surgery from thyroid nodules requiring just follow-up (Rossi et al. 2005). In the group of nonoxyphilic tumors positive reaction with HBME-1 was more common in adenomas with intracapsular invasion and carcinomas, but positive reaction with anti-CD15 – only in carcinomas. Reactivity with these antibodies could mark malignancy. Barroeta et al. (2006) analyzed the diagnostic efficacy of a panel of antibodies to CK19, Gal-3, HBME-1, anti-MAP kinase (ERK), RET, and p16 using a tissue microarray consisting of both benign and malignant FDLT. HBME-1, ERK, and p16 were more specific for malignancy, whereas CK19 and Gal-3 stained benign lesions with a higher frequency and were not specific for malignant FDLT. RET-oncoprotein showed poor sensitivity and specificity.

Gal-3 and CD44v6: In FNAC of thyroid follicular tumors, the positive rates of Gal-3 and CD44v6 were 89% and 74% in follicular carcinoma, respectively, 25% and 30% in follicular adenoma, respectively. Positive staining of either Gal-3 or CD44v6 resulted in a diagnostic sensitivity of 97% and a specificity of 52% for follicular carcinoma among follicular tumors. Immunostaining of Gal-3 or CD44v6 on cytological samples can provide independent information to distinguish follicular carcinoma from adenoma (Maruta et al. 2004; Weber et al. 2004).

13.2.4 Hashimoto's Thyroiditis

Hashimoto's thyroiditis (HT) represents the most common cause of hypothyroidism and nonendemic goiter. In a study of 133 cases of HT, an unexpected expression of Gal-3 was observed in a subset of HT together with the presence of HBME-1, c-met and cyclin-D1 that are also involved in malignant transformation and deregulated cell growth (Gasbarri et al. 2004b). Furthermore, a loss of allelic heterozygosity in a specific cancer-related chromosomal region was demonstrated in some HT harbouring Gal-3-positive follicular cells. This study provided a well-substantiated demonstration that HT may include a spectrum of different thyroid conditions ranging from chronic autoimmune thyroiditis to thyroiditis triggered by specific immune-response to cancer-related antigens (Gasbarri et al. 2004b). Focal PTC like nuclear alterations have been documented in HT. The expression of four genes known to be up-regulated in PTC [*LGALS3* (galectin3), *CITED1, CK19* (cytokeratin 19) and FN1] and HBME1 were re-evaluated. Focal expression of galectin3 (GAL3), CITED1, CK19, HBME1 and FN1 was seen in 87%, 65%, 43%, 26% and 17% of HT, respectively, only in thyrocytes showing PTC-like nuclear alterations. Focal PTC-like immunophenotypic changes in HT suggested the possibility of early, focal premalignant transformation in some cases of HT (Prasad et al. 2004).

13.3 Breast Cancer

Breast tissue expresses a high level of Gal-3 whose expression is down-regulated in breast cancer. The reduced expression of Gal-3 was associated with increasing histologic grade, and thus with the acquisition of invasive and metastatic potential which possibly resulted from reduced extracellular matrix binding and increased cell motility (Idikio 1998).

Galectins in Mammary Carcinoma Cell Lines: Gal-3 is an anti-apoptotic protein that protects T cells, macrophages, and breast carcinoma cells from death triggered by a variety of agents. High levels of Gal-3 are present in a subset of B-cell neoplasms including diffuse large B-cell lymphoma (DLBCL), primary effusion lymphoma (PEL), and multiple myeloma (MM), in both cell lines and patient samples. Galectins are present on mouse mammary carcinoma cells in vitro and in vivo unlike non-malignant cells from the several tissues; and asialo-GM1 ganglioside carbohydrate part – containing probe was the most specific one. Different results concerning the role of Gal-3 in breast cancer malignancy were obtained in studies using cell lines. Galectin expression was reduced during tumor progression in more aggressive forms of spontaneous BLRB mammary carcinomas similarly shown in human breast carcinoma samples. Honjo et al. (2001) showed that the blocking of Gal-3 expression in highly malignant human breast carcinoma MDA-MB-435 cells led to the reversion of transformed cellular phenotype and to significant suppression of tumor growth in nude mice. It was suggested that the expression of Gal-3 is necessary for the maintenance of transformed and tumorigenic phenotype of MDA-MB-435 breast carcinoma cells. Furthermore, Song et al. (2002) showed that Gal-3 enhances the metastatic potential of human breast carcinoma BT 549 cells. It was established that Gal-3 in tumor cells can play the role of survival factor against. Histopathological data, however, led to suggest that galectin expression is hardly a suitable marker of aggressiveness of heterogeneous mammary. It was proposed that galectins that are selectively expressed during mouse mammary carcinoma progression, similarly to human breast carcinomas, seem to be proper targets for asialo-GM1-vectored cytotoxics and the present mouse model might be a relevant model to test novel modes of anti-breast cancer therapy (Moiseeva et al. 2005).

Uptake of Gal-3 in Breast Carcinoma Cells: Mazurek et al. (2005) found that the introduction of wild-type Gal-3 into nontumorigenic, Gal-3-null BT549 human breast epithelial cells conferred tumorigenicity and metastatic potential in nude mice, and that Gal-3 expressed by the cells was phosphorylated. In contrast, BT549 cells expressing Gal-3 incapable of being phosphorylated (Ser6—>Glu Ser6—>Ala) were nontumorigenic. Differentially regulated genes in breast cancers that are also predicted to be associated with phospho-Gal-3 in transformed BT549 cells include C-type lectin 2, insulin-like growth factor-binding protein 5, cathepsins L2, and cyclin D1. These results suggest that phosphorylation of the protein is necessary for regulation of unique sets of genes that play a role in malignant transformation (Mazurek et al. 2005). Gal-3 uptake modulates the adhesion plaques in those cells which express high levels of Gal-3 have thin-dot like plaques that may be suited for rapid adhesion and spreading while cells in which Gal-3 expression is reduced or knocked-down, have thick and elongated plaques which may be suited for a firmer adhesion to the substratum. Recombinant Gal-3 added exogenously reduced the thickness of the adhesion plaques of tumor cells with reduced Gal-3 expression. The report suggested that Gal-3 once externalized, is a powerful modulator of cellular adhesion and spreading in breast carcinoma cells (Baptiste et al. 2007).

Gal-3 in Breast Epithelial-Endothelial Interactions: Gal-3 is required for the stabilization of epithelial-endothelial interaction networks. Co-culture of epithelial cells with endothelial cells results in increase in levels of secreted Gal-3 and presence of proteolytically processed

form of Gal-3 in the conditioned media. Data suggested that Gal-3 expression is associated with specific morphological precursor subtypes of breast cancer and undergoes a transitional shift in expression from luminal to peripheral cells as tumors progressed to comedo-DCIS or invasive carcinomas. Such a localized expression of Gal-3 in cancer cells proximal to the stroma could lead to increased invasive potential by inducing novel or better interactions with the stromal counterparts (Shekhar et al. 2004). In the majority of adenomas, the neoplastic cells show moderate to strong Gal-3 immunoreactivity, but in the majority of adenocarcinomas such immunoreactivity was weak. It was revealed that the progression of canine mammary tumors is associated with low Gal-3 expression (Choi et al. 2004; Johnson et al. 2007). Patients with metastatic cancer commonly show increased serum Gal-3 concentrations, which plays a critical role in cancer metastasis. It highlights the functional importance of altered cell surface glycosylation in cancer progression (Yu et al. 2007).

13.4 Tumors of Nervous System

13.4.1 Galectins and Gliomas

A relationship is assumed to exist between the levels of expression of Gal-3 and the level of malignancy in human gliomas (Bresalier et al. 1997; Gordower et al. 1999; Camby et al. 2001). Bresalier et al. (1997) showed that normal brain tissue and benign tumors did not express Gal-3 but anaplastic astrocytomas (grade 3) and glioblastomas (grade 4 astrocytomas) respectively exhibit intermediate and high level of expression of Gal-3. Moreover, a more significant expression of Gal-3 was associated in metastases than in the primary tumors from which they derived (Bresalier et al. 1997). Though, astrocytic tumor progression is known to be associated with an increased expression of Gal-3, Gordower et al. (1999) showed that the level of Gal-3 expression significantly is decreased in majority of astrocytic tumors. However, Gordower et al. (1999) suggested that human astrocytic tumors are very heterogenous, and in spite of the general decrease in the level of Gal-3 expresssion, some tumor cell clones express a higher level of Gal-3 with increasing level of malignancy (Gordower et al. 1999). Camby et al. (2001) assayed the levels of expression of galectin-1, -3 and -8 in human astrocytic tumors of grades 1–4. The levels of galectin-1 and Gal-3 expression significantly changed during the progression of malignancy in human astrocytic tumors, while that of galectin-8 remains unchanged (Camby et al. 2001). Colonic surgical resections were studied for gal-1 and gal-3. Gal-1 and gal-3 expressed in variable amounts in epithelial cells and the connective tissue of normal colon and the expression of both is significantly increased with degree of dysplasia, suggesting that gal-1 and gal-3 and their binding sites are related to malignant progression, while gal-8 was associated with suppressor activity (Hittelet et al. 2003). It seems that the expression of Gal-3 is highly dependent on non-tumor cells such as endothelial cells or macrophages/microglial cells. This feature can partly explain the conflicting results that have been published on Gal-3 expression in human gliomas (Deininger et al. 2002; Strik et al. 2001; Le Mercier et al. 2010). The regulation of Gal-3 expression by Runx-2, a transcription factor, has been recently suggested to contribute to the malignant progression of glial tumor. Knockdown of Runx2 was shown to be accompanied by a reduction in both Gal-3 mRNA and protein levels, dependent on the glial tumor cell line tested (Vladimirova et al. 2008).

Gal-3 in Distinguishing Gliomas: Cultured Gal-3 deficient glioblastoma cells showed increased motility potential on laminin and modifications in the cytoskeleton reorganization. In addition, c-DNA microarrays and quantitative immunofluorescence analysis showed that Gal-3 deficient U373 cells had an increased expression of integrins-α6 and -β1 proteins known to be implicated in the regulation of cell adhesion (Debray et al. 2004). The distinction of astrocytomas and oligodendrogliomas, mainly pilocytic astrocytomas (PILOs) from infiltrating astrocytomas and oligodendrogliomas (ODs), and high-grade oligodendrogliomas from glioblastomas (GBMs), poses a serious clinical problem. Neder et al. (2004) identified Gal-3 as a possible tool to differentiate them based on gene expression profiles of GBMs. Higher expression of Gal-3 was observed in GBMs and PILOs than in OD, AODs and ASTs. Gal-3 appeared to be differentially expressed in central nervous system tumors, making IHC detection of Gal-3 a useful tool in distinguishing between these gliomas (Neder et al. 2004).

The Interactions Between Galectins and Integrins: Galectins are components of the ECM. The ECM comprises all secreted soluble and insoluble molecules found within the extracellular fluid of the extracellular space. The ECM is not only a static scaffolding for tissue organization but it is involved as well in many regulatory functions like modulation of migration, guidance of axonal growth, synapse formation and cell proliferation. Several reviews have already addressed an in-depth analysis of glioma ECM (Rutka et al. 1999; Wang et al. 2005a; Le Mercier et al. 2010). As emphasized by Uhm et al. (1999), integrins are cell-surface receptors that mediate the physical and functional interactions between a cell and its surrounding ECM. Although classically the role ascribed to integrins has been that of anchoring cells to ECM, the functions of integrins greatly exceed that of mere cell adhesion (Uhm et al. 1999). Within this multifaceted role, integrins have been

shown to be molecular determinants of glioma invasion (Le Mercier et al. 2010; Natarajan et al. 2003; Rutka et al. 1999).

Galectins and integrins closely interact when modulating cell adhesion and/or cell migration. For example, Moiseeva et al. have shown that galectin-1 interacts with the integrin β1 subunit in vascular smooth muscle cells (Fig. 13.1). Gal-3 was also shown to bind to α1β1 integrin and it was suggested that this interaction regulates cell adhesion of various tumor cell lines by preventing α1β1 integrin interaction with the ECM proteins (Ochieng et al. 1998) (Fig. 13.1). Gal-3 also forms a complex with α3β1 integrins and NG2 on the surface of endothelial cells. The subsequent transmembrane signaling via α3β1 has been shown to be responsible for endothelial cell motility and angiogenesis (Fukushi et al. 2004; Deininger et al. 2002). Among other galectins, Gal-8 was also reported to interact with a subgroup of integrins that include α3β1, α6β1, and to a lesser extent with the α4 and the β3 subunits in human carcinoma (1,299) cells. Gal-8 binds α1β1, α3β1 and α5β1 integrins in Jurkat T cells (Carcamo et al. 2006) (Fig. 13.1). These interactions were shown to inhibit cell adhesion and to induce apoptosis (Hadari et al. 2000).

Integrins play a significant role in the malignant progression of cancer through their involvement in cell adhesion, motility and intracellular signaling (Hynes 2002), with an emphasis on the role of β1 integrin subunit in gliomas (Bartik et al. 2008; D'Abaco and Kaye 2007; Le Mercier et al. 2010). As galectins bind integrins, with galectin-1, Gal-3 and galectin-8 all known specifically to modulate β1 integrin function, the understanding of molecular mediators such as galectins and the pathways through which they drive the cell invasion so descriptive of glioblastoma multiforme (GBM) is anticipated to reveal potential therapeutic targets (Fortin et al. 2004).

Galectins and Glioma Cell Migration: Cell migration involves at least three independent but highly coordinated biological processes: (1) cell adhesion to numerous components of the ECM; (2) cell motility, which involves the reorganization of the actin cytoskeleton mainly through modification of the components of the adhesion complex; and (3) invasion that involves the degradation of matrix proteins by tumor-secreted proteolytic enzymes, mainly serine proteases, cathepsins and metalloproteinases (MMPs) (Decaestecker et al. 2007; Lefranc et al. 2005; Rao 2003). Galectins are involved in cell migration at several steps (Stillman et al. 2005). Galectin-1, Gal-3 and to a lesser extent galectin-8, markedly stimulate the migration of glioma cells in vitro. Moreover, biological functions of Gal-3 were modulated by MMPs (Ochieng et al. 1998), which play crucial roles in glioma cell motility and invasion (Rao 2003). However, in contrary to the aforementioned, cultured Gal-3 deficient U373 glioblastoma cells had been shown to have both increased motility potential on laminin and displayed modifications in cytoskeleton reorganization. Indeed, Debray et al. have shown an increased motility of Gal-3 deficient cells cultivated on laminin (Debray et al. 2004; Le Mercier et al. 2010) whereas Camby et al. (2001) had observed an increase of motility when glioma cells were cultivated on plastic pre-coated with Gal-3. Thus, targeting both integrins and galectins represents is a feasible proposition in the future treatment of gliomas. Moreover, impairing galectin-1 expression in vivo in experimental gliomas through the delivery of anti-galectin-1 siRNA augments the therapeutic benefits contributed by temozolomide (Le Mercier et al. 2008).

Gal-3 Ligands in Meningiomas: Hancq et al. (2004) evaluated the discriminatory value of S100 proteins and Gal-3 and its ligand profile with respect to benign and atypical meningiomas. The combination of these three markers enabled an improved discriminatory criterion to be established between the benign and the atypical meningiomas. Study suggested that the Gal-3-binding sites and S100B (and S100A6 to a lesser extent) could play a role in the aggressiveness characterizing atypical meningiomas. During development and progression of pituitary tumors Riss et al. (2003) showed that only lactotroph (PRL) and corticotroph (ACTH) hormone-producing cells and tumors expressed Gal-3. Gal-3 was present in 63.2% PRL adenomas, 83.3% PRL carcinomas, 46.3% ACTH adenomas, and 87.5% ACTH carcinomas, but not in other pituitary adenomas and carcinomas. Riss et al. (2003) suggest that Gal-3 has an important role in pituitary cell proliferation and tumor progression.

13.5 Diffuse Large B-Cell Lymphoma

The WHO classification of lymphomas is based on clinical, morphological, immunohistochemical and genetic criteria. However, each entity displays its own spectrum of clinical aggressiveness. Treatment success varies widely and is not predictable. Gal-3 is an anti-apoptotic protein that protects T cells, macrophages, and breast carcinoma cells from death triggered by a variety of agents. High levels of Gal-3 are present in a subset of B-cell neoplasms including diffuse large B-cell lymphoma (DLBCL), primary effusion lymphoma (PEL), and multiple myeloma (MM), in both cell lines and patient samples. However, Gal-3 could not be detected in Burkitt lymphoma (BL), follicular lymphoma

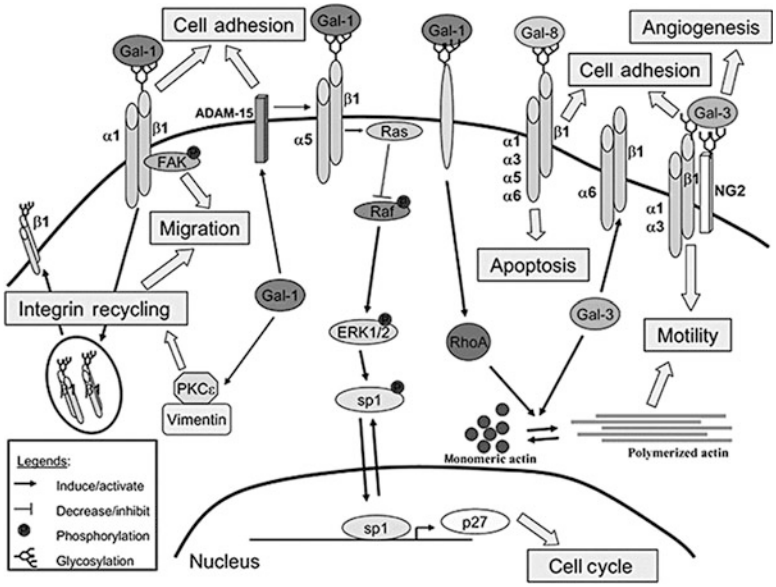

Fig. 13.1 Galectins, integrins and cell migration. The interaction of galectins with integrins modulates cell migration as well as other processes. Galectin-1 (Gal-1) interacts with the β1 integrin subunit inducing the phosphorylation of FAK, which modulates cell migration (Moiseeva et al. 2003). Binding of Gal-1 to integrin is involved in cell adhesion (Moiseeva et al. 1999) and also induces growth inhibition via its interaction with α5β1 (Fischer et al. 2005). Involvement of Ras–MEK–ERK pathway and the consecutive transactivation of Sp1, which induces p27 transcription is shown (Le Mercier et al. 2010). Gal-3 also forms a complex with α3β1 and the proteoglycan NG2 (Fukushi et al. 2004). This interaction regulates endothelial cell motility and angiogenesis. Finally, Gal-3 has been shown to regulate the expression of integrin α6β1 and actin cytoskeleton organization (Debray et al. 2004). However, it is not known with which molecule (s) Gal-3 is interacting to initiate this signaling. Galectin-8 (Gal-8) interacts with several integrins including α1β1, α3β1, α5β1 and α6β1. These interactions are involved in cell adhesion and apoptosis (Hadari et al. 2000). *ERK* extracellular signal-regulated kinase, *FAK* focal adhesion kinase, *MEK* MAP kinase/extracellular signal-regulated kinase kinase (MAPK/ERK Kinase), *PKCε* protein kinase Cε (Adapted with permission from Le Mercier et al. 2010 © John Wiley and Sons)

(FL), marginal zone lymphoma (MZL), MALT lymphoma or B-small lymphocytic lymphoma (B-SLL) cell lines or patient samples. The pattern of expression suggests that aberrantly increased Gal-3 levels in specific B-cell populations may yield a protective advantage during transformation and/or progression of certain B-cell neoplasms (Hoyer et al. 2004).

Non-Hodgkin's and Hodgkin's Lymphomas: Since galectins are involved in oncogenesis and the physiology of immune cells, D'Haene et al. (2005) investigated the expression of galectin-1 and Gal-3 in 25 normal lymphoid tissues, 42 non-Hodgkins and 42 Hodgkins lymphomas. Results showed that in normal lymphoid tissue, lymphocytes do not express galectin-1 and rarely express Gal-3. In contrast, Gal-3 was expressed in 8 of the 16 DLBCL cases and in 1 of the 8 FL cases. Furthermore, Gal-3 mRNA was expressed three times more in the DLBCLs than in the FLs. While the blood vessel walls of the lymphomas expressed galectin-1, the vessel walls of normal lymphoid tissues did not. This expression of galectin-1 in blood vessel walls was correlated with vascular density. This study shows that DLBCL can be distinguished from normal lymphoid tissue and other lymphomas on the basis of Gal-3 expression.

Karpas 299 T-Lymphoma Cells: PUVA treatment first induced G2/M cell cycle arrest resulting in a decrease in the cell proliferation rate in Karpas 299 T-lymphoma cells. Immediately following PUVA treatment, PUVA triggered mitochondrial apoptosis and enhanced the expression of peroxiredoxin, stress protein endoplasmin and Gal-3. Gal-3 was shown to protect mitochondrial membrane integrity and prevent cytochrome C release thereby blocking the effector stage of apoptosis. It was suggested that the elevated level of Gal-3 following PUVA treatment acts in synergy with the constitutively expressed chimeric kinase NPM/ALK to block the apoptosis (Bartosova et al. 2006).

13.6 Gal-3 in Melanomas

Pathogenesis of melanoma is a multi-step process that may include the phases of benign nevi and dysplastic nevi, melanoma, and metastatic melanoma. Dysregulation of cellular

proliferation and apoptosis is probably involved in melanoma progression and response to therapy. Melanocytes accumulate Gal-3 with tumor progression, particularly in the nucleus. The strong association of cytoplasmic and nuclear expression in lesions of sun-exposed areas suggests an involvement of UV light in activation of Gal-3 (Prieto et al. 2006). An increase in serum Gal-3 production has been found in patients with advanced metastatic melanoma. Gal-3 concentration was significantly correlated with both LDH and CRP in the melanoma group. It seemed that at least part of serum Gal-3 might be produced by metastatic melanoma tissue. Gal-3 might play a role in melanoma progression and/or inflammation, and warrants further study (Vereecken et al. 2005, 2006; Vereecken and Heenen 2006). Zubieta et al. (2006) suggested that the range of Gal-3-positive tumor cells in melanoma biopsies varied between 0% and 93% and that of galectin-1-positive tumor cells varied between 5% and 97%. In addition, $23 \pm 27\%$ of tumor-associated lymphocytes were apoptotic. Although these results showed a correlation between Gal-3 expression and apoptosis of tumor-associated lymphocytes, such could not be found with galectin-1. Expression profiling of Gal-3-depleted melanoma cells reveals its major role in melanoma cell plasticity and vasculogenic mimicry (Mourad-Zeidan et al. 2008).

13.7 Head and Neck Carcinoma

Choufani et al. (1999) reported a significantly lower level of Gal-3 and its ligands in head and neck carcinoma (HNSCC) patients than their normal counterparts and the extent of decrease of Gal-3 expression correlated with the increasing level of clinically detectable HNSCCs aggressiveness. This was confirmed with a low differentiation status which is known as an indicator of recurrence rate in HNSCCs (Delorge et al. 2000; Lefranc et al. 2003). With 61 cases of HNSCC as their basis, including 31 oral, 20 laryngeal, and 10 hypopharyngeal lesions, Delorge et al. (2000) showed that the main modifications observed in connection with a loss of differentiation were related to a modification in the levels of both Gal-3/Gal-3-binding site and T-antigen/T-antigen-binding site expressions. Results also suggested that Gal-3 could act as an acceptor site for the T antigen (Delorge et al. 2000).

On the other hand, expression of Gal-3 correlated highly and positively with the level of apoptosis in human cholesteatomas, which is a benign disease, characterized by unstrained growth and accumulation of keratin debris in middle ear cavity. An up-regulation of Gal-3 expression, which is associated with pronounced apoptotic activity, could have a physiologically protective effect against the substantial apoptotic features occurring in recurrent cholestestomas (Sheikholeslam-Zadeh et al. 2001). Honjo et al. (2000) reported that the nuclear expression of Gal-3 markedly decreases during progression of squamous cell carcinomas of tongue from normal to cancerous states, while cytoplasmic expression increased. It was suggested that Gal-3 translocates from nucleus to cytoplasm during neoplastic progression and may serve as a prognostic factor for tongue cancer (He et al. 2004).

The expression of Gal-3 was examined in relation to neoplastic progression of HSCCs and LSCCs. Gal-3 positivity expressed as percentage of cells was significantly higher in LSCCs and HSCCs than in Low_D or High_D, respectively. Increased expression of Gal-3 in HSCCs was accompanied by a shift from the cytoplasmic compartment to the nucleus. In intertumor-type comparison, laryngeal carcinomas presented nuclear presence of Gal-3 only rarely (1 of 58 cases in laryngeal cancer vs. 27 of 79 cases in hypopharyngeal cancer), and a comparatively low labeling index. Results reveal an association between level of presence of Gal-3 and neoplastic progression of HSCCs and LSCCs (Saussez et al. 2008a, b).

Detection of accessible Gal-3-specific ligands is an independent prognostic marker in advanced head and neck squamous cell cancer with therapeutic potential (Plzak et al. 2004). Adenoid cystic carcinoma (ACC) is one of the most common malignant tumors of the salivary glands characterized by multiple recurrences and distant metastasis resulting in significantly worsening prognosis. Seventeen (48.6%) ACC tumor specimens were found Gal-3-positive. Gal-3 reactivity was significantly associated with regional and distant metastasis. There was no statistical significance in the correlation of Gal-3 expression and disease-free survival and overall survival rate. Gal-3 may be used as an indicator in the prediction of metastatic spread in ACC (Teymoortash et al. 2006).

Esophageal Squamous Carcinoma: In patients with esophageal squamous cell carcinoma (ESCC), neither nuclear nor cytoplasmic expression of Gal-3 was a prognostic indicator in ESCC. But elevated expression of Gal-3 in the nuclei but not the cytoplasm may be an important biological parameter related to histological differentiation and vascular invasion in patients with ESCC (Shibata et al. 2005; Saussez et al. 2007a, b).

13.8 Lung Cancer

Lung cancer is the leading cause of cancer deaths in the world. The tumor stage is the most powerful prognostic tool for predicting the survival rates of lung carcinoma patients.

Prognosis of individual patients is difficult in part because of the marked clinical heterogeneity among such patients. The histological expression of Gal-3 was assessed in a panel of lung tumor specimens including small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). Among new prognostic markers, a striking difference in Gal-3 expression was observed between tumors, with high expression in NSCLC (42/47 samples) and low expression in SCLC (negative in 13/18, weak in 5/18). This differential expression of Gal-3 between histological types of lung carcinoma suggests that Gal-3 may have an important influence on tumor cell adhesion and apoptosis (Buttery et al. 2004). Prognostic value of Gal-3 expression in lung adenocarcinomas and squamous cell carcinomas has been studied by Mathieu et al. (2005). In 165 squamous cell carcinomas and 121 adenocarcinomas, immunostained for Gal-3, a large majority of cases displayed Gal-3 expression. Though, the Gal-3 immunohistochemical expression differs between squamous cell carcinomas and adenocarcinomas, the nuclear expression of Gal-3 behaved as a significant prognostic predictor for all the cases as a group (Mathieu et al. 2005).

However, binding of galectin-1 and its expression tended to increase, whereas the parameters for Gal-3 decreased in advanced pT and pN stages. The number of positive cases was considerably smaller among the cases with small cell lung cancer than in the group with non-small-cell lung cancer, among which adenocarcinomas figured prominently with the exception of galectin-1 expression. The survival rate of patients with Gal-3-binding or galectin-1-expressing tumors was significantly poorer than that of the negative cases. The expression and the capacity to bind the adhesion/growth regulatory Gal-3 were defined as an unfavorable prognostic factor not correlated with the pTN stage (Szoke et al. 2005).

13.9 Colon Neoplastic Lesions

Conflicting reports are available regarding expression of Gal-3 in human colonic mucosa and colonic tumors. Some studies suggest decreasing Gal-3 level in colon carcinoma progression (Castronovo et al. 1992; Lotz et al. 1993), whereas other reports presented opposite results (Irimura et al. 1991; Sanjuán et al. 1997; Schoeppner et al. 1995). Castronovo et al. (1992) and Lotz et al. (1993) found low levels of Gal-3 mRNA in human colon cancer tissues relative to levels in normal colonic mucosa. Castronovo et al. (1992) also stated that patients with Dukes' C and D tumors had a lower ratio of primary tumor Gal-3 mRNA to normal tissue mRNA than the patients with Dukes' B tumors. On the other hand, a higher content of Gal-3 was reported by Irimura et al. (1991) in advanced stage of Dukes' D colorectal cancer than in samples of early-stage disease. Though the normal mucosa distant from areas of neoplasia was characterized by weak or negative expression of Gal-3, Schoeppner et al. (1995) demonstrated that cytoplasmic levels of Gal-3 correlated with progression from adenoma to carcinoma. Thus, Gal-3 expression in invasive cancers varied according to the stage in Dukes' scale (Irimura et al. 1991; Schoeppner et al. 1995). Sanjuan et al. (1997) and Schoeppner et al. (1995) observed that normal colonic mucosa usually strongly expressed Gal-3 both in the nucleus (100% of cases) and in cytoplasm (77%). Nuclear and cytoplasmic expression was significantly down-regulated in adenomas (60%, 16%, respectively), whereas cytoplasmic expression of Gal-3 increased in carcinomas again (64%) although it usually did not reach the level of normal mucosa expression. A correlation between increased levels of Gal-3 expression and shorter survival periods, particularly in the case of patients with Dukes' A and B colon tumors, was observed (Nagy et al. 2003; Sanjuán et al. 1997; Schoeppner et al. 1995).

Gal-3 expression has been correlated with progression and metastasis in colon cancer. Results provide strong evidence that Gal-3 plays a role in the ability of colon cancer cells to metastasize to distant sites. Greco et al. (2004) found that (1) the expression of Gal-3 was significantly increased on the surface of cells from adenomas with respect to normal mucosa from the same patient; and (2) Gal-3 overexpression was not related with the presence of K-ras mutation. These results indicated that the evaluation of Gal-3 expression (and of its ligand, 90 kDa) can be of interest in the characterization of nonmalignant and malignant colon cancers (Greco et al. 2004; Endo et al. 2005). The incidence of lymph node and distant metastasis in galectin 3-positive cancer was significantly higher than that in Gal-3-negative cases. It was proposed that Gal-3 expression is an independent factor for prognosis in colorectal cancer (Endo et al. 2005). In colon cancer sera, the major Gal-3 ligand was a 40-kDa band distinct from mucin, carcinoembryonic antigen, and Mac-2 binding protein. The major circulating ligand for Gal-3, which is elevated in the sera of patients with colon cancer, is a cancer-associated glycoform of haptoglobin of 40-kDa which was 10 to 30-fold higher in patients than in healthy subjects (Bresalier et al. 2004). Greco et al. (2004) suggested that a 90 kDa ligand molecule of Gal-3 was increased in the blood from patients with both adenomatous and adenocarcinomatous lesions;

Gal-3 Up-Regulates MUC2 Transcription: Gal-3 and MUC2 in intestine have been correlated with the malignant behavior of colon cancer cells. Down-regulation of Gal-3 expression by antisense transfection resulted in a significant decrease in liver colonizing ability, whereas up-regulation of Gal-3 increased metastatic potential. Moreover, the alterations in Gal-3 levels resulted in parallel changes in

the level of Muc 2 mucin – a major ligand for Gal-3 (Dudas et al. 2002). Gal-3 up-regulates MUC2 protein at the level of transcription through AP-1 activation. Gal-3 responsiveness was found between 1,500 and 2,186 bp upstream of the translation start site, a region that contains one consensus AP-1 binding site in MUC2 promoter constructs. Mutation in the AP-1 site markedly decreased MUC2 promoter activity. Analyses suggested an association between Gal-3, c-Jun, and Fra-1 in forming a complex at the AP-1 site on the MUC2 promoter (Song et al. 2005).

13.10 Expression of Gal-3 in Other Tumors

Vulvar Squamous Lesions: The expression patterns of Gal-3 and the frequency of infiltrating CD1a positive DCs were determined in 82 cases of vulvar tissues, consisting of normal squamous epithelia (NE), vulvar condylomas (VC), high grade vulvar intraepithelial neoplasias (HG-VIN) of common type, and invasive keratinizing squamous cell carcinomas (SCC). Gal-3 expression was cytoplasmic, nuclear or membranous in NE, VCs, and HG-VINs, with negative or weak and occasionally moderate reactivities. In keratinizing SCC, exclusively cytoplasmic staining patterns with moderate reactivity were observed in 59% of cases. Data indicated that qualitative and quantitative changes of Gal-3 expression and infiltration by CD1a positive DCs in vulvar NE, VCs, and HG-VIN lesions, respectively, compared with SCCs play a role in the development of an infiltrative phenotype, and may provide adjunctive criteria in the diagnosis of invasion of vulvar squamous epithelia (Brustmann 2006).

KSHV Downregulation of Gal-3 in Kaposi's Sarcoma: In prostate, ovarian and breast cancer, down regulation of Gal-3 is associated with malignancy and tumor progression. Kaposi's sarcoma (KS) is an angioproliferative tumor of vascular endothelial cells and produces rare B cell lymphoproliferative diseases in the form of primary effusion lymphomas and some forms of multicentric Castleman's disease. Alcendor et al. (2009) found reduced levels of gal-3 expression in a significant fraction of latency associated nuclear antigen (LANA) positive spindle cell regions in human archival KS tissue. Gal-3 protein expression is down-regulated 10-fold in 10-day Kaposi's sarcoma-associated herpesvirus (KSHV) infected dermal microvascular endothelial cells (DMVEC) accompanied by down-regulation of mRNA with a consistent downregulation of Gal-3. Of the galectins assayed, only galectin-1 was also downregulated in KSHV infected DMVEC. Data suggest that KSHV vFLIP and LANA are the viral genes targeting Gal-3 down regulation.

Parathyroid Carcinoma: The diagnosis of parathyroid carcinoma (PC) is difficult and based on morphological features, which are not totally reliable. Hyperplastic and neoplastic parathyroid lesions may present overlapping morphologic features, and several markers have been proposed to distinguish benign from malignant growths. The expressin of Gal-3 in several malignant PC tumors, including follicular carcinomas of the thyroid indicated that Gal-3 immunostaining is a valuable tool to support a diagnosis of PC in highly proliferating tumors affecting a single parathyroid gland (Bergero et al. 2005). Hyperplastic lesions responsible for primary nonfamilial or tertiary hyperparathyroidism, as well as parathyroid adenomas, were negative for Gal-3, as opposed to carcinomas. In addition, secondary and familial primary hyperplasia cases were positive for Gal-3 in approximately two thirds of cases. All hyperplastic lesions (positive or negative for Gal-3) had a low Ki-67 index. Based on these findings, secondary hyperplasia has a low proliferative potential but an unexplained Gal-3 reactivity, which reduces its diagnostic role in differentiating benign from malignant nodules in the context of multiglandular parathyroid diseases (Saggiorato et al. 2006).

Prostate Cancer: Expression of Gal-3 is generally reduced in prostate cancer relative to the level in normal human prostate tissue (Ellerhorst et al. 1999; Pacis et al. 2000). Van den Brûle et al. (2000) demonstrated a clear change in location of Gal-3 in prostate carcinoma cells as compared with non-tumor cells. In general, normal glandular cells expressed Gal-3 in both the nucleus and cytoplasm. Studies suggest that Gal-3 might have anti-tumor activities when present in the nucleus, whereas it could favor tumor progression when expressed in the cytoplasm (Van den Brûle et al. 2000). Study on LNCaP, a human prostate cancer cell line, used to generate transfectants expressing Gal-3 either in the nucleus or in the cytosol, demonstrated that Gal-3 exerts opposite biological activities according to its cellular localization: nuclear Gal-3 plays antitumor functions and cytoplasmic Gal-3 promotes tumor progression (Califice et al. 2004a). He and Baum (2006) observed reduced T-cell migration across endothelial cells induced to increase galectin-1 expression by exposure to prostate cancer cell conditioned medium, compared to T-cell migration across control-treated endothelial cells; the inhibitory effect of galectin-1 on T-cell migration was reversed by specific antiserum. It was indicated that galectin-1-mediated clustering of CD43 contributes to the inhibitory effect on T-cell migration. Inhibition of T-cell migration is an anti-inflammatory activity of galectin-1 (He and Baum 2006).

Gal-3 Inhibits Anticancer Drug-Induced Apoptosis Through Regulation of Bad Protein: Prostate cancer exhibits resistance to anticancer drugs, at least in part due

to enhanced antiapoptotic mechanisms. The expression of exogenous Gal-3 in LNCaP cells, which do not express Gal-3 constitutively, inhibits anticancer drug-induced apoptosis by stabilizing mitochondria. The expression of Gal-3 stimulated the phosphorylation of Ser(112) of Bcl-2-associated death (Bad) protein and down-regulated Bad expression after treatment with cis-diammine-dichloroplatinum. Findings indicated that Gal-3 inhibits anticancer drug-induced apoptosis through regulation of Bad protein and suppression of mitochondrial apoptosis pathway. Therefore, targeting Gal-3 could improve the efficacy of anticancer drug chemotherapy in prostate cancer (Fukumori et al. 2006).

DES-Induced Renal Tumors in Syrian Hamster: The diethylstilbestrol (DES)-induced renal tumors in male Syrian hamster kidney (SHKT) represent a unique animal model for the study of estrogen-dependent renal malignancies. Except galectin-4, all galectins (1, -3, -7, and -8) were expressed in kidney tumors. Small clusters of galectin-1-positive, most likely preneoplastic cells at the corticomedullary junction were already evident 1 week after DES administration. Expression of Galectin-1 and -3 was apparently associated with the first steps of the neoplastic transformation, because small tumorous buds were found to be positive after 1 month of treatment. In contrast, galectins-7 and -8 were detected in large tumors and medium-sized tumors, respectively, thereby indicating an involvement in later stages of DES-induced SHKT (Saussez et al. 2005, 2006).

Gal-3 Inhibits Apoptosis in Bladder Carcinoma Cells: Gal-3 has been shown to regulate CD95, a member of TNF family of proteins in the apoptotic signaling pathway. The generality of this phenomenon has been questioned by studying a different protein [e.g., TNF-related apoptosis-inducing ligand (TRAIL), which induces apoptosis in a wide variety of cancer cells]. Overexpression of Gal-3 in J82 human bladder carcinoma cells rendered them resistant to TRAIL-induced apoptosis, whereas phosphatidylinositol 3-kinase (PI3K) inhibitors (wortmannin and LY-294002) blocked the Gal-3 protecting effect. Because Akt is a major downstream PI3K target reported to play a role in TRAIL-induced apoptosis, Oka et al. (2005) questioned the possible relationship between Gal-3 and Akt. Overall results suggested that Gal-3 involves Akt as a modulator molecule in protecting bladder carcinoma cells from TRAIL-induced apoptosis (Oka et al. 2005).

Solid and Pseudopapillary Tumor of Pancreas: Berberat et al. (2001) showed that Gal-3 was strongly overexpressed at mRNA and protein level in human pancreatic cancer compared to its expression in normal human pancreas cells. On the other hand, metastatic pancreatic cancer cells in lymph nodes and in liver showed strong Gal-3 immunoreactivity, indicating that Gal-3 might have an impact on metastasis formation. Gal-3, containing NWGR antideath motif of the Bcl-2 protein family, is involved in various aspects of cancer progression. Solid pseudopapillary tumors (SPT) of the pancreas are rare neoplasms that occur mostly in young women. Gal-3 is a major factor in the carcinogenesis of pancreatic ductal adenocarcinoma in SPT. Gal-3 is strongly expressed in all SPTs, whereas its level was lowered in metastatic nodules. In contrast, Gal-3 expression was not found in normal pancreatic endocrine cells or in neuroendocrine tumors. Thus, Gal-3 is a useful marker to distinguish SPT from neuroendocrine tumor, and also indicator of behavior because its low expression is associated with metastatic spreading (Geers et al. 2006).

Hepatocellular Carcinoma: Studies demonstrated that normal hepatocytes do not express Gal-3, but it expresses in hepatocellular carcinoma (HCC), independent of prior hepatitis B virus (HBV) infection. However, Gal-3 expression in HCC can be positively influenced by HBV infection through a mechanism that may include the transactivation of the Gal-3 gene promoter. The focal regenerating nodules of cirrhotic tissue also express Gal-3. It is possible that these cells indicate early neoplastic events (Hsu et al. 1999).

Gastric Cancer: In patients with gastric cancer, Gal-3 expression was correlated with nodal status, lymphatic inivasion, pathological stage and histological parameters. On the other hand, Gal-3 expression did not correlate with the expression of Ki-67. By PCR-SSCP-sequence analysis, two single nucleotide polymorphisms (SNPs) were detected in the Gal-3 gene, but none showed mutations. The reduced Gal-3 expression was associated with lymph node metastasis, advanced stage and tumor differentiation in gastric cancer. Gal-3 expression could be a useful prognostic factor in gastric cancer (Okada et al. 2006).

ACTH-Producing Adenomas and Prolactinomas: Gal-3 is expressed in a subset of normal pituitary cells and tumors including PRL, ACTH, and in folliculo-stellate (FS) cells and tumors. Gal-3 has an important regulatory role in pituitary cell proliferation. Gal-3 is associated with functioning ACTH and PRL tumors and is expressed infrequently in silent ACTH adenomas, suggesting that Gal-3 protein and/or gene is altered in non-functioning ACTH tumors. The use of ACTH and Gal-3 immunostaining should help in the diagnosis of silent ACTH adenomas (Jin et al. 2005). Gal-3 is expressed both in invasive prolactinomas and noninvasive prolactinomas. Since significantly higher expression seen in the invasive prolactinomas, Gal-3 expression may be used as a useful indicator to determine the invasiveness and prognosis of prolactinomas (Wang et al. 2005b).

Pheochromocytomas: Malignant and benign pheochromocytomas were analyzed for the expression of Gal-3. One malignant pheochromocytoma with distant metastases showed strong and one malignant undifferentiated pheochromocytoma with local invasion showed partly strong cytoplasmic staining. Nine of ten sporadic and all hereditary benign pheochromocytomas had absent/weak staining. One benign sporadic pheochromocytoma had moderate cytoplasmic staining. The distinct expression in various types of pheochromocytomas is intriguing and requires further investigation (Gimm et al. 2006).

Testicular Tumors: In testicular tumorigenesis, Gal-3 has a dual function according to the histological type of tumors and their hormone dependency. In malignant testicular Sertoli cell tumors, the expression of Gal-3 is down-regulated while, in benign Leydig cell tumors, this expression is maintained, indicating the possible implication of this gene in the development of more aggressive testicular sex cord stromal tumors. In contrast to sex cord stromal tumors, Gal-3 expression is up-regulated in testicular germ cell tumors. A significant elevation of the Gal-3 mRNA level occurs in non-seminomatous testicular germ cell tumors and cell line as compared to normal testes and seminomas, indicating the possible role of this gene in the non-seminomatous differentiation of germ cell tumors (Devouassoux-Shisheboran et al. 2006).

13.11 Gal-3 in Metastasis

Adhesive interactions between the molecules on cancer cells and the target organ are one of the key determinants of the organ specific metastasis. It was shown that β1,6 branched N oligosaccharides which are expressed in a metastasis-dependent manner on B16-melanoma metastatic cell lines, participate in the adhesion process. High metastatic cells showed increased translocation of lysosome associated membrane protein (LAMP1), to the cell surface. LAMP1 on high metastatic cells, carries very high levels of these oligosaccharides, which are further substituted with poly N-acetyl lactosamine (polylacNAc), resulting in the expression of high density of high affinity ligands for Gal-3 on the cell surface. Krishnan et al. (2005) showed that Gal-3 is expressed in highest amount in the lungs as compared to other representative organs and that the lung vascular endothelial cells expressed Gal-3 constitutively on their surface. Gal-3 on the organ endothelium could thus serve as one of the anchors for the circulating cancer cells, expressing high density of very high affinity ligands on their surface, and facilitate organ specific metastasis. However, in liver and lung metastatic cells galectins seem to be expressed within cytoplasm and/or nuclei. Galectin expression correlated directly with aggressive tumor potential in the A/Sn transplantable animal model similar to findings in several human breast carcinoma cell lines.

C4.4A with Gal-3 Influences Laminin Adhesion: C4.4A is a member of the Ly6 family, with low homology to uPAR. It has been detected mainly on metastasizing carcinoma cells and proposed to be involved in wound healing. C4.4A has been regarded as an orphan receptor, whose functional activity has not been fully explored. C4.4A ligands are strongly expressed in tissues adjacent to squamous epithelia. For example, in tongue and esophagus, the expression pattern partly overlaps with laminin (LN) and complements the C4.4A expression that is found predominantly on the basal layers of squamous epithelium. Evidence suggests that association of C4.4A with Gal-3 influences LN (LN1 and LN5) adhesion. C4.4A was described originally as a metastasis-associated molecule (Paret et al. 2005).

Serum Gal-3 in Cancer Cell Endothelial Adhesion: Patients with metastatic cancer commonly show increased serum Gal-3 concentrations, which plays a critical role in cancer metastasis. It highlights the functional importance of altered cell surface glycosylation in cancer progression (Yu et al. 2007). MUC1, a large transmembrane mucin protein that is overexpressed and aberrantly glycosylated in epithelial cancer, is a natural ligand for Gal-3. Recombinant Gal-3 at concentrations similar to those found in the sera of patients with metastatic cancer increased adhesion of MUC1-expressing human breast (ZR-75-1) and colon (HT29-5F7) cancer cells to human umbilical vein endothelial cells (HUVEC). It was indicated that Gal-3, by interacting with cancer-associated MUC1 via oncofetal Thomsen-Friedenreich antigen (Galβ1,3 GalNAc-α(TF)), promotes cancer cell adhesion to endothelium by revealing epithelial adhesion molecules that are otherwise concealed by MUC1 (Yu et al. 2007).

13.12 β1,6 N-acetylglucosaminyltransferase V in Carcinomas

The Golgi enzyme β1,6 N-acetylglucosaminyltransferase V (Mgat5) is up-regulated in carcinomas and promotes the substitution of N-glycan with poly N-acetyllactosamine, the preferred ligand for Gal-3. Transformation is associated with increased expression of β1,6GlcNAc-branched N-glycans, products of Mgat5. The expression of Mgat5 sensitizes mouse cells to multiple cytokines. Gal-3 cross-linked Mgat5-modifies N-glycans on epidermal growth factor and transforming growth factor-β receptors at the cell surface and delayed their removal by constitutive endocytosis. Mgat5 also promoted cytokine-mediated leukocyte

signaling, phagocytosis, and extravasation in vivo. Thus, conditional regulation of N-glycan processing drives synchronous modification of cytokine receptors, which balances their surface retention against loss via endocytosis (Partridge et al. 2004). Lagana et al. (2006) reported that fibronectin fibrillogenesis and fibronectin-dependent cell spreading are deficient in $Mgat5^{-/-}$ mammary epithelial tumor cells and inhibited in $Mgat5^{+/+}$ cells by blocking Golgi N-glycan processing with swainsonine or by competitive inhibition of galectin binding. It appeared that fibronectin polymerization and tumor cell motility are regulated by Gal-3 binding to branched N-glycan ligands that stimulate focal adhesion remodeling, FAK and PI3K activation, local F-actin instability, and α5β1 translocation to fibrillar adhesions.

Both tyrosine-phosphorylated caveolin-1 (pY42Cav1) and Mgat5 are linked with focal adhesions (FAs); their function in this context is unknown. Gal-3 binding to Mgat5-modified N-glycans functions together with pY42Cav1 to stabilize focal adhesion kinase (FAK) within FAs, and thereby promotes FA disassembly and turnover. Expression of the Mgat5/galectin lattice alone induces FAs and cell spreading. Results suggest that transmembrane crosstalk between the galectin lattice and pY42Cav1 promotes FA turnover by stabilizing FAK within FAs previously unknown, interdependent roles for Gal-3 and pY42Cav1 in tumor cell migration (Goetz et al. 2008).

13.13 Macrophage Binding Protein

The 90 K/Mac-2 binding protein (M2BP) is a member of the macrophage scavenger receptor cysteine-rich domain superfamily. Systemic levels of 90 K protein have been correlated with inflammation in many diseases, including asthma. 90 K protein is increased in asthma in blood. Its inhibitory effect on TH2 cytokine transcription suggests that increased 90 K protein expression is an attempt to limit the ongoing inflammation in asthma (Kalayci et al. 2004). Biliary M2BP levels, especially when used in conjunction with biliary CA19-9 levels, showed promise as a novel diagnostic marker for biliary tract carcinoma (Koopmann et al. 2004). The tumor-associated M2BP is highly expressed in lung cancer and the M2BP-specific immunity was observed in many patients with lung cancer. M2BP can be used as a target antigen in cancer immunotherapy. Hence peptides derived from M2BP with an HLA-A24 binding motif were analyzed for their ability to induce M2BP-specific cytotoxic T lymphocytes (CTL). Two CTLs, one induced with M2BP(241–250) (GYCASLFAIL) and the other with M2BP(568–576) (GFRTVIRPF), produced interferon-γ in response to HLA-A24-positive TISI cells pulsed with the same peptide in vitro. Although the CTLs induced with M2BP(241–250) reacted with both peptide-pulsed TISI cells and BT20 cells expressing both M2BP and HLA-A24, the CTLs induced with M2BP(568–576) did not react with BT20 cells. Findings suggested that M2BP (241–250) is naturally processed from the native M2BP molecule in cancer cells and recognized by M2BP-specific CTLs in an HLA-A24 restriction. The M2BP-derived CTL epitope with an HLA-A24 binding motif is expected to be useful as a target antigenic epitope in clinical immunotherapy for lung cancer (Kontani et al. 2004).

M2BP implicated in cancer progression and metastasis is modified by β1-6 branched N-linked oligosaccharides in colon cancer cells; glycans shown to contribute to cancer metastasis. M2BP-His bound to fibronectin, collagen IV, laminins-1, -5, and -10 and Gal-3 (Mac-2) but poorly to collagen I and galectin-1. As expected, binding of M2BP to Gal-3 was dependent on carbohydrate since it was inhibitable by lactose and asiolofetuin. Thus, a possible mechanism by which M2BP may contribute to colon cancer progression is by modulating tumor cell adhesion to extracellular proteins, including Gal-3 (Ulmer et al. 2006).

13.14 Galectinomics

Importantance of Galectins in Malignancy-Associated Processes: Gene expression pattern of human galectins have been demonstrated in tumor cell lines of various histogenetic origin (galectinomics). The presence of mRNAs for human galectins-1, -2, -3, -4, -7, -8, and -9 was monitored in a panel of 61 human tumor cell lines of different origin (breast, colon, lung, brain, skin, kidney, urogenital system, hematopoietic system). The results clearly demonstrate that human tumor cells express more mRNA species for galectins than those for galectins-1 and -3. To derive unequivocal diagnostic and prognostic information, additional monitoring of these so far insufficiently studied family members is essential (Lahm et al. 2001).

The presence and evidence of tumor-associated up-regulation were shown for galectin-1 and -3. This was less clear-cut for galectin-4 and -8. Galectin-7 was expressed in all cell lines; galectin-2 and -9 were detected at comparatively low levels. Galectin-2, -3 and -8 up-regulation was observed in superficial tumors, but not in muscle-invasive tumors. Immunoreactivity correlated with tumor grading for galectin-1, -2 and -8, and disease-dependent mortality correlated with galectin-2 and -8 expression. Binding sites were visualized using labelled galectins. Langbein et al. (2007) demonstrated a complex expression pattern of the galectin network in urothelial carcinomas. Galectin-1, -2, -3 and -8 are both potential disease markers and also possible targets for bladder cancer therapy.

Gastric Tumors: The expression of lactoside-binding lectin L-31 was higher in malignant gastric tumor than in normal tissue in 9/26 cases, similar in 42/26 cases, and lower in 3/26 cases. The higher expression of L-31 in primary cancers and metastases of certain types implicates this lectin in metastatic phenotype, but the presence of L-31 in primary cancer is not sufficient to allow the metastatic propensity of the tumor to be predicted (Lotan et al. 1994). In mucinous carcinomas of the ovary and gastrointestinal tract, cytoplasmic galectin 4 expression was relatively consistent. Meprin-α is an additional useful marker in differentiating primary from secondary mucinous adenocarcinomas of the ovary (Heinzelmann-Schwarz et al. 2007).

Reports suggest that a lack of RUNX3 function contributes to human gastric carcinogenesis. Sakakura et al. (2005) examined RUNX3 expression in clinical samples of peritoneal metastases in gastric cancers. Significant down-regulation of RUNX3 through methylation on the promoter region was observed in primary tumors (75%) as well as in clinical peritoneal metastases of gastric cancers (100%) as compared with normal gastric mucosa. Stable transfection of RUNX3 inhibited cell proliferation slightly, and modest TGF-beta-induced antiproliferative and apoptotic effects. It strongly inhibited peritoneal metastases of gastric cancers in animal model. Microarray analysis identified 28 candidate genes under the possible downstream control of RUNX3 and indicated that silencing of RUNX3 affects the expression of important genes involved in aspects of metastasis including cell adhesion (sialyltransferase 1 and galectin 4), proliferation, apoptosis, and promoting the peritoneal metastasis of gastric cancer. Identification of such genes could suggest new therapeutic modalities and therapeutic targets (Sakakura et al. 2005).

Colorectal cancer: The levels of Gal-3 (L31) in colorectal cancer specimens from primary tumors of patients with distant metastases (Dukes' stage D) were significantly higher than those from patients without detectable metastases (Dukes' stages B1 and B2). Results indicated that the relative amount of the L-31 lectin increases as the colorectal cancer progresses to a more malignant stage (Lotan et al. 1991). Wollina et al. (2002) suggest that galectins-1, -3, and -4 may be involved in early stages of human colon carcinoma development and that galectin-8 is involved in the later stages (Nagy et al. 2003; Wollina et al. 2002). In view of relevant ligands such as Bcl-2 or integrins, the presence of galectins-3 and -8 seems to be related to the loss of proliferation control and change in cell adhesion properties that are involved in clonal expansion and epidermal spread of malignant T cell clones. Successful chemotherapy of CTCL alters galectin expression selectively as shown for liposomal doxorubicin (Wollina et al. 2002). It has been noted that the majority of colon cancers develop from preexisting adenomas. Differentially expressed genes were detected between normal-adenoma and adenoma-carcinoma, and were grouped according to the patterns of expression changes. Down-regulated genes in the sequence included galectin 4. Up-regulated genes included matrix metalloproteinase 23B in carcinoma but not in adenoma, supporting the pathobiological roles in malignant transformation (Lee et al. 2006).

Tumor vasculature: Little is known about galectin expression and regulation in tumor vasculature. Galectin-1/-3/-8/-9 are overexpressed in endothelium. Galectin-2/-4/-12 were detectable at mRNA level, albeit very low. Galectin-8 and -9 displayed alternative splicing. Endothelial cell activation in vitro significantly increased the expression of galectin-1 and decreased the expression of both galectin-8 and galectin-9. Gal-3 expression was unaltered. Although a portion of these proteins is expressed intracellularly, the membrane protein level of galectin-1/-8/-9 was significantly increased on cell activation in vitro, 6-fold, 3-fold, and 1.4-fold, respectively. Data showed that endothelial cells express several members of the galectin family and that their expression and distribution changes on cell activation, resulting in a different profile in the tumor vasculature (Thijssen et al. 2008).

13.15 Mechanism of Malignant Progression by Galectin-3

Galectin-3 Phosphorylation at Ser-6 by Casein Kinase 1: The mechanisms by which Gal-3 contributes to malignant progression are not fully understood. It has been shown that the antiapoptotic activity of Gal-3 is regulated by the phosphorylation at Ser-6 by casein kinase 1 (CK1). How phosphorylation at Ser-6 regulates Gal-3 function, was explored by Takenaka et al. (2004) who generated serine-to-alanine (S6A) and serine-to-glutamic acid (S6E) Gal-3 mutants and transfected them into the BT-549 human breast carcinoma cell line, which does not express Gal-3. BT-549 cell clones expressing wild-type (wt) and mutant Gal-3 were exposed to chemotherapeutic anticancer drugs. Perhaps Ser-6 phosphorylation acts as a molecular switch for its cellular translocation from the nucleus to the cytoplasm and, as a result, regulates the antiapoptotic activity of Gal-3 (Takenaka et al. 2004).

DNA analysis of a human pituitary tumor, breast carcinoma cell lines, and thyroid carcinoma cell lines showed that in cells expressing Gal-3 protein, the *LGALS3* gene was unmethylated, whereas in Gal-3 null cells, the promoter of the *LGALS3* gene was methylated. It indicated that Gal-3 expression is regulated in part by methylation of promoter in pituitary as well as in other tumors (Ruebel et al. 2005). The combination of 5-mc with Gal-3 led to an excellent accuracy level of 96%. Among follicular neoplasia 5-mc, accuracy to

differentiate malignant tumors tends to be higher than Gal-3. These data stress the necessity of epigenetic events evaluation among thyroid nodules and propose global DNA methylation assessment as a potential diagnostic tool to combine with other valuable markers (Galusca et al. 2005). Since Gal-3 is functionally involved in cancer progression and metastasis, it may serve as a possible therapeutic target in the treatment of pituitary tumors.

Gal-3 Regulates a Molecular Switch from N-Ras to K-Ras: Depending on the cellular context, Ras can activate characteristic effectors by mechanisms still poorly understood. Promotion by galectin-1 of Ras activation of Raf-1 but not of phosphoinositide 3-kinase (PI3-K) is one such mechanism. Elad-Sfadia et al. (2004) described a mechanism controlling selectivity of K-Ras4B (K-Ras), an important Ras oncoprotein. It was demonstrated that Gal-3 acts as a selective binding partner of activated K-Ras. Unlike galectin-1, which prolongs Ras activation of ERK and inhibits PI3-K, K-Ras-GTP/Gal-3 interactions promote, in addition to PI3-K and Raf-1 activation, a third inhibitory signal that attenuates active ERK (Elad-Sfadia et al. 2004). Though, the Gal-3 is a selective binding partner of activated K-Ras-GTP and since both proteins are antiapoptotic and associated with cancer progression, Shalom-Feuerstein et al. (2005) questioned the possible functional role of Gal-3 in K-Ras activation. Shalom-Feuerstein et al. (2005) found that overexpression of Gal-3 in human breast cancer cells (BT-549/Gal-3) coincided with a significant increase in wild-type (wt) K-Ras-GTP coupled with loss in wt N-Ras-GTP, whereas the nononcogenic Gal-3 mutant proteins [Gal-3(S6E) and Gal-3(G182A)] failed to induce the Ras isoform switch. Only wt Gal-3 protein co-precipitated and colocalized with oncogenic K-Ras, resulting in its activation with radical alterations in Ras signaling pathway, whereby the activation of AKT and Ral was suppressed and shifted to the activation of extracellular signal-regulated kinase (ERK). These workers suggested that Gal-3 confers on BT-549 human breast carcinoma cells several oncogenic functions by binding to and activation of wt K-Ras, suggesting that some of the molecular functions of Gal-3 are, at least in part, a result of K-Ras activation (Shalom-Feuerstein et al. 2005). Eude-Le Parco et al. (2009) made genetic assessment of importance of Gal-3 in cancer initiation, progression, and dissemination in mice.

13.16 Anti-Galectin Compounds as Anti-Cancer Drugs

To interfere with galectin-carbohydrate interactions during tumor progression, a current challenge is the design of specific galectin inhibitors for therapeutic purposes. Certain galectins directly involved in cancer progression seem to be promising targets for the development of novel therapeutic strategies to combat cancer. Indeed, migrating cancer cells resistant to apoptosis still constitute the principal target for the cytotoxic drugs used to treat cancer patients. Reducing the levels of migration in apoptosis-resistant cancer cells can restore certain levels of sensitivity to apoptosis in restricted-migration cancer cells. Anti-galectin agents can restrict the levels of migration of several types of cancer cell and should therefore be used in association with cytotoxic drugs to combat metastatic cancer. Experimental proof in support of this concept with particular attention to glioblastomas has been provided (Ingrassia et al. 2006). Glioblastomas form the most common type of malignant brain tumor in children and adults, and no glioblastoma patient has been cured to date (Ingrassia et al. 2006).

Galactosides and Lactosides as Inhibitors of Gal-1 and -3: Among aryl 1-thio-β-d-galacto- and lacto-pyranosides carrying a panel of substituents on the phenyl groups, best galectin-1 inhibitors were p-nitrophenyl thiogalactoside 5a for the monosaccharide and o-nitrophenyl thiolactoside 6f or napthylsulfonyl lactoside 8c, both being 20 times better relative to natural ligands. Relative inhibitory properties of these were as low as 2,500 and 40 μM, respectively (Giguere et al. 2006a).

Evaluation of the N acetyllactosamine thioureas as inhibitors against galectins-1, 3, 7, 8N (N-terminal domain), and 9N (N-terminal domain) revealed thiourea-mediated affinity enhancements for galectins-1, 3, 7, and 9N and in particular, good inhibitors against galectin-7 and 9N (K_D 23 and 47 μM, respectively, for a 3-pyridylmethylthiourea derivative), representing more than an order of magnitude affinity enhancement over the parent natural N-acetyllactosamine (Salameh et al. 2006).

Copper(I)-catalyzed addition of alkynes to methyl 3-azido-3-deoxy-1-thio-β-D-galactopyranoside afforded stable and structurally simple 3-deoxy-3-(1H-1,2,3-triazol-1-yl)-1-thio-galactosides carrying a panel of substituents at the triazole C4 in high yields. The 3-(1H-[1,2,3]-triazol-1-yl)-1-thio-galactoside collection synthesized contained inhibitors of the tumor- and inflammation-related Gal-3 with K_D values as low as 107 μM, which is as potent as the natural disaccharide inhibitors lactose and N-acetyllactosamine (Salameh et al. 2005). Among anomeric oxime ether derivatives of β-galactose, the best inhibitor, [E]-O-(β-D galactopyranosyl)-indole-3-carbaldoxime (E-52), had a K_D value of 180 μM, which is 24 times better than methyl β-D-galactopyranoside (K_D = 4,400 μM) and in the same range as methyl lactoside (K_D = 220 μM) (Tejler et al. 2005). Galactosides and lactosides bearing triazoles or isoxazoles, provided specific galectin-1 and -3 inhibitors with potencies as low as 20 μM (Giguere et al. 2006b).

Cumpstey et al. (2005) synthesized compounds that can bind galectins-1, -3, -7, -8N and -9N. An aromatic

nucleophilic substitution reaction between 1,5-difluoro-2,4-dinitrobenzene and a galacto thiol gave 5-fluoro-2,4-dinitrophenyl 2,3,4,6-tetra-O-acetyl-1-thio-β-D-galactopyranoside. The modified forms of these compounds were efficient inhibitors against galectin-7. The best inhibitors against galectin-7 were poor against the other galectins and thus have potential as simple and selective tools for dissecting biological functions of galectin-7.

Synthetic Lactulose Amines: Anticancer Agents: Rabinovich et al. (2006) reported the synthesis of three low molecular weight synthetic lactulose amines (SLA): (1) N-lactulose octamethylenediamine (LDO), (2) N, N′-dilactulose-octamethylenediamine (D-LDO), and (3) N, N′-dilactulose-dodecamethylenediamine (D-LDD). These galectin inhibitors with subtle differences in their carbohydrate structures may be potentially used to specifically block different steps of tumor growth and metastasis (Rabinovich et al. 2006).

In an attempt to block the Gal-3 carbohydrate recognition domain with synthetic peptides and reduce metastasis-associated carcinoma cell adhesion, Zou et al. (2005) demonstrated that carbohydrate-mediated, metastasis-associated tumor cell adhesion could be inhibited efficiently with short synthetic peptides which do not mimic naturally occurring glycoepitopes yet bind to the Gal-3 CRD with high affinity and specificity (Zou et al. 2005). Protein-ligand interactions can be significantly enhanced by the fine-tuning of arginine-arene interactions (Sorme et al. 2005).

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Part V

R-Type Animal Lectins

R-Type Lectin Families

14

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14.1 Ricinus Communis Lectins

In 1888, Peter Hermann Stillmark reported that seed extracts of the poisonous plant Ricinus communis (castor bean) contain a toxin that can agglutinate erythrocytes. This agglutinin was named as "ricin." Other agglutinins were soon discovered in the seed extracts of other species of poisonous plants. In twentieth century, these proteins were recognized as important members of the glycan-binding proteins known as "lectins." The CRD of this lectin is closely related in sequence and three-dimensional structure to the CRDs of a number of other plant lectins. Some of these structural similarities were also noted in a variety of animal and bacterial glycan-binding proteins discussed in this chapter, causing all proteins containing this ricin-type CRD to be classified as R-type lectins Section 14.2.

The R-type domain is an ancient type of protein fold that is found in many glycosyltransferases as well as in bacterial and fungal hydrolases. Interestingly, the R-type CRD is the only one conserved between animal and bacterial lectins (Sharon and Lis 2004). Ricin was the first lectin discovered and it is the prototypical lectin in this category. Two different lectins have been purified from *R. communis* seeds, and in the original nomenclature they were termed RCA-I and RCA-II. RCA-I is an agglutinin but a very weak toxin. RCA-II is commonly called ricin, and it is both an agglutinin and a very potent toxin. The designation RCA-II has now been dropped, but the original name for the agglutinin RCA-I has been retained. The molecular mass of RCA-I is approximately 120 kDa and that of ricin is approximately 60-kDa. Ricin is a type II ribosome-inactivating protein (RIP-II). Although one might predict that RCA-I also would be highly toxic, it has weak activity compared to ricin because it lacks a separate A chain (Fig. 14.1).

14.1.1 Properties of Ricin

14.1.1.1 Ricin Is a Ribosome Inactivating Protein

Ricin is classified as a Type 2 ribosome inactivating protein (RIP). Whereas Type 1 RIPs consist of a single enzymatic protein chain, Type 2 RIPs, also known as holotoxins, are heterodimeric glycoproteins. Type 2 RIPs consist of an A chain that is functionally equivalent to a Type 1 RIP, covalently connected by a single disulfide bond to a B chain that is catalytically inactive, but serves to mediate entry of the A-B protein complex into the cytosol. Both Type 1 and Type 2 RIPs are functionally active against ribosomes in vitro, however only Type 2 RIPs display cytoxicity due to the lectin properties of the B chain. In order to display its ribosome inactivating function, the ricin disulfide bond must be reductively cleaved.

14.1.1.2 Ricin (RCA-II) Is Toxic and Differs from RCA-I

Ricinus communis agglutinin-I (RCA-I) is a tetramer with two ricin heterodimer-like proteins that are noncovalently associated. Each heterodimer in RCA-I contains an A-chain disulfide linked to the galactose-binding B chain. The sequences of the A chain of ricin and the A chain of RCA-I differ in 18 of 267 residues and are 93% identical, whereas the B chains differ in 41 of 262 residues and are 84% identical. All the subunits are N-glycosylated and usually express oligomannose-type N-glycans. The genome of R. communis also encodes several other proteins that have high homology with ricin, and some of these lectins have been designated ricin-A, -B, -C, -D, and -E.

Ricin is synthesized as a single prepropolypeptide with a 35-amino-acid amino-terminal signal sequence, followed by the A-chain domain, a 12-amino-acid linker region, and the B chain. Proteolysis results in cleavage between the A and B chains. Mature ricin contains four intrachain disulfide bonds; a single interchain disulfide bond links A chain to B chain.

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The A chain contains the catalytic activity responsible for the toxicity and the B chain has the glycan-binding activity. The mature A chain has 267 amino acids and the B chain has 262 amino acids. Each B subunit is a product of gene duplications and has two CRDs, each of which is composed of an ancient 40-amino-acid galactose-binding polypeptide region.

The B chain of ricin has two binding sites for sugars, which are about 35 Å apart. Ricin binds to β-linked galactose and N-acetylgalactosamine, whereas RCA-I prefers β-linked galactose. The affinities of these lectins for monosaccharides are quite low (K_D in the range 10^{-3}–10^{-4} M). In contrast, the binding to cells is of much higher affinity (K_D in the range 10^{-7}–10^{-8} M), owing to both increased avidity from the multivalency and enhanced binding to glycans terminating in the sequence Galβ1–4GlcNAc-R. Such sequences represent higher-affinity determinants for lectin binding. In general, ricin and RCA-I both preferentially bind to glycans containing nonreducing terminal Galβ1–4GlcNAc-R or GalNAcβ1–4GlcNAc-R, although they bind weakly to Galβ1–3GlcNAc-R. Neither ricin nor RCA-I binds well to glycoconjugates containing nonreducing terminal α-linked Gal residues. RCA-I is commonly used for glycan isolation and characterization because it is safer than ricin and has higher avidity because of its tetrameric nature. Ricin is often used as a toxin in cell selection for glycosylation mutants, and the A chain of ricin is often used in chimeric proteins as a toxin for specific-cell killing (Stirpe et al. 1992; Lord et al. 1994; Sharon and Lis 2004).

- The tertiary structure of ricin is a globular, glycosylated heterodimer of approximately 60–65 kDa. Ricin toxin A chain and ricin toxin B chain are of similar molecular weight, approximately 32 and 34 kDA respectively.
- **Ricin A Chain** (RTA) is an N-glycoside hydrolase composed of 267 amino acids. It has three structural domains with approximately 50% of the polypeptide arranged into α-helices and β-sheets. The three domains form a pronounced cleft that is the active site of RTA.
- **Ricin B Chain** (RTB) is a lectin composed of 262 amino acids that is able to bind terminal galactose residues on cell surfaces. RTB form a bilobal, barbell-like structure lacking α-helices or β-sheets where individual lobes contain three subdomains. At least one of these three subdomains in each homologous lobe possesses a sugar-binding pocket that gives RTB its functional character.

The gene for ricin A chain has been cloned (Halling et al. 1985). Because ricin is a multimeric glycoprotein, recombinant forms produced in Escherichia coli (such as ricin B chain) are usually poorly active. A chain expressed from recombinant sources will be referred to here as rRTA. The rRTA derived from an *E. coli* expression system was crystallized and refined by X-ray structure (Kim et al. 1992; Mlsna et al. 1993). The structure of the heterodimeric ricin has been solved and refined to 2.5 A resolution (Rutenber et al. 1991). The structures of both the -A (Katzin et al. 1991) and the lectin B chain (Rutenber and Robertus 1991) have been described. The RTA is linked by a disulfide bond to the B chain (RTB). The RTB binds to target cell surfaces via its lectin action, and the disulfide bond keeps RTA, the toxic moiety, tethered until it is taken up by endocytosis. The bond is reduced in the cell permitting enzyme action; it is known that the heterodimer is inactive against ribosomes.

14.1.1.3 Carbohydrate-Binding Module

The R-type domain contained in these proteins is the CRD, which is also termed a carbohydrate-binding module (CBM) and has been placed in the CBM13 family in the CAZy database (carbohydrate-active enzymes database). Figure 14.1 shows that the A chain has eight α-helices and eight β-strands and it is the catalytic subunit, as discussed above. The B chain, which contains R-type lectin domains, has two tandem CRDs that are about 35 Å apart and have a shape resembling a barbell, with one binding domain at each end. Each R-type domain has a three-lobed organization that is a β-trefoil structure (from the Latin trifolium meaning "three-leaved plant") (Fig. 14.2). The β-trefoil structure probably arose evolutionarily through gene fusion events linking a 42-amino-acid peptide subdomain that has galactose-binding activity. The three lobes are termed α, β, and γ and are arranged around a threefold axis. Conceivably, each lobe could be an independent binding site, but in most R-type lectins only one or two of these lobes retain the conserved amino acids required for sugar binding. Sugar binding is relatively shallow in these loops and arises from aromatic amino acid stacking against the Gal/GalNAc residues and from hydrogen bonding between amino acids and hydroxyl groups of the sugar ligands. A characteristic feature of these loops in the R-type domain in ricin is the presence of (QxW)3 repeats (where x is any amino acid), which are found in many, but not all, R-type family members (Hazes 1996).

14.1.2 Other R-Type Plant Lectins

In addition to RCA-I and ricin, other plant lectins with R-type domains include the ricin homolog from *Abrus precatorius* and the bark lectins from the elderberry plant, *Sambucus sieboldiana* lectin (SSA) and *Sambucus nigra* agglutinin (SNA). The SSA and SNA are unusual in that they are the only R-type lectins that bind well to α2–6-linked sialic acid–containing ligands and they do not bind to α2–3-linked sialylated ligands. SSA and SNA are heterotetramers (~140 kD) composed of two heterodimers each containing an A chain (which resembles ricin A chain) disulfide-bonded

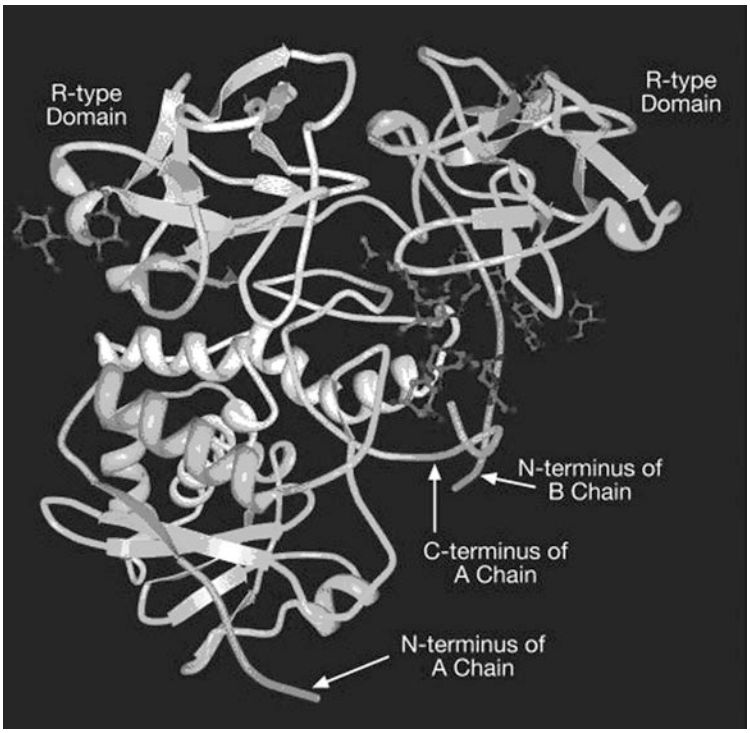

Fig. 14.1 The crystal structure of ricin refined to 2.5 Å. (Adapted by permission from Rutenber et al. 1991© John Wiley and Sons. PDB ID: 2AAI.)

to a B chain (which binds glycans and is an R-type lectin). The A chain in these proteins has very weak RIP-II activity in vitro. SSA and SNA may have the same overall organization as RCA-I (see Fig. 14.1). The toxins abrin, modeccin from Adenia digitata, Viscum album agglutinin (VAA or mistletoe lectin), and volkensin also have R-type domains, belong to the RIP-II class, and kill cells in a manner similar to ricin. There are other R-type plant lectins in the RIP-II class that are not toxic, and these include several proteins from the genus Sambucus (elderberry), such as nigrin-b, sieboldin-b, ebulin-f, and ebulin-r. All of the B subunits of these proteins appear to bind Gal/GalNAc, but they may have some differences in affinity and may recognize different Gal/GalNAc-containing glycoconjugates.

14.2 R-Type Lectins in Animals

The R-type lectin domain is found in several animal lectins, including the mannose receptor (MR) family, discussed in subsequent chapter, and in some invertebrate lectins. EW29 is a galactose-binding lectin from the annelid (earthworm) Lumbricus terrestris. The R-type domain is also found in pierisin-1, which is a cytotoxic protein from the cabbage butterfly Pieris rapae, and in the homologous protein pierisin-2, from Pieris brassicae. Tandem R-type motifs are found in some other R-type family members, including ricin, but the presence of four such motifs is unique, thus far, to pierisin-1. Some proteins with the R-type lectin domain are also enzymes and these are found in both animals and microbes. For example, Limulus horseshoe crab coagulation factor G has a central R-type lectin domain, which is flanked at the amino terminus by a xylanase Z-like domain and at the carboxyl terminus by a glucanase-like domain. This protein also has a subunit that is a serine protease.

14.3 Mannose Receptor Family

There are four known members of MR family in humans, all of which contain an R-type lectin domain; other family members are not predicted. The MR family includes the MR, the phospholipase A2 (PLA2) receptor, DEC-205/MR6-gp200, and Endo180/urokinase plasminogen activator receptor-associated protein (Fig. 14.3). All of these proteins are large type I transmembrane glycoproteins and they contain a single fibronectin type II domain similar to R-type CRDs of ricin, 8–10 C-type lectin domains (CTLDs), and an amino-terminal cysteine-rich domain (East et al. 2002;

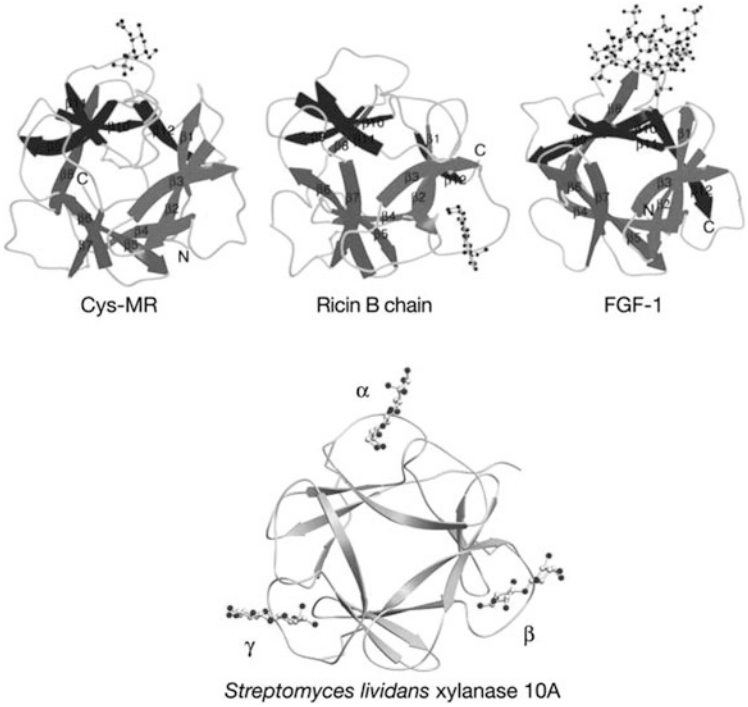

Fig. 14.2 Structures of the β-trefoil R-type domains in different proteins. (*Top*) Cysteine-rich R-type domain of the mannose receptor (MR) in complex with 4-O-sulfated GalNAc; ricin B chain in complex with galactose; and acidic fibroblast growth factor (FGF-1) in complex with sulfated heparan decasaccharide. N and C-termini are labeled, and lobes I, II, and III are indicated in different shades. Each structure is depicted with a bound ligand (4-SO_4-GalNAc for Cys-MR, galactose for ricin B chain, and sulfated heparin decasaccharide for aFGF) (Adapted with permission from Liu et al. 2000 © The Rockefeller University Press). (*Bottom*) *Streptomyces lividans* endo-β1-4xylanase in complex with lactose (Adapted by permission from Notenboom et al. 2002 © American Chemical Society)

Llorca 2008). However, despite the common presence of multiple lectin-like domains, these four endocytic receptors have divergent ligand binding activities, and it is clear that the majority of these domains do not bind sugars. Endo180 binds in a Ca^{2+}-dependent manner to mannose, fucose, and N-acetylglucosamine but not to galactose. This activity is mediated by one of the eight CTLDs, CTLD2. Monosaccharide binding specificity of Endo180 CTLD2 is similar to that of MR CTLD4. However, additional experiments indicate that, unlike the cysteine-rich domain of the MR, the cysteine-rich domain of Endo180 does not bind sulfated sugars. Thus, although Endo180 and the MR are now both known to be mannose binding lectins, each receptor is likely to have a distinct set of glycoprotein ligands in vivo (East et al. 2002; Yan et al., 1997). The mannose receptor acts as a molecular scavenger, clearing harmful glycoconjugates or micro-organisms through recognition of their defining carbohydrate structures. The MR can also bind collagen and that the fibronectin type II domain mediates this activity. Neither of the two types of sugar-binding domain in MR is involved in collagen binding. The fibronectin type II domain shows the same specificity for collagen as the whole receptor, binding to type I, type III and type IV collagens. These are additional roles for this multifunctional receptor which mediates collagen clearance or cell-matrix adhesion (Napper et al. 2006). Details of this family have been discussed in next Chap. 15.

14.4 UDP-Galnac: Polypeptide α-N-Acetylgalactosaminyltransferases

14.4.1 Characteristics of UDP-GalNAc: α-N-Acetylgalactosaminyltransferases

A large homologous family of uridine diphosphate (UDP)-*N*-acetyl-α-D galactosamine(GalNAc): poly-peptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts, EC 2.4.1.41) initiate mucin-type *O*-glycosylation by transferring GalNAc to the hydroxyl group of serine and threonine residues (GalNAcα1-*O*-Ser/Thr) (Fig. 14.3). In *Caenorhabditis elegans,* a total of 11

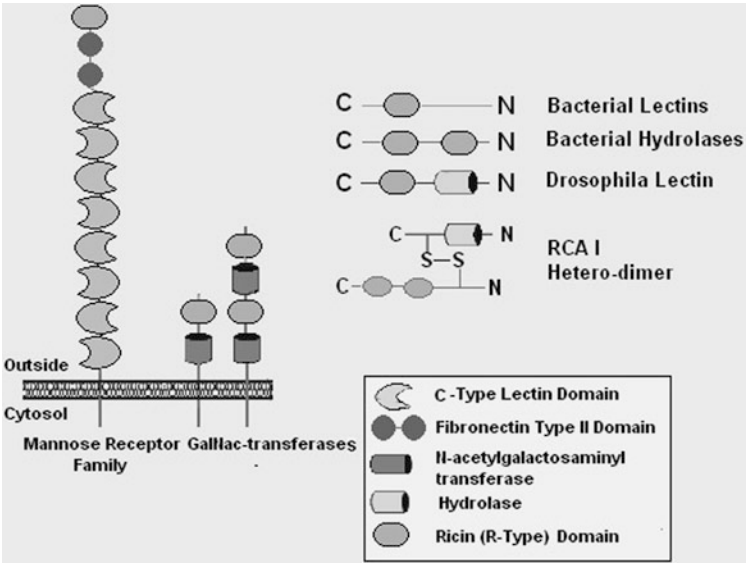

Fig. 14.3 The R-type lectin superfamily. Different groups within the family of animal, bacterial and ricin (plant) are indicated with the domain structures shown In mannose receptor family, DEC-205 contains ten lectin domains where as others contain eight CTLDs

distinct sequence homologs of ppGalNAc-T gene family were cloned, sequenced, and expressed. All clones encoded type II membrane proteins that shared 60–80% amino acid sequence similarity with the catalytic domain of mammalian ppGalNAc-Ts enzymes. Two sets of cDNA clones contained variants that appeared to be produced by alternative message processing. In addition to the existence of ppGalNAc-T enzymes in a nematode organism, the substantial diversity of these isoforms in *C. elegans* suggests that mucin O-glycosylation is catalyzed by a complex gene family, which is conserved among evolutionary-distinct organisms (Hagen and Nehrke 1998). Human and rodent ppGalNAc-T families are said to include 20 distinct isoforms, of which 16 have been characterized (Ten Hagen et al. 2003). Despite the seeming simplicity of ppGalNAc-T catalytic function, it is estimated that there are 24 unique ppGalNAc-T human genes. These ppGalNAcTs may be separated into two general classes: those that can transfer N-acetyl-galactosamine from UDP-GalNAc to unmodified polypeptide acceptors, and those that prefer acceptor glycopeptides containing GalNAc-Ser/Thr (i.e., a set predicted to have a CRD). Cloning of the genes encoding these enzymes revealed that they are large proteins and have a unique multidomain structure. All the enzymes are type II transmembrane proteins and have a carboxy-terminal R-type domain of about 130 amino acid residues and an amino-terminal catalytic domain (Ten Hagen et al. 2001, 2003). The R-type domain has the QxW repeat, and recent evidence shows that these domains in the ppGalNAcTs function as lectins during the catalytic process.

The ppGalNAc-T isoforms display tissue-specific expression in adult mammals as well as unique spatial and temporal patterns of expression during murine development. In vitro assays suggest that a subset of the ppGalNAc-Ts have overlapping substrate specificities, but at least two ppGalNAc-Ts (ppGalNAc-T-T7 and -T9 [now designated -T10]) appear to require the prior addition of GalNAc to a synthetic peptide before they can catalyze sugar transfer to this substrate. Site-specific O-glycosylation by several ppGalNAc-Ts is influenced by the position and structure of previously added O-glycans. Collectively, these observations argue in favor of a hierarchical addition of core GalNAc residues to the apomucin. Various forms of O-glycan pathobiology may be reexamined in light of the existence of an extensive ppGalNAc-T family of enzymes. Studies have demonstrated that at least one ppGalNAc-T isoform is required for normal development in *Drosophila melanogaster* (Ten Hagen et al. 2003). The ppGalNAc-T family is conserved in evolution, and distinct subfamilies of orthologous isoforms with conserved kinetic properties have been identified in vertebrates and invertebrates (Schwientek et al. 2002; Stwora-Wojczyk et al. 2004). Differences in kinetic properties, substrate specificities, and expression patterns of these isoenzymes provide for differential regulation of O-glycan attachment sites and density (Hassan et al. 2000; Ten Hagen et al. 2003). Although ablation of several ppGalNAc-T isoforms in mice have not demonstrated a phenotype (Ten Hagen et al. 2003), the finding that impairment of a single isoform in *Drosophila melanogaster* disrupts devel-

opment (Ten Hagen and Tran 2002; Schwientek et al. 2002), and that the human ppGalNAc-T isoform, GalNAc-T3, is implicated in the disease familial tumoral calcinosis (Topaz et al. 2004) demonstrate the nonredundant function of some ppGalNAc-T isoforms.

The GalNAc-glycopeptide substrate specificity exhibited by some isoforms has been associated with a ricin-like lectin domain found in the C-terminal region of most ppGalNAc-T isoforms (Hassan et al. 2000). This distinct lectin-like domain was originally identified by Hazes (1996) and Imberty et al. (1997). Studies have demonstrated that the catalytic and lectin domains of ppGalNAc-Ts fold into separate domains (Fritz et al. 2004, 2006; Kubota et al. 2006). In agreement with predictions from studies of the role of the lectin domain in directing GalNAc-glycopeptide substrate specificities, Fritz and associates were able to model a glycopeptide into the catalytic pocket and show potential interactions of the GalNAc residues with the carbohydrate-binding sites of the lectin domain.

The ppGalNAc-Ts can be subdivided into three putative domains, each containing a characteristic sequence motif. The 112-amino acid glycosyltransferase 1 (GT1) motif represents the first half of the catalytic unit and contains a short aspartate-any residue-histidine (DXH) or aspartate-any residue-aspartate (DXD)-like sequence. Secondary structure predictions suggest that the GT1 motif forms a 5-stranded parallel β-sheet flanked by 4 α-helices, which resembles the first domain of the lactose repressor. Four invariant carboxylates and a histidine residue are predicted to lie at the C-terminal end of three β-strands and line the active site cleft. Site-directed mutagenesis of murine ppGalNAc-T1 reveals that conservative mutations at these five positions result in products with no detectable enzyme activity. The second half of the catalytic unit contains a motif (positions 310–322) which is also found in β1,4-galactosyltransferases (termed the Gal/GalNAc-T motif). Mutants of carboxylates within this motif express either no detectable activity. Mutagenesis of highly conserved (but not invariant) carboxylates produces only modest alterations in enzyme activity. Mutations in the C-terminal 128-amino acid ricin-like lectin motif do not alter the enzyme's catalytic properties (Amadoetal 1999; Hagen et al. 1999; Ten Hagen et al. 2001).

Hagen et al. (1999) initially investigated the function of the lectin domain of GalNAc-T1, and found that selective mutational disruption of the lectin domain of GalNAc-T1 did not severely affect the catalytic function of the enzyme with peptide substrates. But Bennett et al. (1998) found that the GalNAc-T4 isoform exhibited a unique GalNAc-glycopeptide substrate specificity. Mutational analysis of GalNAc-T4 demonstrated that a mutation in the α-repeat of the C-terminal lectin domain of GalNAc-T4 selectively inactivated the GalNAc-glycopeptide catalytic function of the enzyme, whereas activity with unglycosylated peptides was unaffected, in accordance with the original study of GalNAc-T1 (Hassan et al. 2000). Now, it has emerged that some ppGalNAc-T isoforms in vitro selectively function with partially GalNAc O-glycosylated acceptor peptides rather than with the corresponding unglycosylated peptides. O-Glycan attachment to selected sites, most notably two sites in the MUC1 tandem repeat, is entirely dependent on the glycosylation-dependent function of GalNAc-T4. Furthermore, results suggest that the GalNAc-T4 lectin domain modulates the function of the enzyme through interaction with the GalNAc residues of the glycopeptide substrate. Similar studies with GalNAc-T1 and -T2 suggested that the lectin domains of these may be important for the glycosylation of partially GalNAc-glycosylated peptide substrates (Tenno et al. 2002; Fritz et al. 2006). Direct carbohydrate binding of two ppGalNAc-T lectin domains, GalNAc-T4 and GalNAc-T2, representing isoforms with distinct glycopeptide activity (GalNAc-T4) and isoforms without apparent distinct GalNAc-glycopeptide specificity (GalNAc-T2) suggests that ppGalNAc-T lectins serve to modulate the kinetic properties of the enzymes in the late stages of the initiation process of O-glycosylation to accomplish dense or complete O-glycan occupancy (Wandall et al. 2007).

Using a set of synthetic peptides and glycopeptides it was demonstrated that the lectin domain of ppGalNAcT-2 (hT2) directs glycosylation site selection for glycopeptide substrates. It was found that glycosylation of peptide substrates by glycopeptide transferase ppGalNAcT-10 (hT10) requires binding of existing GalNAcs on the substrate to either its catalytic or lectin domain, thereby resulting in its apparent strict glycopeptide specificity. These results highlight the existence of two modes of site selection used by these ppGalNAcTs: local sequence recognition by the catalytic domain and the concerted recognition of distal sites of prior glycosylation together with local sequence binding mediated, respectively, by the lectin and catalytic domains (Raman et al. 2008).

14.4.2 The Crystal Structure of Murine ppGalNAc-T-T1

The family of ppGalNAcTs is unique among glycosyltransferases, containing a catalytic and a C-terminal lectin domain that were shown to be closely associated. The x-ray crystal structure of a ppGalNAc-T, murine ppGalNAc-T-T1, showed that the enzyme folds to form distinct catalytic and lectin domains. The association of two domains forms a large cleft in the surface of the enzyme that contains a Mn^{2+} ion complexed by invariant D209 and H211 of the "DXH" motif

and by invariant H344. Each of the three potential lectin domain carbohydrate-binding sites (α, β, and γ) is located on the active-site face of the enzyme, suggesting a mechanism by which the transferase may accommodate multiple conformations of glycosylated acceptor substrates. A model of a mucin 1 glycopeptide substrate bound to the enzyme shows that the spatial separation between the lectin α site and a modeled active site UDP-GalNAc is consistent with the in vitro pattern of glycosylation observed for this peptide catalyzed by ppGalNAc-T-T1. The structure also provides a template for the larger ppGalNAc-T family, and homology models of several ppGalNAc-T isoforms predict dramatically different surface chemistries consistent with isoform-selective acceptor substrate recognition (Fritz et al. 2004).

Fritz et al. (2006) described the x-ray crystal structures of human ppGalNAcT-2 (hT2) bound to the product UDP and to UDP and an acceptor peptide substrate EA2 (PTTDSTTPAPTTK). The conformations of both UDP and residues Arg362-Ser372 vary greatly between the two structures. In the hT2-UDP-EA2 complex, residues Arg362-Ser373 comprise a loop that forms a lid over UDP, sealing it in the active site, whereas in the hT2-UDP complex this loop is folded back, exposing UDP to bulk solvent. EA2 binds in a shallow groove with threonine 7 positioned consistent with in vitro data showing it to be the preferred site of glycosylation. The relative orientations of the hT2 catalytic and lectin domains differ dramatically from that of murine ppGalNAcT-1 and also vary considerably between the two hT2 complexes. Indeed, in the hT2-UDP-EA2 complex essentially no contact is made between the catalytic and lectin domains except for the peptide bridge between them. Thus, the hT2 structures reveal an unexpected flexibility between the catalytic and lectin domains and suggest a new mechanism used by hT2 to capture glycosylated substrates. Kinetic analysis of hT2 lacking the lectin domain confirmed the importance of this domain in acting on glycopeptide but not peptide substrates. The structure of the hT2-UDP-EA2 complex also resolves long standing questions regarding ppGalNAcT acceptor substrate specificity (Fritz et al. 2006).

The murine enzyme MT1 does not require the R-type domain for catalysis with either glycopeptide (GalNAc-peptide) or peptide acceptors. In contrast, in human hT2, the lectin domain is important in catalysis with GalNAc-peptide acceptors, but not peptide acceptors, and the lectin domains of both hT2 and hT4 can directly bind N-acetylgalactosamine. In mT1, the R-type domain has the β-trefoil structure and the three-lobe repeating α, β, and γ loops. Mutations in the repeats can abolish binding to GalNAc-peptide acceptors. The ppGalNAcTs are the only known glycosyltransferases that have a lectin and catalytic domain conjoined. The available models suggest that each of the ppGalNAcTs has a somewhat different peptide or glycopeptide acceptor specificity, allowing the assortment of enzymes to be highly efficient at adding N-acetylgalactosamine to a tremendous variety of polypeptide substrates, including long mucin polypeptides of thousands of amino acids, and to specific single serine or threonine residues on membrane and secreted glycoproteins. The presence of the two domains in these enzymes and the multiple family members may promote an efficient processive activity and association of these enzymes with acceptor proteins (Fig. 14.4).

14.4.3 Parasite ppGalNAc-Ts

The ppGalNAc-T from human disease-causing parasite, *Toxoplasma gondii* catalyzes the initial step of mucin-type O-glycosylation, the transfer of GalNAc in O-glycosidic linkage to serine and threonine residues in polypeptides. The 84-kDa type II membrane protein contains a 49-amino acid N-terminal cytoplasmic domain, a 22-amino acid hydrophobic transmembrane domain, and a 680-amino acid C-terminal lumenal domain. Sequence motifs include a glycosyltransferase 1 (GT1) motif containing a DXH sequence, a Gal/GalNAc-T motif, and a region homologous to ricin lectin in a single 5.5-kb ppGalNAc-T transcript. Genomic DNA sequences revealed that this transferase is encoded by 10 exons in a 10 kb region. *T. gondii* demonstrates that this human parasite has its own enzymatic machinery for the O-glycosylation of toxoplasmal proteins (Wojczyk et al. 2003).

A full-length cDNA for ppGalNAc-T from the cestode *Echinococcus granulosus* (Eg-ppGalNAc-T1) was found to code for a 654-amino-acid protein containing all the structural features of ppGalNAc-Ts. Interestingly the C-terminal region of Eg-ppGalNAc-T1 bears a highly unusual lectin domain, considerably longer than the one from other members of the family, and including only one of the three ricin B repeats generally present in ppGalNAc-Ts. The role of the lectin domain in the determination of the substrate specificity of these enzymes suggests that Eg ppGalNAc-T1 would be involved in the glycosylation of a special type of substrate. This transferase is expressed in the hydatid cyst wall and the subtegumental region of larval worms. Therefore it seems to participate in the biosynthesis of O-glycosylated parasite proteins exposed at the interface between E. granulosus and its hosts (Freire et al. 2004).

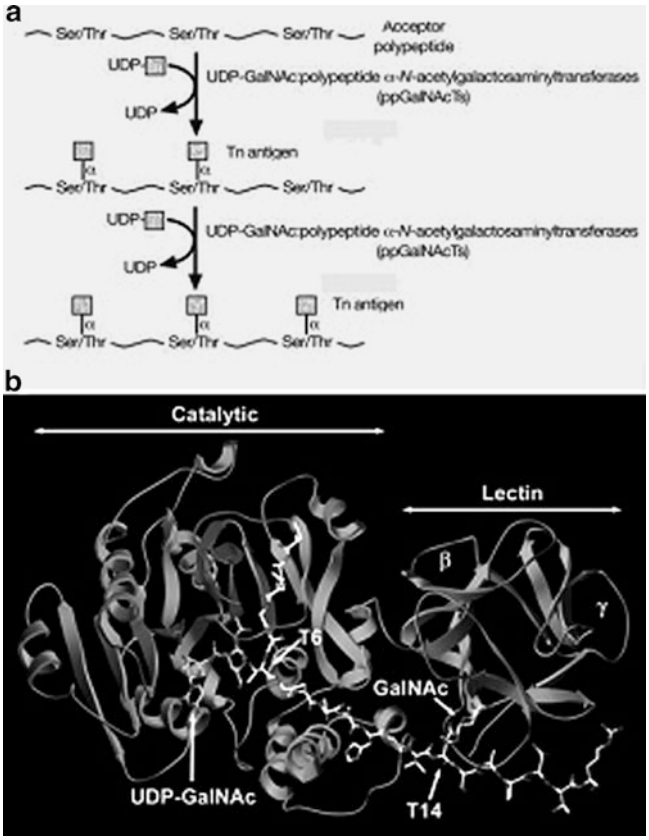

Fig. 14.4 Structure and function of UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferases (ppGalNAcTs). (a) The N-acetylgalactosamine (GalNAc, *yellow* square) transfer reaction of the ppGalNAcT showing an acceptor peptide with Ser and Thr residues and UDP-GalNAc as the donor. Some of the ppGalNAcTs may also prefer to act on the product of this reaction and use peptides with attached *N*-acetylgalactosamine as the acceptor (Cummings and Etzler 2009). **(b) The crystal structure of murine ppGalNAc-T-T1.** Modeled binding of a MUC1 glycopeptide to ppGaNTase-T1 is consistent with its in vitro pattern of glycosylation. Structural alignment of the ppGaNTase-T1 and *S. olivaceoviridis* β-xylanase (PDB ID code 1XYF) lectin domains was used to model GalNAc covalently attached to Thr-14 of a MUC1 peptide (PAPGSTAPPAHGVTSAPDTR, white carbons) into the α site of the ppGaNTase-T1 lectin domain. This docking allowed Thr-6 of the peptide to be positioned within 2.5 Å of the anomeric carbon of the modeled UDP-GalNAc (*gray* carbons). The remainder of the peptide was positioned to avoid overlap with the enzyme. All peptide ϕ and ψ angles are in allowed regions of the Ramachandran plot (Adapted by permission from Fritz et al. 2004 © Nationl Academy of Sciences, USA)

N-Acetylgalactosamine (GalNAc)

14.4.4 Crystal Structure of CEL-III from *Cucumaria echinata* Complexed with GalNAc

CEL-III is a Ca^{2+}-dependent and galactose-specific lectin with two β-trefoil folds from sea cucumber, *Cucumaria echinata*, which exhibits hemagglutinating activity. Six molecules of CEL-III are assumed to oligomerize to form an ion-permeable pore in cell membrane. CEL-III consists of three distinct domains: two carbohydrate-binding domains (1 and 2) at N-terminus that adopt β-trefoil folds as in B-chain of ricin and are members of $(QXW)_3$ motif family; and domain 3, which is a novel fold composed of two α-helices and one β-sandwich. Despite sharing the structure of the B-chain of ricin, CEL-III binds five Ca^{2+} ions at five of the six sub-domains in both domains 1 and 2. Considering the relatively high similarity among the five sub-domains, they are putative binding sites for galactose-related carbohydrates. The paucity of hydrophobic interactions in the interfaces between the domains and biochemical data suggest that these domains rearrange upon carbohydrate binding in erythrocyte membrane. This conformational change may be responsible for oligomerization of CEL-III molecules and hemolysis in the erythrocyte membranes (Uchida et al. 2004; Hatakeyama et al. 2007).

The three-dimensional structure of CEL-III/GalNAc and CEL-III/methyl α-galactoside complexes was solved by X-ray crystallography. In these complexes, five carbohydrate molecules were found to be bound to two carbohydrate-binding domains (domains 1 and 2) located in the N-terminal 2/3 portion of the polypeptide and that contained β-trefoil folds similar to ricin B-chain. The 3-OH and 4-OH of bound carbohydrate molecules were coordinated with Ca^{2+} located at the subdomains 1α, 1γ, 2α, 2β, and 2γ, simultaneously forming hydrogen bond networks with nearby amino acid side chains, which is similar to carbohydrate binding in C-type lectins. The binding of carbohydrates was further stabilized by aromatic amino acid residues, such as tyrosine and tryptophan, through a stacking interaction with the hydrophobic face of carbohydrates. The orientation of bound GalNAc and methyl α-galactoside was similar to the galactose moiety of lactose bound to the carbohydrate-binding site of the ricin B-chain, although the ricin B-chain does not require Ca^{2+} ions for carbohydrate binding. The binding of the carbohydrates induced local structural changes in carbohydrate-binding sites in subdomains 2α and 2β (Hatakeyama et al. 2007).

14.5 Microbial R-Type Lectins

A feature of many microbial glycosidases is the presence of both a catalytic domain and a carbohydrate-binding module (CBM). *Streptomyces lividans* endo-β1–4xylanase 10A (Xyn10A) is a good example of such an enzyme. Xyn10A catalyzes the cleavage of β1–4xylans and can bind to xylan and a variety of small soluble sugars, including galactose, lactose, and xylo- and arabino-oligosaccharides. The catalytic domain is at the amino terminus and the carboxyl terminus has an R-type β-trefoil motif. As mentioned above, the R-type domain CBM represents the CBM13 family in the CAZy database. In Xyn10A, all of the original β-trefoil sugar-binding motifs are retained, along with the conserved disulfide bridges, and evidence suggests that each of the three potential sugar-binding sites in β-trefoil structure interact with sugars and each site may span up to four xylose residues. The binding to monosaccharides is very weak (K_D in the range of 10^{-2}–10^{-3} M), but multivalent binding to polysaccharides can be of very high affinity.

14.5.1 *S. olivaceoviridis* E-86 Xylanase: Sugar Binding Structure

Endo-β-1,4-xylanase 10A (Xyn10A) from *Streptomyces lividans* includes an N-terminal catalytic module and a 130-residue C-terminal family 13 carbohydrate-binding module(CBM13). This latter domain adopts a β-trefoil structure with three potential binding sites (α, β, and γ) for a variety of small sugars, xylooligosaccharides, and xylan polymers. CBM13 binds mono- and oligo-saccharides with association constants of $1-10 \times 10^2$ M^{-1}. The primary function of CBM13 is to bind the polysaccharide xylan, but it retains the ability of R-type lectins to bind small sugars such as lactose and galactose. The association of CBM13 with xylan appears to involve cooperative and additive participation of three binding pockets in each of the three trefoil domains of CBM13, suggesting a novel mechanism of CBM-xylan interaction. It appears to be specific only for pyranose sugars. CBM13 binds insoluble and soluble xylan, holocellulose, pachyman, lichenan, arabinogalactan and laminarin. Site-directed mutation indicates the involvement of three functional sites on CBM13 in binding to soluble xylan. The sites are similar in sequence, and are predicted to have similar structures, to α, β and γ sites of ricin toxin B-chain, which is also in family 14. The binding of CBM13 to soluble xylan involves additive and co-operative interactions between the three binding sites. Analysis of ^{15}N NMR relaxation data revealed that CBM13 tumbles as an oblate ellipsoid and that its backbone is relatively rigid on the sub-nanosecond time scale. In particular, the three binding sites show no distinct patterns of increased internal mobility (Boraston et al. 2000). Chemical shift changes in spectra of CBM13 demonstrated that sugars (L-arabinose, lactose, D-xylose, xylobiose, xylotetraose, and xylohexaose) associate independently with the three binding sites of CBM14. The site-specific association constants showed that L-arabinose, lactose, and D-xylose preferentially bind to α site of CBM13, xylobiose binds equally well to all three sites, and xylotetraose and xylohexaose prefer binding to the β site. Inspection of the crystallographic structure of CBM13 provides a rationalization for these results (Schärpf et al. 2002). Crystal structures of CBM13 in complex with lactose and xylopentaose revealed two distinct mechanisms of ligand binding. CBM13 has retained its specificity for lactose via Ricin-like binding in all of the three classic trefoil binding pockets. However, CBM13 has the ability to bind either the nonreducing galactosyl moiety or the reducing glucosyl moiety of lactose. The mode of xylopentaose binding suggests adaptive mutations in the trefoil sugar binding scaffold to accommodate internal binding on helical polymers of xylose (Notenboom et al. 2002).

In addition to CBM13 as a xylan binding domain (XBD), *S. olivaceoviridis* E-86 contains a $(\beta/\alpha)_8$-barrel as a catalytic domain and a Gly/Pro-rich linker between them. The crystal structure of this enzyme showed that XBD has three similar subdomains, as indicated by the presence of a triple-repeated sequence, forming a galactose binding lectin fold similar to that found in Ricin toxin B-chain. Comparison with the structure of ricin/lactose complex suggests three potential sugar binding sites in XBD. In the catalytic cleft, bound sugars were observed in the xylobiose and xylotriose complex structures. In the XBD, bound sugars were identified in subdomains α and γ in all complexes with xylose, xylobiose, xylotriose, glucose, galactose and lactose. XBD binds xylose or xylooligosaccharides at same sugar binding sites as in Ricin/lactose complex but its binding manner for xylose and xylooligosaccharides is different from the galactose binding mode in ricin, even though XBD binds galactose in the same manner as in the ricin/galactose complex. These different binding modes are utilized efficiently and differently to bind the long substrate to xylanase and ricin-type lectin. Family 13 CBM has rather loose and broad sugar specificities and is used by some kinds of proteins to bind their target sugars. In such enzyme, XBD binds xylan, and the catalytic domain may assume a flexible position with respect to XBD/xylan complex, in as much as the linker region is unstructured (Fujimoto et al. 2002).

14.5.2 The Mosquitocidal Toxin (MTX) from *Bacillus sphaericus*

The mosquitocidal toxin (MTX) from *Bacillus sphaericus* and the apoptosis-inducing pierisin-1 from the cabbage butterfly Pieris rapae are two of the most intriguing members of

the family of ADP-ribosyltransferases. Both are approximately 100 kDa proteins, composed of an N-terminal ADP-ribosyltransferase (~27 kDa) and a C-terminal putative binding and translocation domain (~70 kDa) consisting of four ricin-B-like domains. They both share structural homologies, with an overall amino acid sequence identity of approximately 30% and seem to largely differ with regard to their targets or cell internalization mechanisms. MTX ADP-ribosylates numerous proteins in lysates of target insect cells at arginine residues, whereas pierisin-1 modifies DNA of insect and mammalian cells by ADP-ribosylation at 2′-deoxyguanosine residues resulting in DNA adducts, mutations and eventually apoptosis (Carpusca et al. 2006).

The crystal structure of mosquitocidal toxin from *Bacillus sphaericus* (MTX), determined at 2.5 A resolution, revealed essentially a chain consisting of four ricin B-type domains curling around the catalytic domain in a hedgehog-like assembly. The structure is probably not affected by packing contacts and explains autoinhibition data reported earlier. An analysis of ricin B-type lectin complexes and sugar molecules shows that the general construction principle applies to all four lectin domains of MTX, indicating 12 putative sugar-binding sites. These sites are sequence-related to pierisin, which is known to bind glycolipids. It seems therefore likely that MTX also binds glycolipids. The seven contact interfaces between the five domains are predominantly polar and not stronger than common crystal contacts so that in an appropriate environment, the multi-domain structure would likely uncurl into a string of single domains. The structure of the isolated catalytic domain plus an extended linker was established earlier in three crystal packings, two of which showed a peculiar association around a 7-fold axis. The catalytic domain of the reported MTX closely resembles all three published structures, except one with an appreciable deviation of the 40 N-terminal residues. A comparison of all structures suggests a possible scenario for the translocation of the toxin into the cytosol (Treiber et al. 2008). The crystal structure of MTX catalytic domain is helpful to reveal new insights into structural organization, catalytic mechanisms, and autoinhibition of both enzymes.

14.6 R-Type Lectins in Butterflies

14.6.1 Pierisin-1

Pierisin-like proteins are found in subtribes Pierina, Aporiina and Appiadina. Pierisin from P. rapae is called pierisin-1, and that from P. brassicae is called pierisin-2. Pierisin-1 is a 98-kDa protein comprising 850 aa consisting of N-terminal region (27 kDa) and C-terminal region (71 kDa). The N-terminal region of pierisin-1 has a partial regional sequence similarity with ADP-ribosylating toxins such as the A-subunit of cholera toxin, and disruption of this possible NAD-binding site by site-directed mutagenesis abolishes its apoptosis-inducing activity (Watanabe et al. 1999). Unlike other ADP-ribosyltransferases, the N-terminal region of pierisin-1 targets the N2 amino groups of guanine residues in DNA to yield N2-(ADP-ribos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al. 2001). The C-terminal region of pierisin-1 shares sequence similarity with HA-33, a subcomponent of hemagglutinin of botulinum toxin that binds to sialic acid or galactose moieties on surfaces of neuronal cells (Inoue et al. 1999; Lord et al. 2003). Receptors for pierisin-1 on mammalian cells have been found to be the neutral glycosphingolipids, including globotriaosylceramide and globotetraosylceramide, and their expression levels largely determine sensitivity to the toxic protein (Matsushima-Hibiya et al. 2003). Pierisin-1 is distributed in fat bodies during the final larval instar and is abundantly expressed in fifth instar larvae and early pupae in the cabbage butterfly, Pieris rapae (Watanabe et al. 1998, 2004a). It appears that pierisin-1 may play important roles in induction of apoptosis to remove larval cells in the pupation of Pieris rapae. This protein has potent cytotoxic activity against TMK-1 human gastric cancer cells and various human cancer cell lines, inducing typical apoptotic cell death with characteristic morphological features, DNA fragmentation, and cleavage of poly(ADP-ribose) polymerase (Watanabe et al. 1998; Kono et al. 1999). Among mammalian cell lines tested, human HeLa cells were the most sensitive to the cytotoxic effects of pierisin-1 (Kono et al. 1999). The other cabbage white butterfly, Pieris brassicae, also contains the cytotoxic protein and named pierisin-2. Its amino acid sequence is 91% identical to that of pierisin-1. Pierisin-2 targets DNA, and the structure of the DNA adduct produced by pierisin-2 is the same as that produced by pierisin-1 (Takamura-Enya et al. 2004).

Globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4), two neutral glycosphingolipids showed receptor activities for pierisin-1. Alteration of QXW by site-directed mutagenesis caused marked reduction of pierisin-1 cytotoxicity. Study suggests that pierisin-1 binds to Gb3 and Gb4 receptors at C-terminal region, in a manner similar to ricin, and then exhibits cytotoxicity after incorporation into the cell (Matsushima-Hibiya et al. 2003).

14.6.1.1 Cytotoxic and Apoptotic Activity in Pierisin

ADP-ribosylation is generally known to be a posttranslational modification where the ADP-ribose moiety of b-NAD is transferred to specific proteins. Several types of bacteria have been shown to produce mono(ADP-ribosyl) transferase the acceptors of which are usually specific amino acid residues in proteins in eukaryotic cells. Cholera

toxin ADP-ribosylates arginine residues in G proteins, whereas pertussis toxin ADP-ribosylates a cysteine residue. Diphtheria toxin and Pseudomonas aeruginosa exotoxin A use diphthamide, a modified histidine, as the acceptor amino acid. Clostridium botulinum C3 exoenzyme is an asparagine-specific ADP-ribosyltransferase. Thus, mono(ADPribosyl)ation reactions occur at nitrogen or sulfur atoms in different amino acids to produce N- or S-glycosides.

Pierisin-1 is a potent inducer of apoptosis of mammalian cells; apoptosis is accompanied by cleavage of DNA to nucleosome units and of poly(ADP-ribose) polymerase (Watanabe et al. 1998; Kono et al. 1999). Like diphtheria toxin, Pierisin-1 is also considered to be an ADP-ribosylating toxin. Several spectral analyses and independent syntheses indicated that the acceptor site for ADP-ribosylation is N-2 of guanine base. Cytotoxic activity in extracts of pupae and adults of various kinds of butterflies and moths was tested in vitro against the human gastric carcinoma cell line, TMK-1. Among species examined, cytotoxicity was limited to Pieris rapae, Pieris napi and Pieris brassicae, while with the other butterflies and moths no activity was observed, even at high concentration. The pupae showed the strongest activity. The active principle in the pupae of Pieris rapae was heat-labile and not extractable with organic solvents. This cytotoxic factor was named pierisin (Watanabe et al. 1998) and later Pierisin-1. In addition to human gastric cancer TMK-1 cell line, Pierisin-1 showed cytotoxic effects in nine other human cancer cell lines and human umbilical vein endothelial cells (HUVECs) of the human cells (Kono et al. 1999). After incorporation of pierisin-1 into the cell by interaction of its C-terminal region with the receptor in the cell membrane, the entire protein is cleaved into the N- and C-terminal fragments with intracellular protease, and the N-terminal fragment then exhibits cytotoxicity (Kanazawa et al. 2001). Pierisin-1 is a toxic protein in mice and rats and results in a gradual decrease in body weight due to decreased food intake, relative polycythemia with low serum albumin concentration and atrophy of the thymus, spleen, seminal vesicles and adipose tissue after i.p. administration. It induced diarrhea, fusion and atrophy of the villi and dilatation of the crypts in the small intestine of BALB/c mice. However, oral administration of pierisin-1 at a dose of 10,000 μ/kg body weight did not exert any obvious effects (Shiga et al. 2006). Pierisin-1 has an A small middle dotB structure-function organization like cholera or diphtheria toxin, where the "A" domain (N-terminal) exhibits ADP-ribosyltransferase activity. The target molecule for ADP-ribosylation by pierisin-1 in the presence of β-[adenylate-32P]NAD was found DNA as the acceptor, but not protein as is the case with other bacteria-derived ADP-ribosylating toxins such as such as cholera toxin and pertussis toxin. Thus, the targets for ADP-ribosylation by pierisin-1 were concluded to be 2′-deoxyguanosine residues in DNA. Pierisin-1 efficiently catalyzes the ADP-ribosylation of double-stranded DNA. The ADP-ribose moiety of NAD is transferred by pierisin-1 to the amino group at N2 of the deoxyguanosine base (Takamura-Enya et al. 2001; Kanazawa et al. 2001). These findings opened a new field regarding the biological significance of ADP-ribosylation (Takamura-Enya et al. 2001).

Pierisin-1 induced apoptosis in mammalian cells is accompanied by a release of cytochrome C and activation of a variety of caspases, and this apoptosis was inhibited by over-expression of Bcl-2 (Watanabe et al. 2002; Kanazawa et al. 2002). Pierisin-1 treatment primarily activates ATR pathway and eventually activates ATM pathway as a result of the induction of apoptosis. It was suggested that mono-ADP-ribosylation of DNA causes a specific type of fork blockage that induces checkpoint activation and signaling (Shiotani et al. 2006).

14.6.1.2 Cytotoxicity of Butterflies Extracts Against Cancer Cells

Cells cytotoxicity has been studied in 18 kinds of butterflies against TMK-1 cells. Positive results have been obtained with extracts from Pieris rapae, Pieris brassicae, and Pieris napi among the genus Pieris. However, no cytotoxicity was observed in the other extracts from examined butterflies: Eurema hecabe, Colias erate, and Hebomoia glaucippe of the family Pieridae; Papilio bianor, Papilio helenus, Papilio maackii, Papilio machaon, Papilio protenor, and Papilio xuthus of the family Papilionidae; Dichorragia nesimachus, Vanessa indica, Sasakia charonda, and Hestina japonica of the family Nymphalidae; Celastrina argiolus and Lycaena phlaeas of the family Lycaenidae (Matsumoto et al. 2008). In further study (Matsumoto et al. 2008), crude extracts from 20 other species of Pieridae family were examined for cytotoxicity in HeLa cells and DNA ADP-ribosylating activity. Both activities were detected in extracts from 13 species: subtribes Pierina. All of these extracts contained substances recognized by anti-pierisin-1 antibodies, with a molecular mass of ≈100 kDa close to pierisin-1. Extracts from seven species, Appias lyncida, Leptosia nina, Anthocharis scolymus, Eurema hecabe, Catopsilia pomona, Catopsilia scylla, and Colias erate, showed neither cytotoxicity nor DNA ADP-ribosylating activity, anti-pierisin-1 immune activity. Thus, pierisin-like proteins, showing cytotoxicity and DNA ADP-ribosylating activity, are suggested to be present in the extracts from butterflies not only among the subtribe Pierina, but also among the subtribes Aporiina and Appiadina. These findings offer insight to understanding the nature of DNA ADP-ribosylating activity in the butterfly.

14.6.1.3 Molecular Cloning of Pierisin-1

The pierisin gene encodes an 850-amino acid protein with a molecular weight of 98,081. The expressed protein induced apoptosis in human gastric carcinoma TMK-1 and cervical

carcinoma HeLa cells, like the native protein, indicating functional activity. The deduced amino acid sequence of pierisin showed 32% homology with a 100-kDa mosquitocidal toxin from *Bacillus sphaericus* SSII-1. In addition, pierisin showed regional sequence similarities with ADP-ribosylating toxins, such as the A subunit of cholera toxin. A glutamic acid residue at the putative NAD-binding site, conserved in all ADP-ribosylating toxins, was also found in pierisin. Substitution of another amino acid for glutamic acid 165 resulted in a great decrease in cytotoxicity and induction of apoptosis. Moreover, inhibitors of ADP-ribosylating enzymes reduced pierisin-induced apoptosis. Hence, pierisin might possess ADP-ribosylation activity that leads to apoptosis of the cells (Watanabe et al. 1999).

14.6.1.4 Enzymatic Properties of Pierisin-1

ADP-ribosyltransferase catalyzes the transfer of an ADP-ribosyl moiety of NAD to specific proteins. ADP-ribosyltransferase showed a K_m for NAD of 0.17 mM and kcat of 55 s^{-1} (Watanabe et al. 2004a). Binding of C-terminal region of pierisin-1 to glycosphingolipid Gb3 and Gb4 receptors on cell membrane is necessary for incorporation into cells, while the N-terminal polypeptide catalyzes transfer of the ADP-ribose moiety of NAD at N2 of dG in DNA. Resulting DNA adducts cause mutation if they are present at low levels. If the DNA damage is more severe, the cells undergo apoptosis. Shiotani et al. (2005) examined the repair system for ADP-ribosylated dG adducts using nucleotide excision repair (NER) mutants of CHO cells. It is suggested that the NER system is involved in the repair of ADP-ribosylated dG adducts in DNA. Kawanishi et al. (2007) examined the involvement of NER system in the removal of N2-ADPR-dG in E. coli and human cells and suggested the involvement of the NER system in the repair of N2-ADPR-dG in both E. coli and human cells.

Similar to pierisin-1 and -2, crude extracts from the clams Meretrix lamarckii (shellfish), Ruditapes philippinarum, and Corbicula japonica incubated with calf thymus DNA and β-NAD results in production of N2-(ADP-ribos-1-yl)-2′-deoxyguanosine. The CARP-1 showed no homology with pierisin-1 or -2. However, a glutamic acid residue (E128) at NAD-binding site was conserved in in CARP-1. Although the CARP-1 in the culture medium showed no cytotoxicity against HeLa and TMK-1 cells, introduction of this protein by electroporation induced apoptosis in these cells (Nakano et al. 2006).

14.6.2 Pierisin-2, Pierisin-3 and -4

Pierisin from P. rapae is called pierisin-1, and that from P. brassicae is called pierisin-2. Pierisin-2 is a cytotoxic and apoptosis-inducing protein present in Pieris brassicae and purified from pupae. The cDNA encodes an 850-amino-acid protein with a molecular mass of 97,986. The deduced amino-acid sequence of pierisin-2 was 91% identical with that of pierisin-1. The results from site-directed mutagenesis at Glu165, a conserved residue among ADP-ribosylating enzymes necessary for NAD binding, and from experiments with ADP-ribosylating enzyme inhibitors suggested that pierisin-2 could be considered as an ADP-ribosylating toxin like pierisin-1 (Matsushima-Hibiya et al. 2000). Like pierisin-1, pierisin-2 also catalyzed ADP-ribosylation of dG in DNA to give the same reaction product as demonstrated for pierisin-1. With oligonucleotides as substrates, ADP-ribosylation by pierisin-2 was suggested to occur by one-side attack of the carbon atom at 1 position of the ribose moiety in NAD toward N2 of dG (Takamura-Enya et al. 2004).

Pierisin-like proteins are found in subtribes Pierina, Aporiina and Appiadina. The nucleotide sequences of Pierisin-3 and -4 encode an 850 and an 858 amino acid protein, respectively. The partial peptide sequences of Pierisin-3 and -4 purified from pupae were identical to the deduced amino acid sequence of ORF. Pierisin-3 showed 93% similarity to Pierisin-1 and 64% similarity to Pierisin-4 in amino acid sequences. Pierisin-3 and -4 synthesized in vitro exhibited apoptosis-inducing activity against human cervical carcinoma HeLa and human gastric carcinoma TMK-1 cells. Site-directed mutagenesis at a glutamic acid residue comprising the NAD-binding site resulted in a decrease in cytotoxicity of both proteins. Moreover, proteins with calf thymus DNA and β-NAD resulted in the formation of N2-(ADP-ribos-1-yl)-2′-deoxyguanosine, as in Pierisin-1 and -2. Results suggest apoptosis-inducing ability and molecular evolution of Pierisin-like proteins in family Pieridae (Yamamoto et al. 2009).

14.7 Discoidin Domain and Carbohydrate-Binding Module

14.7.1 The Discoidin Domain

Discoidin domain (DS) (also known as F5/8 type C domain, or C2-like domain) was first identified in discoidin proteins of *Dictyostelium discoideum* and subsequently found in a variety of extracellular and membrane proteins including the blood coagulation Factor V and Factor VIII, milk fat globule protein, neuropilins, neurexin IV, and discoidin domain receptor proteins (Kiedzierska et al. 2007; Baumgartner et al. 1998; Pratt et al. 1999). The DS domain is a structural and functional motif that is appended, singly or in tandem, to various eukaryotic and prokaryotic proteins. The first DS domain in the amoeba *Dictyostelium discoideum* was described as a lectin

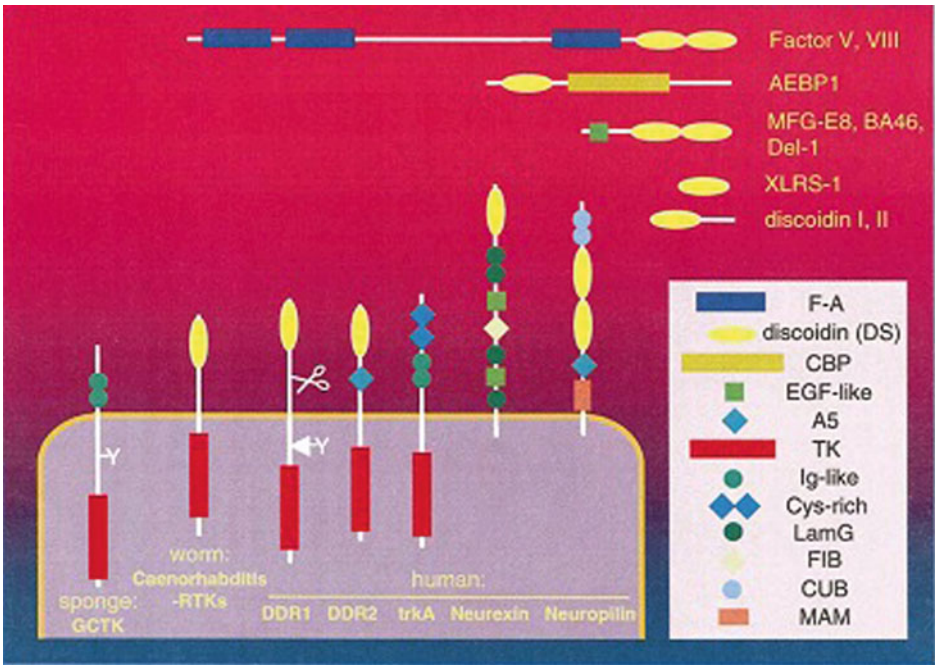

Fig. 14.5 The family of proteins with DS domains. Schematic representation of transmembrane and secreted proteins. Abbreviations for the domains shown are as follows: *F-A* A-domain in Factor V and VIII, *CBP* carboxypeptidase, *EGF* epidermal growth factor, *TK* tyrosine kinase, *A5* homology to A5 antigen, *Ig* immunoglobulin, *LamG* laminin-G, *FIB* fibronectin-like, *CUB* complement binding, *MAM* meprin/A5/PTPmu. The tyrosines in the N-P-X-Y motives of GCTK and DDR1b are highlighted. Proteolytic processing of DDR1 is indicated (Adapted by permission from Vogel 1999 © The Federation of American Societies for Experimental Biology)

with high affinity for galactose and galactose derivatives (Sauer et al. 1997). Since then, high resolution structures of a number of discoidin domains have been determined (Pratt et al. 1999; Macedo-Ribeiro et al. 1999; Lee et al. 2003). In all cases, the domain consists of a 5-strand antiparallel β-sheet that packs against a 3-strand antiparallel β-sheet to form a core barrel-like structure. An intramolecular disulfide bond between conserved cysteine residues at the beginning and end of the discoidin domain stabilizes the three dimensional structure. At the opposite end spikes or loops protrude from the core β-barrel structure to define a groove or cleft that serves as the ligand binding site (Fig. 14.5).

Kiedzierska et al. (2007) outlined the biological role of this module in various eukaryotic proteins. The DS domain binds a wide variety of ligand molecules, including phospholipids, carbohydrates, and partner proteins, thus enabling its cognate protein to participate in various physiological functions such as cellular adhesion, migration, neural development, and nutrition assimilation. Discoidin domain receptors interact with collagen to regulate cell proliferation and extracellular matrix modeling via activation of their tyrosine kinase activities (Vogel et al. 2006). A subgroup of the domain possessing carbohydrate-binding ability is also classified as the carbohydrate-binding module family 32 (CBM32). Co-crystallizations of CBM32 members and their ligands, such as the module of Clostridium perfringens N-acetylglucosaminidase with β-galactosyl-1,4-β-N-acetylglucosamine or the module of Micromonospora viridifaciens sialidase with lactose, demonstrate that the protruding loops form the ligand binding site (Macedo-Ribeiro et al. 1999; Lee et al. 2003). Discoidin proteins from *Dictyostelium discoideum* function as lectins with a high affinity for galactose residues to promote cell aggregation (Poole et al. 1981). Factors, V and -VIII bind phosphatidylserine on the surface of platelets and endothelial cells through their discoidin domains as a crucial step in initiating the blood coagulation cascade (Fuentes-Prior et al. 2002). The discoidin domains of SED1 mediate the interaction of sperm and egg as part of the fertilization process (Ensslin and Shur 2003).

14.7.2 Discoidins from *Dictyostelium discoideum* (DD)

The cell adhesion protein discoidin from cellular slime mould *Dictyostelium discoideum* has a binding site for carbohydrate residues related to galactose. The lectin, that consists of two distinct species (discoidins I and II), is synthesised as the cells differentiate from vegetative to aggregation phase and was originally thought to be involved in intercellular adhesion, but discoidin I is now thought to be

involved in adhesion to the substratum by a mechanism resembling that of fibronectin in animals. The social amoeba *D. discoideum* (Slime mold) adopts a cohesive stage upon starvation and then produces Discoidin I and II, two proteins able to bind galactose and N-acetyl-galactosamine. The carbohydrate-binding protein (discoidin) agglutinated formalinized sheep erythrocytes and synthesized by *D. discoideum* cells when cells were deprived of food. Agglutination of erythrocytes by this protein was inhibited by N-acetyl-D-galactosamine, D-galactose, and Lfucose, but other monosaccharides had little or no effect. It appears to be present on the surface of cohesive but not vegetative slime-mold cells. The possibility that this protein may mediate intercellular adhesion in Dictyostelium has been suggested (Rosen et al. 1973; Kamohara et al. 2001). The discoidin domain is a approximately 150 amino acid motif common in both eukaryotic and prokaryotic proteins and arranged into a β-sandwich fold with several flexible loops. Presumably, the β-sandwich fold is stabilized predominantly by hydrophobic interactions. The variability within the loops has been suggested to account for the diverse binding spectrum of the DS domain. The cell adhesion protein discoidin, a related domain, named discoidin I-like domain (DLD, or D) has been found to share a common C-terminal region of about 110 amino acids with the FA5/8C domain, but whose N-terminal 40 amino acids are much less conserved. Discoidin domain is major domain of many blood coagulation factors, as blood coagulation factors V and VIII. In coagulation factors V and VIII the repeated domains compose part of a larger functional domain which promotes binding to anionic phospholipids on the surface of platelets and endothelial cells. The C-terminal domain of the second FA5/8C repeat (C2) of coagulation factor VIII has been shown to be responsible for phosphatidylserine-binding and essential for activity. It forms an amphipathic α-helix, which binds to the membrane. FA5/8C contains two conserved cysteines in most proteins, which link the extremities of the domain by a disulfide bond. A further disulfide bond is located near C-terminal of second FA58C domain in MFGM Q08431. Ricin B chain and discoidin I share a common primitive protein fold (Robertus and Ready 1984).

Similar domains have been detected in other extracellular and membrane proteins. The X-ray structures of the wild-type and recombinant Discoidin II in unliganded state and in complex with monosaccharides revealed that the protein forms a homotrimer which presents two binding surfaces situated on the opposite boundaries of the structure. The binding sites of N-terminal domain contain PEG molecules that could mimic binding of natural ligand. The C-terminal lectin domain interactions with N-acetyl-D-galactosamine and methyl-β-galactoside have been reported. The carbohydrate binding sites are located at the interface between monomers. Specificity for galacto configuration can be rationalized since the axial O_4 hydroxyl group is involved in several hydrogen bonds with protein side chains. Results highlight the structural differentiation of the DS domain involved in many cell-adhesion processes from the lectin activity of *Dictyostelium discoidins (*Aragão et al. 2008).

14.7.3 Discoidin Domain Receptors (DDR1 and DDR2)

Two mammalian receptor tyrosine kinases (DDR1 and DDR2) have extracellular domains closely related to lectin, discoidin, required for cell aggregation. The subgroup of DDRs is distinguished from other members of the receptor tyrosine kinase (RTK) family by a discoidin homology repeat in their extracellular domains that is also found in a variety of other transmembrane and secreted proteins. Two mammalian receptor tyrosine kinases (DDR1 and DDR2) have extracellular domains closely related to a *D. discoideum* lectin, discoidin, required for cell aggregation. DDR1 and DDR2 are detected as 125- and 130-kDa glycosylated proteins in a Western blot of lysates from overexpressing cells (Alves et al. 1995). The mammalian DDR receptors bind and get activated by specific types of collagen. Stimulation of DDR receptor tyrosine kinase requires the native triple-helical structure of collagen and occurs over an extended period of time. Collagen activation of DDR1 induces phosphorylation of a docking site for Shc phosphotyrosine binding domain, whose presence is controlled by alternative splicing. Activation of DDR2 by collagen results in the up-regulation of matrix metalloproteinase-1 expression. Thus the discoidin-related DDR tyrosine kinases are novel collagen receptors with the potential to control cellular responses to the extracellular matrix (Vogel et al. 1997).

The cDNAs of RTK subfamily have been cloned from different species by many laboratories. They represent two distinct genes, which have now been renamed DDR1 (Vogel et al. 1997) and DDR2 (Alves et al. 1995; Lai and Lemke 1994; Karn et al. 1993; Playford et al. 1996). It is surprising to note that the closest relative to the tyrosine kinase domain of DDR1 is found in the genome of the marine sponge *Geodia cydonium*. The multiple sequence alignment shows that the catalytic core region of DDR1 is 59% identical to the Geodia tyrosine kinase called GCTK (61% for DDR2), whereas the closest mammalian RTK subfamily, the neurotrophin receptors, are 55–58% related (Gamulin et al. 1997). A similar Ig-domain repeat is found in the mammalian nerve growth factor receptors, which are the second-most-related RTK subfamily to the Geodia sequence. Thus far, five isoforms of the DDR1 protein have been

characterized, that arise by alternative splicing (Alves et al. 2001). The longest DDR1 transcript codes for the full-length 919 amino acid long receptor (c-isoform), whereas the a- or b-receptor isoforms lack 37 or 6 amino acids in the juxtamembrane or kinase domain, respectively (Alves et al. 1995). The DDR1b protein is the predominant isoform expressed during embryogenesis, whereas the a-isoform is upregulated in certain human mammary carcinoma cell lines (Johnson et al. 1993; Perez et al. 1996). Results suggest that the discoidin-related DDR tyrosine kinases are novel collagen receptors with the potential to control cellular responses to the extracellular matrix (Vogel 1999, 2001). The revelation that collagens function as ligands for DDR1 and DDR2 suggests that some of the other mammalian DS domains may interact with matrix proteins as well.

14.7.4 Earth Worm (EW)29 Lectin

The galactose-binding lectin EW29 from the earthworm Lumbricus terrestris is composed of two homologous domains, both of which are members of the R-type lectin family. The EW29 shows haemagglutination activity and is composed of a single peptide chain that includes two homologous domains: N-terminal and C-terminal domains (14,500 Da) and show 27% identity with each other. A truncated mutant of EW29 comprising the C-terminal domain (rC-half) has haemagglutination activity by itself. In order to clarify how rC-half recognizes ligands and shows haemagglutination activity, X-ray crystal structures of rC-half in complex with D-lactose and N-acetyl-D-galactosamine have been determined. The structure of rC-half is similar to that of the ricin B chain and consists of a β-trefoil fold; the fold is further divided into three similar subdomains referred to as subdomains α, β and γ, which are gathered around the pseudo-threefold axis. The structures of sugar complexes demonstrated that subdomains α and γ of rC-half bind terminal galactosyl and N-acetylgalactosaminyl glycans. The sugar-binding properties are common to both ligands in both subdomains and are quite similar to those of ricin B chain-lactose complexes. These results indicate that the C-terminal domain of EW29 uses these two galactose-binding sites for its function as a single-domain-type haemagglutinin (Suzuki et al. 2009; Hemmi et al. 2009). The truncated mutant rC-half comprising C-terminal domain was crystallized by the hanging-drop vapour-diffusion method. The crystal belonged to space group P4(3)2(1)2, with unit-cell parameters a = b = 61.2, c = 175.6 A, and diffracted to beyond 1.9 A resolution. Matthews coefficient calculations suggested that this crystal contained two molecules per asymmetric unit (Suzuki et al. 2004). Since ricin B chain and discoidin I share a common primitive protein fold (Robertus and Ready 1984), presence of β-trefoil fold in discoidin domain containing lectins is subject of further investigations.

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Mannose Receptor Family: R-Type Lectins

15

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15.1 R-Type Lectins in Animals

R-type lectins exist ubiquitously in nature and mainly bind to galactose unit of sugar chains. Originally found in plant lectin, Ricin, the R-type lectin domain is found in several animal lectins, including the members of mannose receptor (MR) family, and in some invertebrate lectins (discussed in Chap. 14). The R-type domain contained in these proteins is the CRD, which is also termed a carbohydrate-binding module (CBM) and has been placed in the CBM13 family in the CAZy database (carbohydrate-active enzymes database). While the A chain in ricin has eight α-helices and eight β-strands, and is the catalytic subunit, the B chain contains R-type lectin domains, has two tandem CRDs that are about 35 Å apart and have a shape resembling a barbell, with one binding domain at each end. Each R-type domain has a three-lobed organization that is a β-trefoil structure (from the Latin trifolium meaning "three-leaved plant"). The β-trefoil structure probably arose evolutionarily through gene fusion events linking a 42-amino-acid peptide subdomain that has galactose-binding activity. The three lobes are termed α, β, and γ and are arranged around a threefold axis (See Fig. 14.2; Chap. 14). Conceivably, each lobe could be an independent binding site, but in most R-type lectins only one or two of these lobes retain the conserved amino acids required for sugar binding. The R-type domain is also found in pierisin-1, which is a cytotoxic protein from the cabbage butterfly Pieris rapae, and in the homologous protein pierisin-2, from Pieris brassicae. Tandem R-type motifs are found in some other R-type family members. For example, Limulus horseshoe crab coagulation factor G has a central R-type lectin domain, which is flanked at the amino terminus by a xylanase Z-like domain and at the carboxyl terminus by a glucanase-like domain. In this chapter we will restrict our discussion to R-type lectins of mannose receptor family, which comprises also of endocytic receptors.

15.2 Mannose Receptor Lectin Family

There are four known members of the MR family in humans, all of which contain an R-type lectin domain. In addition, the members of mannose receptor family have a unique structural composition due to the presence of multiple C-type lectin-like domains within a single polypeptide backbone. The four members of the mannose receptor family [the mannose receptor, the M-type phospholipase A_2 receptor, DEC-205 and Endo180 (or urokinase plasminogen activator receptor-associated protein)] share a common extracellular arrangement of an amino-terminal cysteine-rich domain related to R-type domain, followed by a fibronectin type II domain and 8–10 C-type lectin-like CRD domains within a single polypeptide. In addition, all have a short cytoplasmic domain, which mediates their constitutive recycling between the plasma membrane and the endosomal apparatus, suggesting that these receptors function to internalize ligands for intracellular delivery (See Fig. 14.3; Chap 14). However, despite the common presence of multiple lectin-like domains, these four endocytic receptors have divergent ligand binding activities, and it is clear that the majority of these domains do not bind sugars. All of the MR family members except DEC-205 recycle back to the cell surface from early endosomes, but DEC-205 recycles from late endosomes. However, each receptor has evolved to have distinct functions and distributions. These receptors are unusual among animal lectins in that they can bind ligands in either a "cis" or "trans" fashion, which means they can bind to cell-surface glycoconjugates on the same cell or to those on other cells and to soluble ligands.

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15.3 The Mannose Receptor (CD206)

15.3.1 Human Macrophage Mannose Receptor (MMR)

The CD206, also known as macrophage MR (MMR) is the best characterised member of the family of four endocytic molecules that share a common domain structure; a cysteine-rich (CR) domain related to the R-type CRD of ricin, a fibronectin-type II (FNII) domain and tandemly arranged C-type lectin-like domains (CTLD, eight in the case of MR). The MR and other members of this family are among the few mammalian glycan-binding proteins that have two separate lectin motifs (C-type and R-type) in the same molecule. This group is also unusual in that it is the only known lectin group in mammals with more than two C-type lectin domains in the same molecule. Only CTLDs 4 and 5 of the MR have been shown to bind glycans in a Ca^{2+}-dependent manner and to bind mannose, N-acetylglucosamine, and fucose. Each protein is a recycling plasma membrane receptor with a cytoplasmic domain that mediates clathrin-dependent endocytosis and uptake of extracellular glycan-containing ligands. The MR is the heavily glycosylated endocytic receptor. Glycosylation differentially affects both MR lectin activities. The glycosylation of MR, terminal sialylation in particular, could influence its binding properties at two levels: (1) it is required for mannose recognition; and (2) it modulates the tendency of MR to self-associate, effectively regulating the avidity of the CR domain for sulfated sugar ligands (Su et al. 2005). The MR is unusual protein in sense that it is the only member of the MR family that can function both in clathrin-dependent endocytosis and in the phagocytosis of nonopsinized microbes and large ligands.

The MR of macrophages (MMR), epithelial, and endothial cells acts as a molecular scavenger, binding to and internalizing a variety of pathogenic microorganisms and harmful glycoproteins. The macrophage MR is a 175–180 kDa type I transmembrane glycoprotein. The MR is expressed at high levels on hepatic endothelial cells and Kupffer cells as well as on many other endothelial and epithelial cells, macrophages, and immature dendritic cells (DCs). The MR was originally discovered in the 1980s in rabbit alveolar macrophages as a membrane protein that bound mannose-containing ligands as well as pituitary hormones such as lutropin and thyrotropin, which have 4-O-sulfated N-acetylgalactosamine residues on N-glycans (Leteux et al. 2000). The MR is part of the innate immune system and facilitates the phagocytosis of mannose-rich pathogens. It also assists leukocytes in responding appropriately to antigens by promoting trafficking to the germinal center and is also involved in antigen presentation.

The primary structure of the mannose receptor reflects its diverse carbohydrate specificity. Size and shape parameters indicate that the receptor is a monomeric, elongated and asymmetric molecule. Domain organization of the CRD-4 monomer in MR represents two possible conformations: extended and U-shaped. Hydrodynamic coefficients predicted for modeled receptor conformations are consistent with an extended conformation with close contacts between three pairs of CRDs. The N-terminal cysteine-rich domain and the fibronectin type II repeat appear to increase the rigidity of the molecule. The rigid, extended conformation of the receptor places domains with different functions at distinct positions with respect to the membrane. An N-terminal cysteine-rich domain mediates recognition of sulfated *N*-acetylgalactosamine, which is the terminal sugar of the unusual oligosaccharides present on pituitary hormones (Fiete et al. 1998; Napper et al. 2001). The extracellular domains are linked to a transmembrane region and a small cytoplasmic domain. The CRDs of the extracellular region mediate calcium-dependent binding to sugars that are commonly found on microorganisms, but rarely seen in sufficient density in terminal positions of mammalian oligosaccharides (Weis et al. 1998; Drickamer and Taylor 1993).

The gene for the human MMR is divided into 30 exons. The first three exons encode the signal sequence, the NH2-terminal cysteine-rich domain, and the fibronectin type II repeat, while the final exon encodes the transmembrane anchor and the cytoplasmic tail. The intervening 26 exons encode the eight CRDs and intervening spacer elements. The pattern of intron positions and comparison of sequences of CRDs suggests that these domains evolved by duplication (Kim et al. 1992).

15.3.2 Structure-Function Relations

Understanding the molecular basis of cell surface ligand recognition and endosomal release by the MR requires information about how individual domains interact with sugars as well as the structural arrangement of the multiple domains. The NH2-terminal cysteine-rich domain and the fibronectin type II repeat are not necessary for endocytosis of mannose-terminated glycoproteins. The CRDs 1–3 have at most very weak affinity for carbohydrate, where as, of the eight C-type CRDs, CRDs 4–8 are required for binding and endocytosis of mannose/GlcNAc/fucose-terminated ligands, but only CRD-4 has demonstrable sugar binding activity in isolation. CRD 4 shows the highest affinity binding and has multispecificity for a variety of monosaccharides. As the main mannose-recognition domain of MR (CRD4) is the central ligand binding domain of the receptor, analysis of this domain suggests ways in which multiple CRDs in whole receptor might interact with each other (Feinberg et al. 2000;

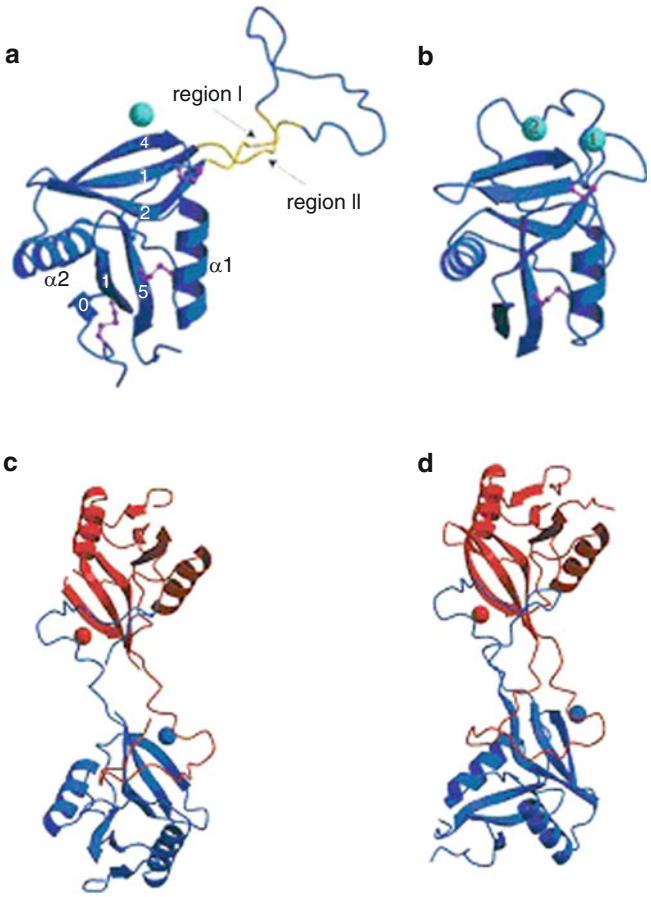

Fig. 15.1 Structure of CRD-4 monomer and comparison to rat Mannose-binding protein A. (a), Ribbon diagram of CRD-4. Disulfide bonds are shown in *pink ball-and-stick* representation, and the Ca^{2+} is shown as a *bluegreen sphere*. The two segments that connect the extended loop to the core of the CRD, region I (residues 701–708) and region II (residues 729–734), are shown in *yellow*. (**b**). Ribbon diagram of the MBP-A CRD. Ca^{2+} site 1 is the auxiliary site, and Ca^{2+} site 2 is the principal site. **Figs 14.1c and 14.1d: Domain-swapped dimer structure. (c)**. Two molecules of copy A of CRD-4, related by a crystallographic twofold rotational symmetry axis in the lattice. (**d**). crystallographic dimer of copy **C**. The two protomers are shown in *blue* and *red*. The distal part of the extended loop of the partner protomer in the dimer, which forms part of the Ca^{2+}-binding site, is shown in *red*, next to the *blue* core of the other protomer (Adapted by permission from Feinberg et al. (2000) © The American Society for Biochemistry and Molecular Biology)

Mullin et al. 1997). However, CRD 4 alone cannot account for the binding of the receptor to glycoproteins. At least 3 CRDs (4, 5, and 7) are required for high affinity binding and endocytosis of multivalent glycoconjugates. In this respect, the MR is like other carbohydrate-binding proteins, in which several CRDs, each with weak affinity for single sugars, are clustered to achieve high affinity binding to oligosaccharides (Taylor et al. 1992). The overall structure of CRD-4 (Fig. 15.1a) is similar to other C-type CRDs, containing two α helices and two small antiparallel β sheet in MBP (Fig. 15.1b). The core region of the CRD-4 domain, consisting of β strands 1–5 and the two α helices, superimposes on the equivalent residues of the rat MBP-A CRD. The principal difference resides in the position of helix α2, which is the most variable element of secondary structure among the C-type lectin-like folds.

15.3.2.1 Domain-Swapped Dimer Structure

Extensive interactions occur between the extended loops formed by residues 701–734 of two CRD-4 molecules related by a crystallographic twofold rotational symmetry axis (Fig. 15.1c, d). Most strikingly, the most distal portion of the loop (residues 708–728) forms the upper portion of the Ca^{2+}- and sugar-binding site of the partner molecule. This domain swapping (3D domain swapping) is a mechanism for forming oligomeric proteins from their monomers), Bennett et al. (1995) produces a dimer in which each end, comprised of residues 625–700 and 735–768 from one polypeptide chain and residues 708–728 from a crystal symmetry-related molecule, has the compact fold typical of C-type CRDs (Fig. 15.1a). This "hybrid molecule" was referred to as CRD4 monomer-like (CRD4-M) (Feinberg et al. 2000). Comparison of CRD4-M with MBP (Fig. 15.1b) and E-selectin and other C-type CRDs reveals a remarkable similarity in the structure of these loops. The two crossover segments that connect residues 708–728 to the core of the CRD, residues 701–707 and residues 729–734, referred to as regions I and II, respectively, are examples of "hinge loops," which are segments of polypeptide that link the swapped domain to the remainder of the molecule and which have different conformations in the monomer and dimer (Feinberg et al. 2000).

Although the basic C-type lectin fold is preserved, a loop extends away from the core of the domain to form a domain-swapped dimer in the crystal (Fig. 15.1c, d). Combined studies on ligand binding, site-directed mutagenesis, and NMR indicated that CRD-4 of the mannose receptor has specificity for mannose, GlcNAc, and fucose like the C-type CRDs of rat serum and liver mannose-binding proteins (MBPs) (Drickamer and Taylor 1993; Weis et al. 1998; Weis et al. 1991; Wu et al. 1996). Some aspects of binding of sugar and Ca^{2+} by CRD-4 are similar to those of the mannose binding protein (MBP) CRDs, but others are different (Wu et al. 1996) (Fig. 15.1a). The structure likely represents an endosomal form of the domain formed when Ca^{2+} is lost from the auxiliary calcium site3 (Feinberg et al. 2000). Transfection of the mannose receptor cDNA into Cos-I cells is necessary for receptor-mediated endocytosis of mannose-rich glycoconjugate as well as phagocytosis of yeasts. Deletion of the cytoplasmic tail results in a mutant receptor that is able to bind but not ingest the ligated pathogens, suggesting that the signal for phagocytosis is contained in the cytoplasmic tail (Ezekowitz et al. 1990).

Size and shape parameters derived from sedimentation and diffusion coefficients suggest that the receptor is a monomeric, elongated and asymmetric molecule. Proteolysis experiments indicated the presence of close contacts between several pairs of domains and exposed linker regions separating CRDs 3 and 6 from their neighboring domains. Hydrodynamic coefficients predicted for modeled receptor conformations are consistent with an extended conformation with close contacts between three pairs of CRDs. The N-terminal cysteine-rich (CR) domain and the fibronectin type II repeat appear to increase the rigidity of the molecule. The rigid, extended conformation of the receptor places domains with different functions at distinct positions with respect to the membrane (Napper et al. 2001). The crystal structures of CR alone and complexed with 4-sulfated-*N*-acetylgalactosamine showed that CR folds into threefold symmetric ß-trefoil shape resembling fibroblast growth factor. The sulfate portion of 4-sulfated-*N*-acetylgalactosamine and an unidentified ligand found in the native crystals binds in another pocket in the third lobe (Liu et al. 2000).

15.3.2.2 Ca^{2+} and Monosaccharide Binding at CRD-4 in MR

CRD-4 requires two Ca^{2+} for sugar binding, like the CRD of rat MBP-A. The binding site for one Ca^{2+} which ligates to the bound sugar in MBP-A, is conserved in CRD-4 where as the auxiliary Ca^{2+} binding site is not. Mutation of the four residues at positions in CRD-4 equivalent to the auxiliary Ca^{2+} binding site in MBP-A indicated that only one, Asn^{728}, is involved in the ligation of Ca^{2+}. Sequence comparisons with other C-type CRDs suggest that the proposed binding site for the auxiliary Ca^{2+} in CRD-4 of the MR is unique. Proton NMR spectra of methyl glycosides of mannose, GlcNAc, and fucose in the presence of CRD-4 and site-directed mutagenesis indicated that a stacking interaction with Tyr^{729} is also involved in binding of sugars to CRD-4. C-5 and C-6 of mannose interact with Tyr^{729}, whereas C-2 of GlcNAc is closest to this residue, indicating that these two sugars bind to CRD-4 in opposite orientations. Sequence comparisons with other mannose/GlcNAc-specific C-type CRDs suggest that use of a stacking interaction in the binding of these sugars is probably unique to CRD-4 of the MR (Mullin et al. 1997).

15.3.3 Cell and Tissue Distribution

Immune Cells: MR expression is detectable on monocytes cultured for 3 days (macrophages). In the thymus and lymph node, MR-positive branched cells (macrophages and DCs) were detected in connective tissue, thymus cortex (not medulla), and in the T cell area (not the B cell area) of lymph nodes (Noorman et al. 1997). Mononuclear phagocytes comprise the majority of interstitial cells in the mouse dermis. These cells express the mouse macrophage galactose-/N-acetylgalactosamine-specific-lectin (mMGL)/CD301 as well as the MR/CD206 (Dupasquier et al. 2006; van Vliet et al. 2005). MR positive APCs are present in several peripheral organs: skin, liver, cardiac and skeletal muscle and tongue. MR positive cells in salivary gland, thyroid and pancreas co-express MHC class II and the myeloid markers, macrosialin and sialoadhesin, but not the DC markers CD11c or DEC-205. MR and MHC class II co-localized, implying that antigen capture may be the primary role of MR in these cells. The tissue and sub-cellular distribution of MR suggest that it is appropriately located to serve as a high efficiency antigen uptake receptor of APC (Linehan 2005). The macrophage MR is significantly upregulated in nasal polyposis (NP) compared to patients with chronic rhinosinusitis (CS) without NP or turbinate tissue of controls (Claeys et al. 2004). The MR is up-regulated on dexamethasone-treated (immunosuppressed) macrophages, and down-regulated on LPS-activated macrophages (Dupasquier et al. 2006). Embryonic and adult mouse tissues express MR from early embryogenesis till adulthood. The MR is first observed on embryonic day 9 on cells that line blood island vessel walls in the yolk sac. Thus, the MR, expressed on tissue macrophages, is also expressed on subsets of vascular and lymphatic endothelial cells. The MR may be a marker of the so-called reticuloendothelial system (Takahashi et al. 1998).

Non-Immune Organs: Studies demonstrate that Pneumocystis (Pc) -mediated IL-8 release by human alveolar macrophages (AM) requires the coexpression of MR and toll likr receptor 2 (TLR2) and supports the concept that combinatorial interactions of macrophage innate receptors provide specificity of host defense cell responses to infectious challenge (Tachado et al. 2007). A mannose-specific receptor has also been found on retinal pigment epithelial cells (RPE). Human RPE expresses a MR on its apical surface (as does the rat RPE) and that this receptor is similar to the human macrophage MR. It seems that a MR is involved in the phagocytosis of rod outer segments by rat and human retinal pigment epithelium. Glycoproteins having oligosaccharides with terminal sequence SO_4-4-GalNAcβ1,4GlcNAcβ1,4Man-(S4GGnM) are rapidly removed from circulation by a S4GGnM-specific receptor (S4GGnM-R) expressed at the surface of hepatic endothelial cells. The S4GGnM-R from rat liver is closely related to the macrophage MR from rat lung both antigenically and structurally (Fiete et al. 1997). Squamous cell cancers of the head and neck and breast cancer specimens containing peritumoral vessels express MR, and the intratumoral lymph vessels often expressed them in both tumor types (Irjala et al. 2003).

Astrocytes and Microglia: Expression of MR has been demonstrated in brain, both in its soluble and membrane forms. Astrocytes and microglia, two types of glial cells that can be turned into immune-competent cells, are the main sites of expression in vivo and in vitro. Rodent Schwann cells (SC) in primary cultures take up MR ligand mannosyl/man-BSA-FITC in a highly specific manner which suggest that SC express MR in a prospectively functional state and suggest an antigen-presenting function of SC, compatible with a role in infectious/inflammatory states of the peripheral nervous system (Baetas-da-Cruz et al. 2009). MR mediates in vitro pinocytosis by astrocytes and microglia and phagocytosis by microglia. Regional expression of MR in glial and neuronal cells strongly suggests that this receptor plays an important role in homeostasis during brain development and/or neuronal function (Régnier-Vigouroux 2003). Being a differentiation marker and a relevant glycoprotein for the phagocytic and endocytic function of macrophages, the presence of MR in microglia suggests that MR could participate in multiple physiologic and pathologic conditions in the CNS, including inflammation, ischaemia, and neurodegenerative diseases.

Studies demonstrate that the expression and function of MR are inversely regulated by anti-and pro-inflammatory compounds. As observed for macrophages, IFNγ decreases, where as IL-4 increases MR expression. Consequently, the rates of pinocytosis were strongly up-regulated by IL-4 and inhibited by IFNγ. Down-regulation of the MR by IFN-γ is concomitant with the induction of the invariant chain, which is also induced by GM-CSF + IL-4. MR-expressing astrocytes may act as scavenger not only in CNS development but also in defense, against soluble and particulate mannosylated pathogens, presenting fragments there of at strategic locations in the CNS (Burudi et al. 1999; Zimmer et al. 2003).

Role in HIV-1 Neuropathogenesis: Both microglia and astrocytes are susceptible to HIV-1 infection. The HIV-1 in the CNS causes a variety of neurobehavioral and neuropathological disorders. Microglial MR is down-regulated by LPS and up-regulated by dexamethasone, as described for peripheral macrophages (Marzolo et al. 1999). Unlike microglia that express and utilize CD4 and chemokine co-receptors CCR5 and CCR3 for HIV-1 infection, astrocytes fail to express CD4. Results demonstrate the direct involvement of human MR in HIV-1 infection of astrocytes and suggest that HIV-1 interaction with MR plays an important role in HIV-1 neuropathogenesis. It gives a notion that humanMR is capable of eliciting intracellular signaling upon ligand binding (Liu et al. 2004; Lopez-Herrera et al. 2005).

15.3.4 Ligands

Multiple exogenous ligands (e.g. viruses, bacteria and fungi) as well as endogenous ligands (e.g. lutropin, myeloperoxidase and thyroglobulin) have been identified for the mannose-binding region of MR. Two distinct lectin activities have been described: (1) the cysteine-rich (CR) domain recognises sulphated carbohydrates while (2) the CTLDs mediate binding to mannose, fucose or N-acetylglucosamine. FNII domains are known to be important for collagen binding and this has been studied in the context of two members of the MR family, Endo180 and the phospholipase A2 receptor. Whilst no exogenous ligands have been reported for CR domain, the cysteine-rich R-type domain of the MR binds sulfated glycans and also N-glycans on pituitary glyco-hormones (e.g. lutropin) containing 4-SO4-GalNAcβ1–4GlcNAcβ1–2Manα1-R. Other ligands for the MR include chondroitin-4-sulfate proteoglycans on leukocytes that also contain 4-SO4-GalNAc β1-R residues and perhaps sulfated glycans, such as those containing 3-O-sulfated galactose, blood group 3-O-sulfated Le^x, and 3-O-sulfated Le^a. The cysteine-rich domain in MR also mediates recognition of selected glycoforms of membrane receptors expressed by metallophili macrophages in the spleen. The cysteine-rich R-type domain in the MR binds with relatively low affinity to sulfated monosaccharides (K_D in the range of 10^{-3}–10^{-4} M), but oligomeric forms of the protein probably display much higher avidity for glycoproteins with multiple sulfated glycans. Although the cysteine-rich R-type domain binds sulfated glycans, the C-type lectin domains bind unsulfated glycans and may also play a role in glycoprotein homeostasis and clearance. The full-length soluble form of MR was able to bind simultaneously polysaccharide via the carbohydrate recognition domains and sulfated oligosaccharide via the cysteine-rich domain (Zamze et al. 2002).

The MR can also bind collagen and the fibronectin type II domain mediates this activity. Neither of the two types of sugar-binding domain in the receptor is involved in collagen binding. Fibroblasts expressing the mannose receptor adhere to type I, type III and type IV collagens, but not to type V collagen, and the adherence is inhibited by isolated MR fibronectin type II domain. The fibronectin type II domain shows the same specificity for collagen as the whole receptor, binding to type I, type III and type IV collagens. MR is able to bind and internalise collagen in a carbohydrate-independent manner and that MR deficient macrophages have a marked defect in collagen IV and gelatin internalisation. These results have major implications at the molecular level as there are now three distinct ligand-binding sites described for MR (Martinez-Pomares et al.

2006). These results suggest additional roles for this multifunctional receptor in mediating collagen clearance or cell-matrix adhesion.

The *Mycobacterium tuberculosis* (M.tb) envelope is highly mannosylated with phosphatidyl-myo-inositol mannosides (PIMs), lipomannan, and mannose-capped lipoarabinomannan (ManLAM). It was demonstrated that recognition of M.tb PIMs by host cell C-type lectins is dependent on both the nature of the terminal carbohydrates and degree of acylation. Subtle structural differences among the PIMs impact host cell recognition and response and are predicted to influence the intracellular fate of M.tb. For example, higher-order PIMs preferentially associated with MR. In contrast, the lower-order PIM(2)f associated poorly with MDMs and did not bind to COS-1-MR. In contrast with the MR, the PIM(2)f and lipomannan were recognized by DC-SIGN comparable to higher-order PIMs and ManLAM, and the association was independent of their degree of acylation (Torrelles et al. 2006). Glyco-peptidolipids (GPLs) can function to delay phagosome-lysosome fusion and GPLs, like ManLAM, work through the MR to mediate this activity (Sweet et al. 2010; Torrelles et al. 2006).

15.3.5 Functions of Mannose Receptor

15.3.5.1 Role in Immunity

Genetic deletion in mice has highlighted the role of MR in the clearance of endogenous glycoproteins. Therefore MR has been implicated in both homeostatic processes and pathogen recognition. However, the function of MR in host defence is not yet clearly understood as MR-deficient animals do not display enhanced susceptibility to pathogens bearing MR ligands. Moreover, the MR mediates uptake of soluble but not of cell-associated antigen for cross-presentation. In vivo, MR deficiency impaired endocytosis of soluble OVA by DC and concomitant OT-I cell activation. Findings demonstrate that DC use the MR for endocytosis of a particular Ag type intended for cross-presentation (Burgdorf et al. 2006). This scenario is even more complex when considering the role of MR in innate immune activation as, even though no intracellular signaling motif has been identified at its cytoplasmic tail, MR has been shown to be essential for cytokine production, both pro-inflammatory and anti-inflammatory. Furthermore, MR might interact with other canonical pattern recognition receptors in order to mediate intracellular signaling. Gazi and Martinez-Pomares (2009) have summarised recent observations relating to MR function in immune responses and focused on its participation in phagocytosis, antigen processing and presentation, cell migration and intracellular signaling. The MR also functions in adaptive immunity through its ability to deliver antigens to MHC class II compartments and through its cleavage and release as a soluble protein into blood. It is speculated that the membrane-bound MR may bind antigens through its C-type lectin domains and then, following proteolytic cleavage, the soluble MR bound to its cargo may move to germinal centers where it may bind via its R-type domain to macrophages and dendritic cells expressing ligands such as sialoadhesion or CD45 that may contain sulfated glycans.

15.3.5.2 Phagocyte-Bacteria Interactions

The macrophage MR recognizes carbohydrates on the cell walls of infectious organisms. After ligation of mannose-rich glycoconjugates or pathogens, the macrophage MR mediates endocytosis and phagocytosis of the bound ligands by macrophages. The receptor-mediated uptake may either arm the macrophage to contribute to oxidant-mediated tissue damage or may function to clear extracellular myeloperoxidase during the resolution phase of the inflammatory process. Where as human keratinocytes appear to kill *Candida* in presence of MR (Szolnoky et al. 2001), Lee et al. (2003) suggested that MR is not required for the normal host defense during disseminated candidiasis or for the phagocytosis of *C. albicans*.(Lee et al. 2003; Le Cabec et al. 2005). One proposed function for MR found on macrophages and hepatic endothelial cells is to enhance the uptake and process glycoprotein antigens for presentation by MHC class II molecules. Incubation of RNase A (non-glycosylated) or RNAse B (mannosylated) with the transfected cells resulted in identical stimulation of ribonuclease-specific T cells, indicating that endocytosis of the glycosylated protein by the MR does not enhance presentation of this antigen (Napper and Taylor 2004).

A nonredundant role of MR has been demonstrated in the development of $CD4^+$ T-cell responses to *Cryptococcal* mannoproteins (MP) and protection from *C. neoformans* (Dan et al. 2008). *Trichuris muris* is a natural mouse model of the human gastrointestinal nematode parasite *Trichuris trichiura*. Macrophages accumulate in the large intestine of mice during infection and known to express MR. Infection of MR knockout mice with *T. muris* reveals that this receptor is not necessary for the expulsion of the parasite because MR knockout mice expel parasites with the same kinetics as wild-type animals and have similar cytokine responses in the mesenteric lymph nodes. This work suggests that, despite binding components of *T. muris* secretory products, the MR is not critically involved in the generation of the immune response to this parasite (deSchoolmeester et al. 2009).

MR, specifically, recognizes mannose residues on the surface of *Leishmania* parasites. Infection of mouse peritoneal macrophages with *Leishmania donovani* results in decrease in MR activity (Basu et al. 1991). Akilov et al. (2007) demonstrated that host MR is not essential for blocking IFN-γ/LPS-induced IL-12 production and MAPK

activation by *Leishmania*. Thus, the MR is not essential for host defense against *Leishmania* infection or regulation of IL-12 production (Akilov et al. 2007). It was proposed that MR is a binding receptor, which requires a partner to trigger phagocytosis in some specialized cells such as macrophages.

Although alveolar macrophage MR activity is down-regulated in individuals infected with HIV, and that functional MR is shed from the macrophage cell surface, *P. carinii* enhances the formation of soluble MR by macrophages in vitro. Soluble MR was detected in cell-free alveolar fluid from humans infected with HIV and/or *P. carinii* (Camner et al. 2002; Swain et al. 2003). Macrophages recognize and subsequently kill *Klebsiella* expressing Man-α 2/3-Man or Rha-α 2/3-Rha sequences in their capsular polysaccharides by (a) MMRs, and (b) opsonization by the lung surfactant protein A (SP-A), which binds to the capsular polysaccharides of *Klebsiella* and to SP-A receptors on the macrophages (Keisari et al. 1997). MR contributes equally to TLR2 in proinflammatory cytokine production by human monocytes in response to *P. aeruginosa* infection. MR follows the same kinetics and colocalizes with TLR2 in the endosome during in vivo infection of human macrophages with *P. aeruginosa* (Xaplanteri et al. 2009).

15.3.5.3 Macrophage MR in HIV Binding

A role for MMR in the binding and transmission of HIV-1 by macrophages has been suggested (Nguyen and Hildreth 2003). Nonetheless, alveolar macrophage MR activity is down-regulated in individuals infected with HIV, and that functional MR is shed from the macrophage cell surface (Camner et al. 2002). As expected, mannan bound to the CRDs of MR dimers mostly in a calcium-dependent fashion. Surprisingly, gp120-mediated binding of HIV to dimers on MR-transfected Rat-6 cells and macrophages was calcium-independent, and only partially blocked by mannan and partially inhibited by *N*-acetylgalactosamine 4-sulfate. Thus gp120-mediated HIV binding occurs via calcium-dependent, calcium-independent CRDs and CR domain at the C terminus of MR dimers, presenting a much broader target for potential inhibitors of gp120-MR binding (Lai et al. 2009).

15.3.5.4 MR as Endocytic Receptor During Entry of Other Viruses

Influenza viruses showed marked differences in their ability to infect murine macrophages, including resident alveolar and peritoneal macrophages. The hierarchy in infectivity of the viruses resembles their reactivity with mannose-binding lectins. The possible involvement of MR in infection of macrophages by influenza virus has been suggested (Reading et al. 2000). Macrophages (MΦ) and mononuclear phagocytes are major targets of infection by dengue virus (DV), The MMR binds to all four serotypes of DV and specifically to the envelope glycoprotein. Pre-treatment of human monocytes or MΦ with type 2 cytokines (IL-4 or IL-13) enhances their susceptibility to productive DV infection. These findings indicate a new functional role for the MR in DV infection (Miller et al. 2008).

When human MR cDNA is transfected into Cos cells, these usually non-phagocytic cells express cell surface MR and bind and ingest MR ligands such as zymosan, yeast, and *Pneumocystis carinii* (Kruskal et al. 1992). Interaction of MR with Fc receptors is critical for the development of crescentic glomerulonephritis (CGN) in mice. In mouse model of CGN, MR-deficient ($MR^{-/-}$) mice were protected from CGN despite generating humoral and T cell responses similar to those of WT mice, but they demonstrated diminished macrophage and MC Fc receptor-mediated functions, including phagocytosis and Fc-mediated oxygen burst activity. Results demonstrate that MR augments Fc-mediated function and promotes MC survival (Chavele et al. 2010)

15.3.5.5 MR Regulates Cell Migration

MR has been shown to bind and internalize carbohydrate and collagen ligands and to have a role in myoblast motility and muscle growth. Since the related Endo180 (CD280) receptor has also been shown to have a promigratory role, it was likely that MR may be involved in regulating macrophage migration and/or chemotaxis. Contrary to expectation, bone marrow-derived macrophages (BMM) from MR-deficient mice showed an increase in random cell migration and no impairment in chemotactic response to a gradient of CSF-1 (Sturge et al. 2007).

Marttila-Ichihara et al. (2008) showed that migration of lymphocytes from the skin into the draining lymph nodes through the afferent lymphatics is reduced in MR-deficient mice, while the structure of lymphatic vasculature remains normal in these animals. Moreover, in a tumor model the primary tumors grow significantly bigger in $MR^{-/-}$ mice than in the wild-type (WT) controls, whereas the regional lymph node metastases are markedly smaller. Adhesion of both normal lymphocytes and tumor cells to lymphatic vessels is significantly decreased in MR-deficient mice. Thus, MR on lymphatic endothelial cells is involved in leukocyte trafficking and contributes to the metastatic behavior of cancer cells. Blocking of MR may provide a new approach to controlling inflammation and cancer metastasis by targeting the lymphatic vasculature.

15.3.6 Mouse Mannose Receptor

In mouse macrophages, the newly synthesized receptor has a Mr of 157 kDa that rapidly matures to a protein with a Mr of

172 kDa. Both forms of receptors are tightly associated with cell membranes. The receptor is found in a number of mouse macrophage cell types but not found in mouse fibroblasts. The transcript encoding this lectin is present in a number of highly endothelialized sites as well as in chondrocytes in cartilaginous regions of the embryo. Receptor-ligand binding was Ca^{2+} and pH dependent. D-mannose and L-fucose partially inhibit receptor binding to the ligand D-mannose-BSA (Blum et al. 1991; Wu et al. 1996). Cysteine-rich domain of the murine MR binds to macrophages from splenic marginal zone and lymph node subcapsular sinus and to germinal centers: Ligands for cysteine-rich (CR) domain of the murine MR have been detected in murine tissues. In naive mice, the CR-Fc, a Fc chimeric protein bound to sialoadhesin$^+$, F_4/80low/$^-$, macrosialin$^+$ macrophages (Mφ) in spleen marginal zone (metallophilic Mφ) and lymph node subcapsular sinus. These results confirmed the identification of the CR region of the mannose receptor as a lectin (Martinez-Pomares et al. 1996, 1999). MR is localized with CR ligands in Lyve-1$^+$ cells lining venous sinuses. These cells form a physical barrier for blood cells as they need to migrate through sinuses in order to exit the splenic parenchyma and, in this way, contribute to the unique filtration function of this organ. Furthermore, unlike murine spleen, CD68$^+$ red pulp MΦ lack MR expression. However, results of Martinez-Pomares et al. (2005) suggest an unexpected contribution of MR to splenic function through the recognition of sulphated ligands that could influence the filtering capability of this organ (Martinez-Pomares et al. 2005).

Murine MR is a member of type C lectins family with a cysteine-rich domain, a fibronectin type 2 domain, eight type C lectin domains, a transmembrane domain, and a short cytoplasmic carboxyl terminus. Genomic analysis suggests it to be a conserved protein with human sequence homology with the murine form. It expresses as a large transcript in a number of human and murine tissues and tumor cells, and an alternatively spliced smaller transcript with a divergent 5′ sequence, expressed specifically in the human fetal liver. The gene encoding this lectin is interrupted by a large number of introns; the intron structure was similar to macrophage MR (Wu et al. 1996). The 854 bp of rat MR promoter sequence revealed one Sp1 site, three PU.1 sites, and a potential TATA box (TTTAAA)-33 bp 5′ of the transcriptional start site. The promoter was most active in the mature macrophage cell line NR8383 although the promoter also showed activity in the monocytic cell line RAW. Mutation in TTTAAA sequence to TTGGAA, resulting in decrease in activity, suggested that the promoter contains a functional TATA box. Though, the transcription factors Sp1, PU.1, and USF bound to the MR promoter, but only PU.1 and USF contributed to activation. Comparison of the rat, mouse, and human promoter sequences demonstrated that some binding sites are not conserved. Transcription of the rat MR is regulated by binding of PU.1 and a ubiquitous factor at an adjacent site, similar to other myeloid promoters (Egan et al. 1999).

Paracrine Control of Macrophage MR: The macrophage MR is progressively up-regulated as bone marrow precursor cells mature into macrophages and thus serves as a marker of differentiation. Prostaglandins E (PGE) are known inhibitors of monocyte and macrophage precursor proliferation, an effect often associated with cellular maturation. Prostaglandins accelerate macrophage MR expression and hence the differentiation of macrophage precursor cells, suggesting that a paracrine mechanism may exist to regulate macrophage MR expression and function (Schreiber et al. 1990). IL-4 up-regulates total cell-associated MR (cMR), correlating with enhanced surface expression. The influence of IL-10 showed an effect similar to IL-4. In both cases, enhanced cMR levels translated into increased production of the soluble form of the receptor (sMR). These data support a role for MR in T helper cell type 2 cytokine-driven, immune responses and suggest a non-macrophage contribution to sMR production in vivo (Martinez-Pomares et al. 2003). The induction of cyclooxygenase-2 (COX-2) and the production of PGE_2 in response to pathogen-associated decorated with mannose moieties were studied in human monocytes and monocyte-derived macrophages (MDM). It showed that mannan is a strong inducer of COX-2 expression in human MDM, most likely by acting through the MR route. Because COX-2 products can be both, proinflammatory and immunomodulatory, these results disclose a signaling route triggered by mannose-decorated pathogen-associated molecular patterns, which can be involved in both the response to pathogens and the maintenance of homeostasis (Fernández et al 2005).

PPARγ Promotes MR Gene Expression in Murine Macrophages: The Macrophage MR expression is induced in mouse peritoneal macrophages following exposure to PPARγ ligands or to IL-13 via a PPARγ signaling pathway. This novel signaling pathway controlling the macrophage MR surface expression involves the endogenous PPARγ ligand produced by phospholipase A2 activation that may be an important regulator of macrophage MR expression by IL-13 (Coste et al. 2003). Ligand activation of the PPARγ in macrophages promotes uptake, killing of *C. albicans*, and reactive oxygen intermediates production triggered by the yeasts through macrophage MR over-expression.

15.3.7 Interactions of MR with Branched Carbohydrates

15.3.7.1 Inhibitory Potency of Monosaccharides of MR

The relative inhibitory potency of monosaccharides of human placental MR was found as: L-Fuc > D-Man > D-Glc > D-GlcNAc > Man-6-P >> D-Gal >> L-Rha >> GalNAc. The inhibitory potency of mannose, however, increased by two orders of magnitude when linear oligomer was used. Oligomers containing α1-3- and α1-6-linked mannose residues were more inhibitory than oligomers, which contained α1-2- and α1-4-linked mannoses. Linear or branched oligomannosides larger than three units did not have a significant influence on their inhibitory potency; rather, potency was found to decrease in comparison with oligomannosides with three units. Compared to linear oligomers, inhibition of binding was maximum using branched mannose oligosaccharides, α-D-Man-bovine serum albumin conjugates, or mannan. A model is discussed in which branched ligand is bound to spatially distinct sites on the human MR (Kery et al. 1992).

15.3.7.2 Di-Mannoside Clusters Target MR Whereas Lewis Clusters Target DC-SIGN

Dimannoside clusters, recognized by the MR with an affinity constant close to 10^6 liter mol^{-1} have very low affinity for DC-SIGN (less than 10^4 liter mol^{-1}). Conversely, Lewis clusters had higher affinity toward DC-SIGN than toward the MR. Dimannoside clusters are efficiently taken up by human DCs as well as by rat fibroblasts expressing the MR but not by HeLa cells or rat fibroblasts expressing DC-SIGN; DC-SIGN-expressing cells take up Lewis clusters. This suggested that ligands containing di-mannoside clusters can specifically target the MR, whereas ligands containing Lewis clusters will target DC-SIGN (Frison et al. 2003; Guo et al., 2004).

The MR is involved in prevention of LPS-induced acute lung injury after administration of mannose in mice. Mannose also prevented the inflammatory cell accumulation, and inhibited production of cytokines. Furthermore, mannose receptor was up-regulated after mannose administration. Studies reveal involvement of MR and impaired NF-kB activation in the mannose prevention of acute lung injury, and implicate MR as a potential therapeutic target during acute lung injury (Xu et al. 2010).

15.3.7.3 Tissue-Type Plasminogen Activator-Receptor Interaction

Tissue-type plasminogen activator (t-PA) in blood is cleared by the liver partially through a mannose-specific uptake system. The MR (175 kDa) from bovine AM is specifically bound to t-PA and showed saturable binding in presence of Ca^{2+}. Mannose-albumin was an effective inhibitor, whereas galactose-albumin did not have a significant effect. Among monosaccharides, D-mannose and L-fucose were the most potent inhibitors whereas D-galactose and N-acetyl-D-galactosamine were ineffective. t-PA, deglycosylated by endoglycosidase H, did not interact with the receptor. The MR binding to t-PA, probably occurs through its high mannose-type oligosaccharide (Otter et al. 1991). The interaction of ^{125}I-t-PA with isolated rat parenchymal and endothelial liver cells was studied by Otter et al. (1992). It was concluded that (1) the mannose receptor and LDL receptor-related protein (LRP) appear to be the sole major receptors responsible for tPA clearance and (2) therapeutic levels of tPA can be maintained for a prolonged time span by co-administration of the receptor antagonists (Biessen et al. 1997; Otter et al. 1992).

15.3.8 Mannose Receptor-Targeted Drugs and Vaccines

Targeting antigens to endocytic receptors on professional antigen-presenting cells (APCs) represents an attractive strategy to enhance the efficacy of vaccines. Such APC-targeted vaccines have an exceptional ability to guide exogenous protein antigens into vesicles that efficiently process the antigen for MHC class I and class II presentation. The MR and related C-type lectin receptors are particularly designed to sample antigens, much like pattern recognition receptors, to integrate the innate with adaptive immune responses. In fact, a variety of approaches involving delivery of antigens to the MR have demonstrated effective induction of potent cellular and humoral immune responses. Yet, although several lines of evidence in diverse experimental systems attest to the efficacy of targeted vaccine strategies, it is becoming increasingly clear that additional signals, such as those afforded by adjuvants, may be critical to elicit sustained immunity. Therefore, MR-targeted vaccines are likely to be most efficacious in vivo when combined with agents that elicit complementary activation signals. A better understanding of the mechanism associated with the induction of immune responses as a result of targeting antigens to the MR, will be important in exploiting MR-targeted vaccines not only for mounting immune defenses against cancer and infectious disease, but also for specific induction of tolerance in the treatment of autoimmune disease (Gupta et al. 2009; Irache et al. 2008; Keler et al. 2004).

15.4 Phospholipase A2-Receptors

The PLA2 receptor was discovered as a receptor for phospholipase A2 neurotoxins in snake venoms and was referred to as the M-type PLA2 receptor to distinguish it from the

neuronal or N-type PLA2 receptor. It can occur as both a long form that is a type I trans-membrane glycoprotein with a domain structure like the MR and a shorter form that is secreted.

15.4.1 The Muscle (M)-Type sPLA2 Receptors

15.4.1.1 Molecular Cloning and Chromosomal Localization

Cloning of 180 kDa PLA2 receptor from rabbit skeletal muscle revealed that it is a membrane protein with a N-terminal cysteine-rich domain, a fibronectin type II domain, eight repeats of a carbohydrate recognition domain, a unique transmembrane domain, and a intracellular C-terminal domain. The 1458-residue PLA2 receptor, expressed in transfected cells, binds venom PLA2 (vPLA2) with very high affinity (K_D = 10–20 pM). It also tightly binds the two structural types of sPLA2s, i.e. pancreatic PLA2 and synovial PLA2 (K_D = 1–10 nM) (Lambeau and Lazdunski 1999). Cloning of sPLA2 receptors from human kidney revealed two transcripts. One encodes for a transmembrane form of the sPLA2 receptor and the other one is an alternatively processed transcript, caused by polyadenylation occurring at a site within an intron in the C terminus part of the transcriptional unit. This transcript encodes for a shortened secreted soluble sPLA2 receptor lacking the coding region for the transmembrane segment. Soluble and membrane-bound human sPLA2 receptors both bind sPLA2 with high affinities, although the binding properties of the human receptors are different from those obtained with the rabbit membrane-bound sPLA2 receptor. The 180-kDa human sPLA2 receptor gene has been mapped in the q23–q24 bands of chromosome 2. Cloned 180-kDa sPLA2 receptors have the same structural organization as the macrophage mannose receptor, a membrane protein involved in the endocytosis of glycoproteins, and the phagocytosis of microorganisms bearing mannose residues on their surface (Ancian et al. 1995b).

The M-type 180 kDa sPLA2 receptor has been cloned in rabbit, bovine, and humans (Lambeau and Lazdunski 1999; Ancian et al. 1995a). The cloned receptors are homologous to the macrophage MR (Ezekowitz et al. 1990), as well as to DEC-205, a protein involved in the presentation of antigens. Interestingly, all of these proteins are predicted to share the same structural organization, *i.e.* a large extracellular region composed of an N-terminal cysteine-rich domain, a fibronectin like type II domain, eight (Lambeau et al. 1994; Taylor et al. 1992) or ten repeats of a carbohydrate recognition domain (CRD), followed by a unique transmembrane domain and a short intracellular C-terminal domain (Jiang et al. 1995). This latter domain contains a consensus sequence for the internalization of ligand-receptor complexes and is thought to confer to these receptors their endocytic properties (Ancian et al. 1995a; Kruskal et al. 1992).

15.4.1.2 Structural Elements

Specific membrane receptors for sPLA2s were initially identified with snake venom sPLA2s called OS1 and OS2 (Lambeau et al. 1991). One of these sPLA2 receptors (muscle M-type, 180 kDa) has a very high affinity for OS1 and OS2 and a high affinity for pancreatic and inflammatory-type mammalian sPLA2s, which might be the natural endogenous ligands for PLA2 receptors. The binding of pancreatic sPLA2 mutants to the M-type receptor showed that residues within or close to the Ca^{2+}-binding loop of pancreatic sPLA2 are crucially involved in the binding step. Although the presence of Ca^{2+} is essential for the enzymatic activity, it is not required for binding to the receptor. These residues include Gly-30 and Asp-49, which are conserved in all sPLA2s. Leu-31 is also essential for binding of pancreatic sPLA2 to its receptor. Conversion of pancreatic prophospholipase to phospholipase is essential for the acquisition of binding properties to the M-type receptor (Lambeau et al. 1995).

Binding Domain for sPLA2: The rabbit M-type receptor for sPLA2s has a large extracellular domain of 1,394 amino acids, composed of an N-terminal cysteine-rich domain, a fibronectin-like type II domain, and eight carbohydrate recognition domains (CRDs). It is thought to mediate some of the physiological effects of mammalian sPLA2s, including vascular smooth muscle contraction and cell proliferation, and is able to internalize sPLA2s. Site-directed mutagenesis studies showed that a snake venom sPLA2 (OS1), binds to the receptor via its CRDs and that deletion of CRD 5 completely abolishes the binding of sPLA2s. Moreover, a receptor lacking all CRDs but CRD 5 was still able to bind OS1 although with a lower affinity. Deletion of CRDs 4 and 6, surrounding the CRD 5, slightly reduced the affinity for OS1, thus suggesting that these CRDs are also involved in the binding of OS1. The M-type sPLA2 receptor and the macrophage MR are predicted to share the same tertiary structure. The p-Aminophenyl-α-D-mannopyranoside bovine serum albumin, a known ligand of the macrophage MR, binds to the M-type sPLA2 receptor essentially via CRDs 3–6 (Nicolas et al. 1995).

Extended and Bent Conformations of MR Family: M-type receptor for sPLA2s is a membrane protein with a N-terminal cystein-rich domain, a fibronectin-like type II domain, eight repeats of a carbohydrate recognition domain, a unique transmembrane and an intracellular C-terminal. When expressed in transfected cells, the rabbit M-type receptor binds both the inflammatory-type and the

pancreatic-type msPLA2′s with fairly high affinities (K_D ~ 1–10 nM) suggesting that the sPLA2 receptors for vPLA2′s are the normal targets of endogenous msPLA2′s involved in a variety of diseases. Residues within or close to the Ca^{2+} binding loop of pancreatic-type PLA2 are crucially involved in the binding step although the presence of Ca^{2+} which is essential for the enzymatic activity is not required for binding to the receptor. The domain in charge of sPLA2 binding in the M-type receptor has been identified (Llorca 2008). Llorca (2008) reviewed three dimensional structures of the receptors of MR family and their functional implications. Recent research has revealed that several members of this family can exist in at least two configurations: an extended conformation with the N-terminal cysteine rich domain pointing outwards from the cell membrane and a bent conformation where the N-terminal domains fold back to interact with C-type lectin-like domains at the middle of the structure. Conformational transitions between these two states seem to regulate the interaction of these receptors with ligands and their oligomerization.

15.4.1.3 Phospholipases A2 as Ligand of sPLA2 Receptors

Phospholipases A2 (PLA2s) form a large family of structurally related enzymes that catalyse the hydrolysis of the sn-2 acyl bond of glycerophospholipids to produce free fatty acids and lysophospholipids. Venom vPLA2s show a variety of toxic effects in animals including neurotoxicity, myotoxicity, hypotensive, anticoagulant, and proinflammatory effects. Snake venoms are known for decades to contain a tremendous molecular diversity of PLA2s, which can exert a myriad of toxic and pharmacological effects. Because PLA2 products are important for cell signaling and the biosynthesis of biologically active lipids, including eicosanoids and platelet-activating factor, PLA2s are generally considered as key enzymes that control the release of lipid mediator precursors. Secretory sPLA2s form a large family of structurally related enzymes which are widespread in nature. Mammalian cells also express a variety of sPLA2s with 12 distinct members identified so far, in addition to the various other intracellular PLA2s. On the other hand, mammalian secretory PLA2′s (sPLA2′s) are now implicated in many biological functions besides digestion, such as airway and vascular smooth muscle contraction, cell proliferation, and in a variety of diseases associated with inflammation such as rheumatoid arthritis, endotoxic shock, and respiratory distress syndrome. More speculative results suggest the involvement of one or more sPLA2s in promoting atherosclerosis and cancer.

High level PLA2 activity is found in serum and biological fluids during the acute-phase response (APR). Extracellular PLA2 in fluids of patients with inflammatory diseases such as sepsis, acute pancreatitis or rheumatoid arthritis is also associated with propagation of inflammation. PLA2 activity is involved in the release of both pro- and anti-inflammatory lipid mediators from phospholipids of cellular membranes or circulating lipoproteins. PLA2 may thus generate signals that influence immune responses. PLA2 treatment of differentiating monocytes in the presence of granulocyte/macrophage colony-stimulating factor and IL-4 yielded cells with phenotypical and functional characteristics of mature DC. The effects of PLA2 on DC maturation were mainly dependent on enzyme activity and correlated with the activation of NF-kB, AP-1 and NFAT. The transient increase in PLA2 activity seems to generate signals that promote transition of innate to adaptive immunity during the APR (Perrin-Cocon et al. 2004). Scanning of nucleic acid databases indicated that several sPLA2s are also present in invertebrate animals like *Drosophila melanogaster* as well as in plants.

The variety of effects, so mentioned, are apparently linked to the existence of a diversity of very high affinity receptors (K_D values as low as 1.5 pM) for these toxic PLA2s (Lambeau and Gelb 2008). Several different types of receptors (N and M) have been identified for vPLA2′s. The identification of a variety of membrane and soluble proteins that bind to sPLA2s suggests that the sPLA2 enzymes also function as high affinity ligands. Venom sPLA2s and group IB and IIA mammalian sPLA2s have been shown to bind to membrane and soluble mammalian proteins of the C-type lectin superfamily (M-type sPLA2 receptor and lung surfactant proteins), to pentraxin and reticulocalbin proteins, to factor Xa, and proteoglycans including glypican and decorin, a mammalian protein containing a leucine-rich repeat and to N-type receptors. Venom sPLA2s also associate with three distinct types of sPLA2 inhibitors purified from snake serum that belong to the C-type lectin superfamily, to the three-finger protein superfamily and to proteins containing leucine-rich repeats (Cupillard et al. 1999; Valentin and Lambeau 2000; Wu et al. 2006). On the basis of their different molecular properties and tissue distributions, each sPLA2 is likely to exert distinct functions by acting as an enzyme or ligand for specific soluble proteins or receptors, among which the M-type receptor is the best-characterized target. Results reaffirmed that the mouse M-type receptor is selective for only a subset of mouse sPLA2s from the group I/II/V/X structural collection. Binding of mouse sPLA2s to a recombinant soluble mouse M-type receptor leads in all cases to inhibition of enzymatic activity, and the extent of deglycosylation of the receptor decreases yet does not abolish sPLA2 binding. The two mouse sPLA2s (group IB and group IIA) have relatively high affinities for the mouse M-type receptor, but they can have much lower affinities for receptors from other animal species, indicating species specificity for sPLA2 binding to M-type receptors (Cupillard et al. 1999; Rouault et al. 2007).

A number of competitive inhibitors have been developed against the inflammatory-type human group IIA (hGIIA) sPLA2 with the aim of specifically blocking its catalytic activity and pathophysiological functions. The sPLA2:M-receptor interactions suggest that the therapeutic effects of sPLA2 inhibitors may be due not only to inhibition of enzymatic activity but also to modulation of binding of sPLA2 to the M-type receptor or other protein targets (Boilard et al. 2006).

15.4.1.4 Functions of M-type sPLA2 Receptor

The rabbit M-type sPLA2 receptor is a multifunctional protein, which seems to mediate the physiological effects of group I sPLA2, including smooth muscle contraction and cell proliferation. This receptor binds with high affinity to pancreatic group I and inflammatory group II sPLA2s as well as various sPLA2s from snake venoms. The rabbit M-type sPLA2 receptor is able to promote cell adhesion on type I and IV collagens most probably via its N-terminal fibronectin-like type II domain. It also shows that binding of sPLA2s to a recombinant soluble form of this receptor is associated with a noncompetitive inhibition of phospholipase A2 activity (Ancian et al. 1995a).

Endocytic Properties: Rabbit myocytes express M-type sPLAs receptor at high levels. Internalization of the receptor was shown to be clathrin-coated pit-mediated, rapid (ke = 0.1 min^{-1}), and ligand-independent. Analysis of the internalization efficiency of the mutants suggested that the NSYY motif encodes the major endocytic signal, with the distal tyrosine residue playing the key role (Zvaritch et al. 1996). The membrane-bound form can function as an endocytic receptor to internalize phospholipase A2 ligands.

Regulation of Proinflammatory Cytokines: In animal cells, secreted phospholipase A2s are important in the degradation of phospholipids and the release of arachidonic acid, which is the precursor for prostaglandins, leukotrienes, thromboxanes, and prostacyclins. The murine PLA2 receptor binds to sPLA2-X enzyme, where as the human PLA2 receptor may bind to several different phospholipase A2 isozymes. Although from early studies it was thought that the PLA2 receptor might be involved in clearance of phospholipase A2s, murine knockouts for PLA2 receptor showed unusual phenotype of resistance to endotoxic shock. This suggested that the PLA2 receptor might be important in regulating production of proinflammatory cytokines by soluble phospholipase A2s. Thus, the PLA2 receptor might function in signal transduction mediated by phospholipase A2 binding. Some of the C-type lectin domains in the PLA2 receptor function by binding phospholipase A2 ligands, rather than binding directly to glycan ligands. The fibronectin type II domain binds to collagen, which is a feature shared by this domain in other MR family members, except DEC-205.

Upregulation of PLA2 and M-Type Receptor in Rat ANTI-THY-1 Glomerulonephritis: Treatment of rat glomerular mesangial cell (GMC) cultures with pancreatic sPLA2-IB results in an enhanced expression of sPLA2-IIA and COX-2, possibly via binding to its specific M-type sPLA2 receptor. During glomerulonephritis (GN), shortly after induction of anti-Thy 1.1-GN, expression of sPLA2-IB was up-regulated in the kidney with strongest sPLA2-IB protein expression on infiltrated granulocytes and monocytes, and markedly upregulated expression of M-type receptor on resident glomerular cells. It suggested that both sPLA2-IB and the M-type sPLA2 receptors are involved in the autocrine and paracrine amplification of the inflammatory process in different resident and infiltrating cells (Beck et al. 2006).

15.4.2 Neuronal or N-Type PLA2 Receptor

The sPLA2 found in PLA2 honey bee venom is neurotoxic and binds to N-type PLA2 receptor with high affinity. Mutations in the interfacial binding surface, in the Ca^{2+}-binding loop and in the hydrophobic channel lead to a dramatic decrease in binding to N-type receptors, whereas mutations of surface residues localized in other parts of the sPLA2 structure do not significantly modify the binding properties. Neurotoxicity experiments show that mutants with low affinity for N-type receptors are devoid of neurotoxic properties, even though some of them retain high enzymatic activity. These results provide evidence for the surface region surrounding the hydrophobic channel of bee venom sPLA2 as the N-type receptor recognition domain (Nicolas et al. 1997). The bee venom PLA2 specifically binds to a single polypeptide with a mass of approximately 180 kDa. Moreover, mannose-BSA and the bee venom PLA2 bound to the same site on macrophages. Results suggested that bee venom PLA2 binding to macrophages is mediated through MR (Mukhopadhyay and Stahl 1995).

15.5 DEC-205 (CD205)

15.5.1 Characterization

DEC-205 (also known as Ly75 and designated as CD205) is an endocytic receptor with ten membrane-external, contiguous C-type lectin domains. DEC-205 is a 205-kDa protein of MR family that is expressed by dermal dendritic cells and, at a lower level, by epidermal Langerhans cells (Hawiger et al.

2001; Witmer-Pack et al. 1995). It is also expressed on some epithelial cells, on bone marrow stroma, and by endothelial cells. Cloning of DEC-205 gene revealed its relationship to MR and its unique characteristic of having ten tandem C-type lectin domains, rather than the eight found in other MR family members**.** Anti-DEC-205/CD205 antibodies are useful for identifying DCs in human splenic white pulp and its border region with the red pulp (Pack et al. 2008). Unlike mouse DEC-205, which is reported to have predominant expression on DC, human DEC-205 was detected at relatively high levels on myeloid blood DC and monocytes, at moderate levels on B lymphocytes and at low levels on NK cells, plasmacytoid blood DC and T lymphocytes (Kato et al. 2006). None of the ten CTLDs in DEC-205 have the conserved amino acids known to be important in carbohydrate binding, and thus far there is no evidence that these domains bind glycans. There is also no evidence that the cysteine-rich R-type domain at the amino terminus binds to glycan ligands.

The mouse DEC-205 cDNA predicts a molecular structure which has a marked similarity to the MMR. The full coding region of human DEC-205 cDNA from the Hodgkin's disease-derived L428 cell line predicted protein structure of 1722 amino acids consisting of a signal peptide, cysteine-rich domain, fibronectin type II domain, ten carbohydrate recognition-like domains, transmembrane domain, and a cytoplasmic tail. Human DEC-205 is 77% identical to the mouse protein with completely conserved cysteines. The DEC-205 gene was mapped to chromosome band 2q24. The 7.8 and 9.5 kb DEC-205 transcripts are present in myeloid, B lymphoid and Hodgkin's disease-derived cell lines. Immature blood DC contained a barely detectable amount of DEC-205 transcripts but these were markedly increased upon differentiation/activation (Kato et al. 1998). The multi-domain structure of mouse and human DEC-205 was completely conserved in hamster with the overall identity of approximately 80%. DEC-205 transcripts were detected in the thymus and bone marrow cells cultured in the presence of mouse GMC-SF and interleukin-4 in which the DEC-205 expression was up-regulated in the course of cultures. Hamster DEC-205 was mainly detected on cell membrane and shown to mediate the uptake of FITC-conjugated ovalbumin (Maruyama et al. 2002).

15.5.2 Functions of DEC-205

Studies suggest that CD205 has two distinct functions – one as an endocytic receptor on immature dendritic cells and a second as a non-endocytic molecule on mature dendritic (Butler et al. 2007). DEC-205 is important in the recognition and internalization of antigens for presentation to T cells. Upon endocytosis, DEC-205 internalizes to late endosomes/lysosomes and recycles to the surface. Expression of DEC-205 is enhanced in both types of cells upon cell maturation induced by inflammatory stimuli (Hawiger et al. 2001; Jiang et al. 1995; Mahnke et al. 2000). The DEC-205 is rapidly taken up by means of coated pits and vesicles, and is delivered to a multi-vesicular endosomal compartment that resembles the MHC class II-containing vesicles implicated in antigen presentation (Mahnke et al. 2000).

In the steady state, DEC-205 represents a specific receptor for DCs to induce peripheral tolerance to soluble antigens for both $CD4^+$ (Hawiger et al. 2001) and $CD8^+$ T cells (Bonifaz et al. 2002). The key observations are that DCs have efficient receptor based mechanisms to enhance presentation on MHC class I products in vivo, and that these operate in the steady state, and finally, the consequence of presentation is peripheral tolerance in the $CD8^+$ compartment by a deletional mechanism (Bonifaz et al. 2002, 2004; Steinman et al. 2003). Although its ligands await identification, the endocytic properties of CD205 make it an ideal target for those wishing to design vaccines and targeted immunotherapies. Unlike other members of the MMR family, CD205 was up-regulated upon dendritic cell maturation. Furthermore, a small amount of the CD205-DCL-1 fusion protein was detected in mature DC.

15.6 ENDO 180 (CD280)/uPARAP

15.6.1 Urokinase Receptor (uPAR)-Associated Protein

Urokinase receptor (uPAR)-associated protein is a member of macrophage MR protein family and known as Endo180 or urokinase receptor-associated protein (uPARAP) and was discovered independently by several groups. It was found to be part of a trimolecular cell-surface complex with urokinase plasminogen activator (uPA) and its receptor (uPAR). It was also discovered as a novel antigen on macrophages and human fibroblasts. The plasminogen activation cascade system, directed by urokinase and the urokinase receptor, plays a key role in extracellular proteolysis during tissue remodeling. To identify molecular interaction partners of these trigger proteins on the cell, Behrendt et al. (2000) observed a specific tri-molecular complex on addition of pro-urokinase to human U937 cells. This complex included the urokinase receptor, pro-urokinase, and an unknown, high molecular weight urokinase receptor-associated protein. Further analysis identified the novel protein as the human homologue of a murine membrane-bound lectin with hitherto unknown function. The protein, designated uPARAP, is the fourth member of the macrophage MR protein family. The large extracellular domain of Endo 180 contains an N-terminal cysteine-rich domain, a single fibronectin type

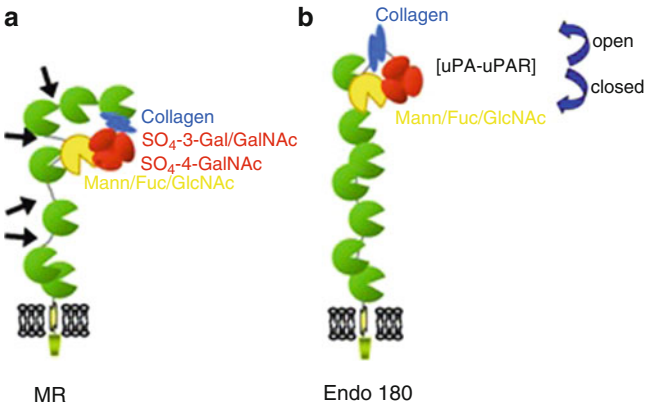

Fig. 15.2 The structural organization of mannose receptor (a) and Endo 180 (b). Globular conformation of the mannose receptor family members where the CysR domain (*red*) interacts with the physiologically active CTLD (*yellow*) and corresponding to CTLD4 in theMR (**a**) and CTLD2 in Endo180 (**b**). The FNII domain has been colored *blue*, while the non-functional CTLDs are shown in *green*. This conformation seems to be receptive to ligand binding and it can interact with several substrates, which are indicated with the corresponding color beside the domain responsible for the interaction. Regions accessible for proteolytic cleavage for the MR have been identified by Napper et al, (2001) and they are highlighted with *black arrows*. In an acidic environment it is predicted that Endo180 switches to a more conformation. For the MR, conformational changes could modify its overall compactness by either an opening or closing of the observed conformation (Adapted by permission from Boskovic et al. (2006) © The American Society for Biochemistry and Molecular Biology)

II domain (a putative collagen-binding site) and eight C-type lectin-like domains. The second of these lectin-like domains has been shown to mediate Ca^{2+} −dependent mannose binding. Endo180 like MR is expressed on macrophages. But it is also expressed on fibroblasts and chondrocytes, some endothelial cells, and tissues undergoing ossification. The cysteine-rich R-type domain at the amino terminus of Endo180 has been shown to be unable to bind sulfated glycans. The function of cysteine-rich domain is unknown. Targeted deletion of Endo180 exons 2–6 in mice showed that this mutation results in the efficient expression of a truncated Endo180 protein that lacks the cysteine-rich domain, the FNII domain and CTLD1. This mutation does not disrupt the C-type lectin activity that is mediated by CTLD2, but results in cells that have a defect in collagen binding and internalization and an impaired migratory phenotype (East et al. 2003).

Despite similarities between Endo180 and the MR, conflicting reports have been published on the three-dimensional arrangement of these receptors. Single particle electron microscopy of the MR and Endo180 display distinct three-dimensional structures, which are, however, conceptually very similar: a bent and compact conformation built upon interactions of the CysR domain and the lone functional CTLD. Studies indicated that, under a low pH mimicking the endosomal environment, both MR and Endo180 experience large conformational changes (Fig. 15.2) (Boskovic et al. 2006).

15.6.2 Interactions of Endo180

Although Endo180 and the MR are now both known to be mannose binding lectins, each receptor is likely to have a distinct set of glycoprotein ligands in vivo (East and Isacke 2002). The distribution and post-translational processing is consistent with Endo180 functioning to internalize glycosylated ligands from the extracellular milieu for release in an endosomal compartment (Sheikh et al. 2000).

The Endo180 interacts with ligand-bound uPAR, uPA, matrix metalloprotease-13 (MMP-13), and collagen V on the cell surface. Endo180 binds to the carboxy-terminal region of type I collagen, and collagens type II, IV, and V. In vitro assays showed that Endo180 binds both to native and denatured collagens and the binding is mediated by the fibronectin type II domain. The restricted expression of Endo180 in both embryonic and adult tissue indicates that Endo180 plays a physiological role in mediating collagen matrix remodeling during tissue development and homeostasis; and the observed receptor upregulation in pathological conditions may contribute to disease progression (Thomas et al. 2005; Wienke et al. 2003). Cross-linking studies have identified Endo180 as an uPAR associated protein and this interaction could be blocked by collagen V. This collagen binding reaction at the exact site of plasminogen activation on the cell may lead to adhesive functions as well as a contribution to cellular degradation of collagen matrices (Behrendt et al. 2000).

The uptake and lysosomal degradation of collagen by fibroblasts constitute a major pathway in the turnover of connective tissue. Studies suggest a central function of Endo180 in cellular collagen interactions (Engelholm et al. 2003). During post-implantation development in mice, Endo180 was expressed in all tissues undergoing primary ossification, including the developing bones that ossify intramembranously, and developing long bones undergoing endochondral ossification. Osteoblasts also expressed uPAR. Besides bone-forming tissues, uPARAP/Endo180 was detected in a mesenchymal condensation of the midbrain and in the developing lungs. Endo180 receptor is strongly expressed in chondrocytes in the articular cartilage of young mice. Expression of Endo180 in articular cartilage chondrocytes of young, but not old, mice and the reciprocal expression of Endo180 and its ligands in the growth plate suggest that this receptor is involved in cartilage development but not in cartilage homeostasis. Endo180 does not appear to play a role in the development or progression of murine osteoarthritis (Howard et al. 2004; Howard and Isocke 2002), though it is involved in the clearance of uPA:uPAR

complex as well as other possible ligands during benign and malignant tissue remodeling (Schnack Nielsen et al. 2002).

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Part VI

I-Type Lectins

I-Type Lectins: Sialoadhesin Family

16

G.S. Gupta

16.1 Sialic Acids

Sialic acids are a family of α-keto acids with nine-carbon backbones. Sialic acids are acidic monosaccharides typically found at the outermost ends of the sugar chains of animal glycoconjugates. Sialic acids belong to most important molecules of higher animals and also occur in some microorganisms. Their structural diversity is high and, correspondingly, the mechanisms for their biosynthesis complex. Sialic acids are involved in a great number of cell functions. They are bound to complex carbohydrates and occupy prominent positions, especially in cell membranes. However, receptors or adhesion molecules mediating such functions between eukaryotic cells were unknown until 1985, when it was found that the members of the selectin family mediate adhesion of leukocytes to specific endothelia through binding to sialylated glycans like sialyl Lewis. Sialic acids are expressed abundantly in animals of deuterostome lineage (primarily in echinoderms and vertebrates), but their expression in another major group of animals, the protostomes (including nematodes, arthropods, and mollusks), is inconspicuous. They are found mostly at distal positions of oligosaccharide chains of glycoproteins and glycolipids and are thus exposed to the extracellular environment, allowing them to be recognized during initial contact of cells with various pathogenic agents such as viruses, bacteria, protozoa, and toxins. The marked structural complexity of sialic acids can thus be interpreted as a result of evolutionary arms race between the hosts and the pathogens.

Various proteins can recognize and bind to Sialic acids family of monosaccharides. Particular attention is focused on the evolving information about sialic acid recognition by certain C-type lectins (the selectins), I-type lectins, and a complement regulatory protein (the H protein). The last two instances are examples of the importance of the side chain of sialic acids and the effects of natural substitutions (e.g., 9-O-acetylation) of this part of the molecule. Accumulated evidence show that sialic acids function in cellular interactions either by masking or as a recognition site. Due to their cell surface location these acidic molecules shield macromolecules and cells from enzymatic and immunological attacks and thus contribute to innate immunity. In comparison to the masking role, sialic acids also represent recognition sites for various physiological receptors, such as selectins and siglecs, as well as for toxins and microorganisms. The recognition function of sialic acids can be masked by O-acetylation, which modifies the interaction with receptors. Many viruses use sialic acids for the infection of cells. Since sialic acids play also a decisive role in tumor biology, they modulate biological and pathological cellular events in a sensitive way. Thus, they are most prominent representatives of mediators of molecular and cellular recognition (Schauer 2004).

Most mammalian cell surfaces display two major sialic acids (Sias), N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Humans lack Neu5Gc due to a mutation in cytidine-5′-monophosphate (CMP)-Neu5Ac hydroxylase, which occurred after evolutionary divergence from great apes. Sonnenburg et al. (2004) described an apparent consequence of human Neu5Gc loss: domain-specific functional adaptation of Siglec-9 (CD33-related Siglecs = CD33rSiglecs). While recombinant human Siglec-9 showed recognition of both Neu5Ac and Neu5Gc, in striking contrast, chimpanzee and gorilla Siglec-9 strongly preferred binding Neu5Gc. Reports also indicated that endogenous Sias (rather than surface Sias of bacterial pathogens) are the functional ligands of CD33r-Siglecs and suggested that the endogenous Sia landscape is the major factor directing evolution of CD33rSiglec binding specificity. Such domain-specific divergences should be taken into consideration in upcoming comparisons of human and chimpanzee genomes (Sonnenburg et al. 2004).

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16.2 Sialic Acid-Binding Ig-Like Lectins (I-Type Lectins)

The immunoglobulin superfamily (IgSF), integrins, cadherins, and selectins comprise distinct categories of cell adhesion molecules. Of these IgSFs, the selectins function through glycoconjugate-mediated interactions, and members of the other families function either through homophilic or heterophilic protein–protein recognition. Proteins other than antibodies and T-cell receptors that mediate glycan recognition via Ig-like domains are called "I-type lectins or Siglecs (Sia-recognizing Ig-superfamily lectins)." The Ig superfamily is defined by their structural similarity to immunoglobulins. The siglecs mediate cell–cell interactions through recognition of specific sialylated glycoconjugates as their counter receptors (Gabius et al. 2002; May et al. 1998). Sialoadhesins recognizing sialylated glycan structures represent the best characterized subgroup. The majority of these proteins are involved in protein-protein binding as receptors, antibodies or cell adhesion molecules and capable of carbohydrate-protein interactions. In contrast to the selectins, these proteins are associated with diverse biological processes, i.e. hemopoiesis, neuronal development and immunity. Their properties, carbohydrate specificities and potential biological functions have been vividly discussed.

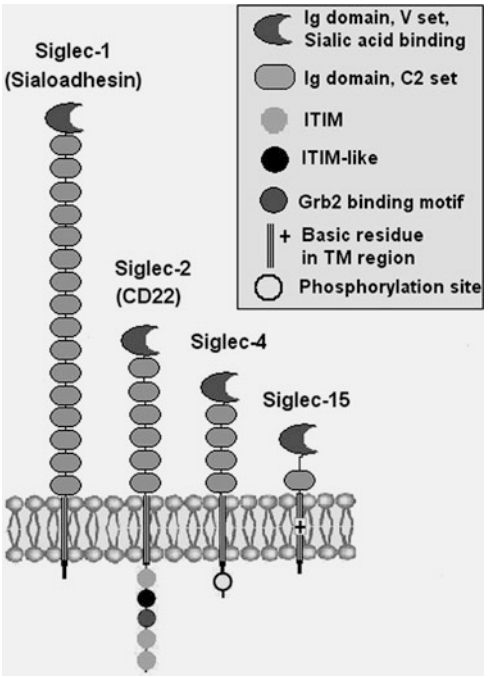

Fig. 16.1 Domain organization of known Siglecs of Sialoadhesin family in humans: Sialoadhesin (Siglec-1), Siglec-2 (CD22), Siglec-4 (Mylin-associated glycoprotein: MAG), and Siglec-15. Most Siglecs have one or more tyrosine-based signaling motifs. Exceptions are Sn, Siglec-14, Siglec-15. Some siglecs having no defined cytoplasmic domain have positively charged amino acid within TM domain, that can associate with DAP12 (DNAX activation protein-12) ITAM containing adaptor

16.2.1 Two Subsets of Siglecs

Siglecs are type 1 transmembrane proteins which comprise a sialic acid-binding N-terminal V-set domain, variable numbers of C2-set Ig domains, a transmembrane region and a cytosolic tail. Over the past few years, several novel siglecs have been discovered through genomics and functional screens. On the basis of their sequence similarities and evolutionary conservation, two primary subsets of siglecs have been identified:

1. **First subset** of the sialoadhesin (Sn) family, classified by sequence homology to members of IgSF, exhibits protein–carbohydrate recognition. In addition to Sn (Siglec-1), the subset includes CD22 (Siglec-2), MAG (myelin-associated glycoprotein) (Siglec-4a), Schwann cell myelin protein (Siglec 4b), Siglec-15, all of which are well-conserved in mammals (Fig. 16.1).
2. **Second**, rapidly evolving, subset is designated the CD33-related siglecs. In humans, these include CD33 and siglecs-5, -6, -7 (7/p75/AIRM1), -8, -9, 10, -11, -14 and -16, whereas, in mice, they comprise murine CD33 and siglecs-E, -F, -G and –H (Angata 2006; Floyd et al. 2000; Crocker and Redelinghuys 2008; Varki and Angata 2006; Yu et al. 2001). Thirteen functional Siglecs [sialoadhesin (siglec-1), CD22 (siglec-2), MAG, CD33 (Siglecs-3) and Siglecs-5-13 (CD33-related-Siglecs)] have been identified in great apes, of which humans lack Siglec-13 (Angata et al. 2004). Along with sialoadhesin, Siglec-2–4 and Siglec 5–16 molecules form the Siglec family of sialic acid-binding lectins (van den Berg et al. 2001; Crocker and Redelinghuys 2008) (see Chap. 17; Fig. 17.1).

The "CD33-related" siglecs have molecular features of inhibitory receptors and may be important in regulating leucocyte activation during immune and inflammatory responses (Crocker 2002; Varki and Angata 2006). Siglecs from both types show specificity for sialic acid-containing glycans that are found on the nonreducing ends of oligosaccharide chains of *N*- and *O*-linked carbohydrates or on glycosphingolipids. Siglecs are further distinguished by their specificity for sialic acids that possess α(2,3), α(2,6), or α(2,8) linkages to an extended oligosaccharide structure, as well as distinguish the structures of the extended glycans themselves (Crocker et al. 2007). With the notable exception of MAG, which is expressed in the nervous system, siglecs are differentially expressed on various subsets of leucocytes where they

play a role in the positive and negative regulation of immune and inflammatory responses (McMillan and Crocker 2008; Crocker et al. 2007).

Most Siglecs with two exceptions (Siglec-4/MAG and Siglec-6) are expressed on various white cells of immune system; Siglec-4/MAG is expressed exclusively in the nervous system (Crocker et al. 2007; Lock et al. 2004; Varki and Angata 2006; Zhang et al 2004) . In particular, CD33-related Siglecs show marked inter-species differences even within the same order of mammals (Angata and Binkman-vander Linden 2004). Although the biological functions of Siglecs expressed in the immune system are not yet fully understood, in vitro and in vivo analyses suggest that these lectins are involved in coupling glycan recognition to immunological regulation. On the other hand, the precise functional importance of sialic acid-binding property of Siglecs is not well understood, although the phenotypic similarity between Siglec-2 null mice and ST6Gal-I (Galβ1–4GlcNAc α2–6 sialyltransferase) null mice suggests that sialic acid binding does affect the signaling activity of this Siglec

I-type lectins are abundant in nervous system and have been implicated in a number of morphogenetic processes as fundamental as axon growth, myelin formation and growth factor signaling. The structural and functional properties of I-type lectins expressed in neural tissues have been reviewed with a main focus on MAG/Siglec-4 (Angata and Binkman-Vander Linden 2002). As endocytic receptors, siglecs provide portals of entry for certain viral and bacterial pathogens, as well as therapeutic opportunities for targeting innate immune cells in disease. Three Siglec family members appear to be tightly restricted in expression to specific cell populations within hematopoietic lineage: sialoadhesin/Siglec-1 to macrophages, CD33/Siglec-3 to cells of the myelomonocytic lineage, and CD22/Siglec-2 to B cells. Likewise MAG/Siglec-4 is only expressed on oligodendrocytes in the central nervous system and on Schwann cells in the peripheral nervous system. For Sn and MAG, specific cell populations which bear the cognate "ligand" have been identified: Sn preferentially interacts with cells of the granulocytic lineage. Additionally, for each Siglec family member, certain sialic acid ligand preferences have been determined (Patel et al. 1999) (Fig. 16.2).

Human mast cells (MC) derived from $CD34^+$ peripheral blood precursors express mRNA for CD22 (Siglec-2), CD33 (Siglec-3), Siglec-5, Siglec-6, Siglec-8 and Siglec-10 and surface expression of these proteins except CD22 and Siglec-10, whose levels were low or undetectable. However, expression of CD22 and Siglec-10 was mostly cytoplasmic. $CD34^+$ precursor cells from peripheral blood constitutively expressed surface CD33, Siglec-5 and Siglec-10. Phenotypic analysis of LAD-2 MC yielded a similar pattern of Siglec expression except that CD22 expression was particularly prominent (Yokoi et al. 2006).

16.2.2 Siglecs as Inhibitory Receptors

Each Siglec has a distinct expression pattern suggesting that these molecules play unique roles in the cells expressing them. Siglecs differ from traditional Ig superfamily members in several ways. Although their extracellular domains contain a variable number of C2-set Ig domains, unlike other Ig superfamily members, Siglecs possess an NH_2-terminal V-set Ig domain that binds sialylated structures (May et al. 1998; Yamaji et al. 2002, Zaccai et al. 2003; Alphey et al. 2003; Dimasi et al. 2004). In addition, many Siglecs have potential tyrosine phosphorylation sites in the context of an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tails, suggesting their involvement in intracellular signaling pathways and in endocytosis. In fact, Siglecs-3 and -7 have been shown to be capable of transmitting negative regulatory signals upon cross-linking by specific antibodies. Although the function of other family members remains unknown, the presence of ITIM sequences in the cytoplasmic tails of some of human siglecs renders them potential inhibitory receptors. Human CD33 has been characterized as an inhibitory receptor by virtue of its ability to bind tyrosine phosphatases SHP-1 and SHP-2 (Ulyanova et al. 1999, 2001). In case of CD22/Siglec-2, a negative regulatory role was further proven by the studies using genetically engineered mice, which suggests that Siglecs possess signal transduction activity. Evidence of signaling has been shown for several human Siglecs (Crocker and Varki 2001; Ikehara et al. 2004; Mingari et al. 2001).

16.2.3 Binding Characteristics of Siglecs

The common feature of Siglec proteins is their recognition of sialic acid residues on cell surface glycoproteins and glycolipids, which is mediated by the amino-terminal V-set domain of a siglec (May et al. 1998). Although recognition of sialic acid residues is a hallmark feature of all siglecs, the specificity of binding has been shown for certain family members in terms of their preferences for the position of a sialic acid residue on N-linked oligosaccharides as well as in the binding of ligands expressed on different cell types (Crocker et al. 1997). Each siglec is expressed in a highly restricted fashion (Angata et al. 2001; Yu et al. 2001; Floyd et al. 2000; Patel et al. 1999; Crocker and Redelinghuys 2008), implying a specific function for each family member. Indeed, it is believed that sialoadhesin is involved in regulation of macrophage function (Crocker et al. 1997); MAG is implicated in myelinogenesis (Umemori et al. 1994), while CD22 serves to inhibit signaling through B-cell antigen receptor via binding of SHP-1 tyrosine phosphatase to its ITIMs (Cyster and Goodnow 1997; Doody et al. 1995), and

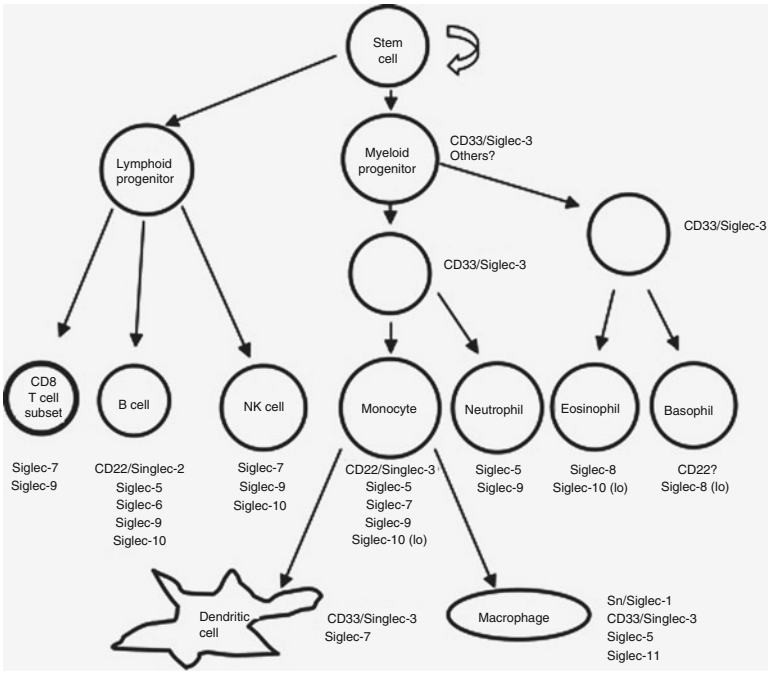

Fig. 16.2 Cell-type specific expression of Siglecs in the hematopoietic and immune cells of humans. The figure shows the distribution of human Siglecs on various cell types (Modified from Crocker and Varki (2001)). Note that expression patterns of Siglecs on bone marrow precursor cell types has not been well studied, except in the case of CD33/Siglec-3. In a few instances, expression is not found in all humans studied (e.g., Siglec-7 and -9 on a small subset of T cells) (Adapted by permission from Varki and Angata (2006) © Oxford University Press)

the lectin function of CD22 is thought to be important in recruiting CD22 to the B-cell antigen receptor (Tedder et al. 1997). Mouse Siglecs (mSiglecs) orthologous to hSiglec-1 (Sn), hSiglec-2 (CD22), and hSiglec-4 (MAG) have been characterized, with latter two including phenotypes with gene disruptions (Otipoby et al. 1996; O'Keefe et al. 1996; Nitschke et al. 2001) .

Siglecs have a different binding pattern and each member shows a distinct specificity for the type of sialic acid with which it interacts. Sialic acids occur naturally in about 30–40 forms, and it has been shown that Sn and MAG are specific for N-acetyl neuraminic acid (Collins et al. 1997), whereas CD22 can bind either N-glycolyl or N-acetyl neuraminic acid (Sjoberg et al. 1994). The glycerol and carboxylate side chains are also essential for binding by Sn and MAG (Collins et al. 1997). The linkage of sialic acid to underlying oligosaccharide chain defines another level of specifity for siglec family members. CD22 is highly specific for α2,6-linked sialic acids (Powell and Varki 1994), whereas sialoadhesin, MAG, and CD33 prefer glycans terminated in α2,3-linked sialic acids (Kelm et al. 1994). The affinities of members of the siglec family for free oligosaccharides are weak (32 μM for CD22; Powell et al. 1995); ~1 mM [Sn and MAG) and for many other lectins (May et al. 1998). Unlike L-selectin and Langerin, which also bind to sulphated analogues of sialyl-Le^x, the siglecs do not give detectable binding signals with sulphated analogues that are lacking sialic acid. The sulphate groups, however, modulate the Siglec binding to the sialyl-Le^x sequence in positive or negative fashion (Campanero-Rhodes et al. 2006).

16.2.4 Siglecs of Sialoadhesin Family

In addition to sialoadhesin or Siglec-1, the subset includes CD22 (Siglec-2), MAG (myelin-associated glycoprotein) (Siglec-4a), Schwann cell myelin protein (Siglec 4b), Siglec-15, all of which are well-conserved in mammals. Sialoadhesin is a macrophage receptor where as CD22 is expressed by B lymphocytes. Both proteins share sequence similarity with MAG, an adhesion molecule of oligodendrocytes and Schwann cells that has been implicated in the process of myelination. Sialoadhesin, CD22, and MAG mediate cell adhesion by binding to cell-surface glycans that contain sialic acid. Whereas sialoadhesin binds equally to the sugar moieties NeuAc α 2—> 3Gal β1—> 3(4) GlcNAc or NeuAc α2—> 3Gal β1—> 3GalNAc, MAG recognizes only NeuAc α2—> 3Gal β1—> 3GalNAc, where as CD22 binds specifically to NeuAc α2—> 6Gal β1—> 4GlcNAc. Moreover, the recognition of sialylated glycans on the surface of particular cell types leads to the selective binding: such as Sn to neutrophils, MAG to neurons and CD22 to lymphocytes (Kelm et al. 1994; Angata 2006; Crocker 2005; Crocker and Zhang 2002). CD22 is a well-characterised B cell restricted siglec that has been shown to mediate both sialic acid-dependent and -independent signaling functions in B cell regulation.

16.3 Sialoadhesin (Sn)/Siglec-1 (CD169)

16.3.1 Characterization of Sialoadhesin/ Siglec-1

Sialoadhesin is a member of Ig superfamily with 17 extracellular domains. The rodent sialoadhesin gene, *Sn*, is located to chromosome 2 F-H1. The human sialoadhesin gene, *Sn*, is localized to the conserved syntenic region on human chromosome 20p13. Hence, the sialoadhesin gene is not linked to the other members of the Sialoadhesin family, CD22, MAG, and CD33, which have been independently mapped to the distal region of mouse chromosome 7 and to human chromosome 19q13.1–3 (Mucklow et al. 1995). The predicted protein sequences of human and mouse Sn are about 72% identical, with the greatest similarity in the extracellular region, which comprises 17 Ig domains (1 variable domain and 16 constant domains) in both species. The expression pattern of human Sn was found to be similar to that of the mouse receptor, being absent from monocytes and other peripheral blood leukocytes, but expressed strongly by tissue macrophages in the spleen, lymph node, bone marrow, liver, colon, and lungs. High expression was also found on inflammatory macrophages present in affected tissues from patients with rheumatoid arthritis (Hartnell et al. 2001).

N-Terminal Sialoadhesin Glycopeptide Domain Mediates Ligand Binding: In Sn, sialic acid binding region has been characterized in detail by X-ray crystallography, nuclear magnetic resonance, and site-directed mutagenesis. Studies indicate that this receptor is likely to function as a macrophage accessory molecule in a variety of cell-cell and cell-extracellular matrix interactions and mediates cell surface interactions through binding of sialylated glycoconjugates. Studies have shown that the membrane distal V-set Ig domain of sialoadhesin contains sialic acid binding site (Vinson et al. 1996). Site-directed mutagenesis of a subset of non-conservative mutations disrupted sialic acid-dependent binding without affecting binding of mAbs directed to two distinct epitopes of Sn. A CD8α-based molecular model predicts that these residues form a contiguous binding site on GFCC'C" β-sheet of the V-set domain centered around an arginine in F strand. A conservative mutation of this arginine to lysine also abolished binding. This amino acid is conserved among all members of Sn family and is therefore likely to be a key residue in mediating sialic acid-dependent binding of sialoadhesins to cells (Vinson et al. 1996). However, the N-terminal Sn domain can mediate sialic acid-binding on its own. The structure of N-terminal Sn domain, in complex with a sialic acid-contains heptapeptide, (Ala-Gly-His-Thr (Neu5Ac)-Trp-Gly-His). The affinity of Sn for this ligand is four times higher than the affinity for the natural linkage 2,3′-sialyllactose (Bukrinsky et al. 2004).

There is a human Siglec-like molecule (Siglec-L1) that lacks a conserved arginine residue known to be essential for optimal sialic acid recognition by known Siglecs. Loss of arginine from an ancestral molecule was caused by a single nucleotide substitution that occurred after common ancestor of humans with great apes but before the origin of modern humans. The chimpanzee Siglec-L1 ortholog preferentially recognizes N-glycolylneuraminic acid, which is a common sialic acid in great apes and other mammals. Reintroducing the ancestral arginine into human molecule regenerates the same properties. Thus, a single base pair mutation that replaced arginine on human Siglec-L1 is likely to be evolutionarily relative to the previously reported loss of N glycolylneuraminic acid expression in the human lineage. It seems that additional changes in the biology of sialic acids have taken place during human evolution (Angata et al. 2001).

16.3.2 Cellular Expression of Sialoadhesin

Stromal macrophages in lymphohemopoietic tissues express macrophage-restricted plasma membrane receptors involved in nonphagocytic interactions with other hemopoietic cells (originally named sheep erythrocyte receptor). Sn is first detected on fetal liver macrophages on day 18 of development. In spleen and bone marrow, Sn appears between day 18 and birth, in parallel with myeloid development. Sialoadhesin is differentially regulated compared with the erythroblast receptor and F4/80 antigen, that it is not required for fetal erythropoiesis, and that its induction on stromal macrophages is delayed until the onset of myeloid and lymphoid development. Resident peritoneal macrophages do not express high levels of Sn in vitro unless an inducing element found in normal mouse serum is present, but such macrophages did not acquire Sn-independent EbR activity (Van den Berg et al. 1996; Hartnell et al. 2001; van den Berg et al. 2001a). Sn can also be induced on macrophages present at sites of inflammation in both humans and rodents (Jiang et al. 2006; Lai et al. 2001). During chronic inflammation, as occurs during autoimmune disease, high levels of Sn are found on macrophages in inflammatory infiltrates, where it is suggested to mediate local cell-cell interactions (van den Berg et al. 2001a). Among haemopoetic cells, Sn binds preferentially to mature granulocytes (Crocker et al. 1997). It has been proposed that in lymphoid tissues Sn may act as a lymphocyte adhesion molecule (Van den Berg et al. 1992), and its selective expression on macrophages in the marginal zone of the

spleen suggests a role in antigen presentation to B cells (Steiniger et al. 1997). However, in chronic inflammatory conditions, such as atherosclerosis and rheumatoid arthritis, Sn is expressed at high levels on inflammatory macrophages. Sn is also detected in intracellular vesicles that were apparently taken up by macrophages. Surprisingly, Sn is also found at contact points of macrophages with other macrophages, sinus-lining cells and reticulum cells, suggesting that it also mediates interactions with these cell types (Schadee-Eestermans et al. 2000). Transcriptional and protein levels of Sn on monocytes in coronary artery disease patients are significantly increased compared with healthy controls, but increased Sn had no correlation with level of serum lipids. Sialoadhesin may be considered as a potential non-invasive indicator for monitoring disease severity and a biomarker for predicting the relative risk of cardiovascular events (Xiong et al. 2009).

Microglia, the resident macrophages of the CNS, reside behind blood–brain barrier and do not express Sn. Microglia and macrophage population in cicumventricular organs, choroid plexus and leptomeninges are exposed to plasma proteins and some macrophages express Sn at these sites. Injury to the CNS, which damages the blood–brain barrier, induces Sn expression on a proportion of macrophages and microglia within the parenchyma (Perry et al. 1992). Macrophages from mesenteric and axillar lymph nodes exhibited higher activity than those from spleen. Quantity of Sn present in lymph node macrophages was 25-fold higher than in splenic macrophages. It suggested that macrophages express high levels of unmasked Sn in lymph nodes. The unmasked forms on these macrophages are available for Sn-dependent adhesive functions, unlike the masked forms on the majority of splenic macrophages (Nakamura et al. 2002).

16.3.3 Ligands for Sialoadhesin

16.3.3.1 Specificity for Sialylated Glycans

Surface proteins on tolerogenic, immature dendritic cells and regulatory T cells are highly α2,6-sialylated, suggesting a glycan motif of tolerogenic cells which might serve as ligand for inhibitory siglecs on the surface of effector cells (Jenner et al. 2006). Sn contains 17 extracellular Ig-like domains, which recognizes oligosaccharides/glycoconjugates containing terminal oligosaccharides NeuNAc2,3-Gal 1,3-GalNAc (disaccharide α-D-Neu5Ac-(2—> 3)-β-D-Gal) or NeuNAc2,3-Gal 1,3-GlcNAc in N- and O-linked glycans, and as such mediates adhesive interactions with lymphoid and myeloid cells (Van den Berg et al. 1992; Crocker et al. 1995). Sn specifically binds to α-2—> 3-sialylated N-acetyl lactosamine residues of glycan chains. The experimental and theoretical STD values indicate that a combined modeling/STD NMR approach yields a reliable structural model for the complex of Sn with α-D-Neu5Ac-(2—> 3)-β-D-Gal-(1—> 4)-D-Glc 1 in aqueous solution (Bhunia et al. 2004). Since, each Siglec exhibits a unique specificity for sialylated glycans, Sn prefers 2,3-linked sialic acids of the Neu5Ac rather than the Neu5,9(Ac)2 or Neu5Gc types (Angata and Brinkman-Vanden Linden 2002; Bhunia et al. 2004). On the other hand, CD22, a B cell-restricted receptor with seven Ig-like domains, selectively recognizes oligosaccharides terminating in NeuAc α2-6Gal in N-glycans. In humans, the amino-terminal V-set Ig-like domain in both proteins is both necessary and sufficient to mediate sialic acid-dependent adhesion of correct specificity. In contrast in murine CD22, only constructs containing both the V-set domain and the adjacent C2-set domain were able to mediate sialic acid-dependent binding (Nath et al. 1995).

The structural diversity of sialic acids influences cell adhesion mediated by molecules like Sn and CD22 in murine macrophages and B-cells. It was shown that the 9-O-acetyl group of Neu5,9Ac2 and the N-glycoloyl residue of Neu5Gc interfere with Sn binding. In contrast, CD22 binds more strongly to Neu5Gc compared to Neu5Ac. Of two synthetic sialic acids tested, only CD22 bound the N-formyl derivative, whereas a N-trifluoroacetyl residue was accepted by Sn (Kelm et al. 1994). Sn-deficient ($Sn^{-/-}$) mice suggest a role for Sn in regulating cells of the immune system rather than in influencing steady-state hematopoiesis (Oetke et al. 2006).

Using a series of synthetic sialic-acid analogues either on resialylated human erythrocytes or as free α-glycosides in hapten inhibition, siglecs required hydroxy group at C-9 for binding, suggesting hydrogen bonding of this substituent with the binding site. Besides, remarkable differences were found among the proteins in their specificity for modifications of N-acetyl group. Whereas Sn, MAG and SMP do not tolerate a hydroxy group as in N-glycolylneuraminic acid, they bind to halogenated acetyl residues. Study indicates that interactions of hydroxy group at position 9 and the N-acyl substituent contribute significantly to the binding strength (Kelm et al. 1998).

16.3.3.2 CD43 as T Cell Counter-Receptor for Sn

Evidences indicate that cell adhesion molecules of Ig family use GFCC'C β-sheet of membrane-distal V-set domains that bind structurally different ligands. Such surface is favored for cell-cell recognition (van der Merwe et al. 1996). Sn has been shown to bind several membrane receptors via both sialic acid-dependent and -independent mechanisms; for instance, the sialomucins leukosialin (CD43) on T cells and MUC-1 on breast cancer cells are putative sialic acid-dependent counter receptors, whereas the macrophage mannose receptor, which is present on several types of myeloid cells (Martinez-Pomares et al. 1996), and the mouse macrophage galactose-type C-type lectin 1 (Kumamoto et al. 2004)

binds Sn in a sialic acid-independent manner. Among major glycoproteins (85, 130, 240 kDa) from a murine T cell line (TK-1), CD43 from COS cells supported increased binding to immobilized Sn. Sn-binding glycoproteins were identified as the sialomucins CD43 and P-selectin glycoprotein ligand 1 (PSGL-1 or CD162), corresponding to 130- and 240-kDa respectively. Further more, Sn bound to different glycoforms of CD43 expressed in CHO cells, including unbranched (core 1) and branched (core 2) *O*-linked glycans, that are normally found on CD43 in resting and activated T cells. Results suggest CD43 as a T cell counter receptor for Sn (van den Berg et al. 2001b). The nature of the sialoglycoprotein recognized by Sn on breast cancer cells was a major band of ~240 kDa, and was shown to be the epithelial mucin, MUC1 (Nath et al. 1999).

16.3.4 Sialoadhesin Structure

Domain deletion and site-directed mutagenesis studies (Vinson et al. 1996) have indicated that functionally important sialic acid–binding portion of the molecule is localized on the N-terminal V-set Ig domain. Molecular cloning of murine Sn showed that it has 17 Ig-like domains. The most similar proteins in the database were CD22, MAG, Schwann cell myelin protein and CD33. Low angle shadowing and electron microscopy showed that Sn consisted of a globular head region of approximately 9 nm and an extended tail of approximately 35 nm. Sialoadhesin specifically recognizes oligosaccharide sequence Neu5Ac α2,3Gal β1,3GalNAc in either sialoglycoproteins or gangliosides. Findings imply that specific sialoglycoconjugates carrying this structure may be involved in cellular interactions between stromal macrophages and subpopulations of haemato-poietic cells and lymphocytes. (Crocker et al. 1991, 1994).

1. Proton NMR analysis of Sn: The molecular interactions between Sn and sialylated ligands have been investigated by using proton NMR. Addition of ligands to 12 kDa N-terminal Ig-like domain of Sn results in resonance shifts in the protein. The results indicated that α2, 3-sialyl-lactose and α2,6-sialyl-lactose bind respectively 2- and 1.5-fold more strongly than does α-methyl-N-acetylneuraminic acid (α-Me-NeuAc). The resonances corresponding to the methyl protons within the N-acetyl moiety of sialic acid undergo up-field shifting and broadening, reflecting an interaction of this group with Trp-2 in Sn as observed in co-crystals of the terminal domain with bound ligand. Affinities of mutant and wild-type forms of Sn in which the first three domains were fused to the Fc region of human IgG, revealed that substitution of Arg^{97} by alanine completely abrogates interaction with α-Me-NeuAc, whereas a conservative substitution with lysine resulted in a 10-fold decrease in affinity. These results confirm the critical importance of conserved arginine in interactions between sialosides and members of Ig-like lectins (Crocker et al. 1999).

2. Crystallographic and in silico analysis of sialoadhesin: The X-ray crystal structures of N-terminal domain of Sn provides important insights into how this transmembrane-spanning receptors functions. A functional fragment of Sn, comprising the N-terminal Ig domain, was expressed in CHO cells as both native (SnD1) and selenomethionyl (Se-SnD1) stop protein. SnD1 in absence and presence of its ligand, 2,3 sialyllactose and Se-SnD1 in absence of ligand have been crystallized. The ligand-free crystals of SnD1 and Se-SnD1 were isomorphous, of space group P3(1)21 or P3(2)21, with unit cell dimensions a = b 38.9 Å, c = 152.6 Å, α = β = 90º, γ = 120º, and diffracted to a maximum resolution of 2.6 A. Cocrystals containing 2,3 sialyllactose diffracted to 1.85 A at a synchrotron source and belong to space group P2(1)2(1)2(1), with unit cell dimensions a = 40.9 Å, b = 97.6 Å, c = 101.6 Å, α = β = γ = 90º (May et al. 1997).

The predicted V-set N-terminal domain of sialoadhesin (SnD1), defined as residues 1–119, contains no O- or N-linked glycosylation sites, and in isolation as a soluble fragment remains competent to bind sialic acids. The structure of the functional N-terminal domain from the extracellular region of sialoadhesin SnD1 was solved in presence of its ligand 3′ sialyllactose to a resolution of 1.85 Å. The structure conforms to the V-set Ig-like fold but contains several distinctive features, including an intra–β sheet disulphide and a splitting of the standard β strand G into two shorter strands. These features appear important in adapting the V-set fold for sialic acid–mediated recognition. Analysis of the complex with 3′ sialyllactose highlights three residues, conserved throughout the siglec family, as key features of sialic acid–binding template. The complex provides information for a heterotypic cell adhesion interaction (May et al. 1998) (Fig. 16.3).

The crystal structure of SnD1 in complex with 2,3-sialyllactose has informed the design of sialic acid analogs (sialosides) that bind Siglecs with significantly enhanced affinities and specificities. Binding assays against sialoadhesin (Siglec-1), CD22 (Siglec-2), and MAG (Siglec-4) showed a 10–300-fold reduction in IC_{50} values (relative to methyl-α-Neu5Ac) for three sialosides bearing aromatic group modifications of the glycerol side chain: Me-α-9-N-benzoyl-amino-9-deoxy-Neu5Ac (BENZ), Me-α-9-N-(naphthyl-2-carbonyl)-amino-9-deoxy-Neu5Ac (NAP), and Me-α-9-N-(biphenyl-4-carbonyl)-amino-9-deoxy-Neu5Ac (BIP). Zaccai et al. (2007) determined the crystal structures of SnD1 in complex with 2-benzyl-Neu5NPro and 2-benzyl-Neu5NAc. These structures reveal that SnD1 undergoes very few structural changes on ligand binding and detail how two novel classes of sialic acid

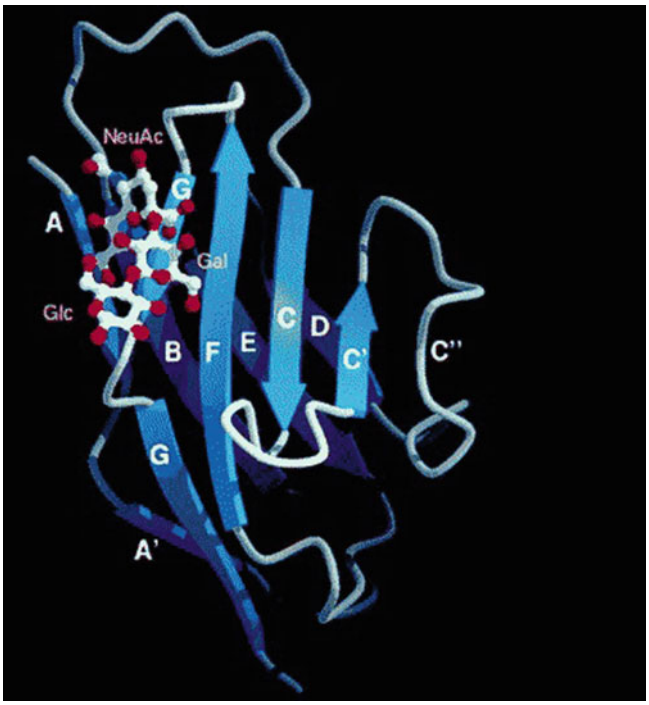

Fig. 16.3 Structure of N-Terminal domain of Sialoadhesin in complex with 3′ Sialyllactose. Each strand is labeled. The 3′ sialyllactose lies along strand G and makes interactions with residues from the A, G, and F strands (Adapted by permission from May et al. (1998) © Elsevier)

analogs bind, one of which unexpectedly can induce Siglec dimerization. In conjunction with in silico analysis, crystal structures of sialosides in complex with SnD1 suggest explanations for the differences in specificity and affinity, providing further ideas for compound design of physiological and potentially therapeutic relevance (Zaccai et al. 2003; 2007).

16.3.5 Regulation of Sialoadhesin

In bone marrow, IL-4 is a potent regulator of sialic acid-specific receptor implicated in macrophage-hemopoietic cell interactions (McWilliam et al. 1992). In bone marrow stromal macrophages, Sn promotes the interactions with developing myeloid cells, and by a subset of tissue macrophages helps in antigen presentation and activation of tumor-reactive T cells. Growth of Ehrlich tumor (ET), a murine mammary carcinoma - may modify the Sn expression by bone marrow macrophages (Kusmartsev et al. 1999). Treatment of bone marrow cells with IFN-γ improved adhesive properties of macrophages, but did not modulate expression of Sn. IL-1 and TNF-α had no effect while combined treatment with these cytokines enhanced binding of sheep erythrocytes to macrophages. Administration of LPS or combined with IFN-γ and TNF-α increased the number of macrophages adhering to plastic and stimulated expression of Sn. Combined treatment with IFN-γ and TNF-α stimulated production of NO by bone marrow macrophages. Blockade of NO synthesis had no effect on adhesive properties of macrophages and expression of Sn (Kusmartsev et al. 2003). IL-13, expressed by activated lymphocytes, markedly suppressed NO release and to a lesser extent secretion of TNF-α by macrophages (Doyle et al. 1994). Glucocorticoids (GC) induce Sn expression on freshly isolated rat macrophages and macrophage cell line R2 in vitro. The cytokines IFN-β, IFN-γ, IL-4, and LPS, although unable to induce Sn expression by themselves, were able to enhance GC-mediated induction of Sn. Effect of GC on Sn expression on rat macrophages can be enhanced by IFN-β, T cell-derived cytokines, or LPS (van den Berg et al. 1996).

16.3.6 Functions of Sialoadhesin

Siglecs are expressed in a highly cell type–specific fashion and appear to be involved in discrete functions ranging from control of myeloid cell interactions (sialoadhesin/CD33; Freeman et al. 1995) to activation of B cells (CD22, Cyster and Goodnow 1997) and regulation of neuronal cell growth and maintenance of myelination in nervous system (MAG) (Li et al. 1994).

Cell-Cell Interactions: The affinity of Sn for sialosides is low (10^{-3} M range), and high-avidity binding requires receptor and ligand clustering. This low affinity, together with a highly extended extracellular domain are key features in permitting Sn to mediate cell–cell interactions, particularly in the plasma microenvironment where the poorly clustered glycans of plasma glycoproteins are unable to compete efficiently with the highly clustered cell-associated glycans involved in avid Sn-binding. Furthermore, cell–cell and cell–matrix interactions are accentuated further by the extension of the N-terminal V-set domain beyond the reach of shorter *cis*-interacting inhibitory siglecs closer to the plasma membrane. Although the expression and features of Sn could potentially influence many macrophage cellular interactions relating to homoeostasis and immunity (Munday et al. 1999), studies on Sn-deficient mice suggest that these may be more important in the regulation of adaptive immune responses. Crocker et al. (2007) reviewed and indicated that Sn-deficient mice exhibit reduced $CD4^+$ T-cell and inflammatory responses in a model of autoimmune uveoretinitis and reduced $CD8^+$ T-cell and macrophage recruitment in models of inherited demyelinating neuropathy in both the central and peripheral nervous systems (Jiang, et al. 2006). Subtle effects on T-cells and IgM

antibody levels were also seen in Sn-deficient mice maintained under specific pathogen-free conditions (Oetke et al. 2006). The sialomucin CD43 on T-cells (van den Berg et al. 2001b) and mucin-1 on breast cancer cells have both been identified as putative Sn counter-receptors.

The rat murine and rat alveolar macrophages express Sn and that sialic acid-dependent receptor (SAR) and sheep erythrocyte receptor (SER)-like activities are mediated by Sn. The mouse and rat Sn on macrophages can function as a lymphocyte adhesion molecule. Sn has been implicated in cellular interactions of stromal macrophages with developing myeloid cells. In all assays, Sn exhibited specific, differential binding to various murine cell populations of hemopoietic origin. In rank order, sialoadhesin bound neutrophils > bone marrow cells = blood leukocytes > lymphocytes > thymocytes. Single-cell analyses confirmed that Sn selectively bound myeloid cells in complex cell mixtures obtained from the bone marrow and blood. This gives the notion that Sn is involved in interactions with granulocytes at different stages of their life histories (Crocker et al. 1995). Thus, Sn provides the example of a macrophage-restricted lymphocyte adhesion molecule (van den Berg et al. 1992). Umansky et al. (1996) described the functional role of SER^+ spleen macrophages in antigen processing and presentation to T lymphocytes. In two syngeneic murine tumor systems (ESb-MP and lacZ transduced ESbL T-lymphoma cells), it was suggested that in situ-activated SER^+ macrophages contribute to host resistance against metastasis (Umansky et al. 1996). The in vivo interactions of T lymphocytes in the Tn syndrome with CD22 are not likely to be affected, whereas adhesion mediated by Sn or MAG could be strongly reduced (Mrkoci et al. 1996).

Importantly, Sn-mediated interactions appear to be important for effective killing of tumor cells by CTLs in a murine tumor model in vivo (van den Berg et al. 1996). Although Sn function is not known, it is involved in the attachment and internalization of certain viruses (Hartnell et al. 2001; Vanderheijden et al. 2003), and has the potential to endocytose sialylated bacteria such as *Neisseria meningitidis* (Jones et al. 2003). In addition, Sn has been known to promote adhesion of macrophages to T cells and to other cell types such as neutrophils and macrophages (Muerkoster et al. 1999). Because Sn is expressed on subsets of inflammatory macrophages, it can serve as a marker of activation. Anti-inflammatory treatments, for instance with IL-11, are associated with reduction in overall tissue damage with a selective decrease in the number of Sn^+ macrophages (Lai et al. 2001).

Generation of Activated Sn-Positive Microglia During Retinal Degeneration: The retina contains a rich network of myeloid-derived cells (microglia) within the retinal parenchyma and surrounding vessels. Their response and behavior during inflammation and neurodegeneration has been examined during the onset of photoreceptor degeneration in the rods of mouse and to assess their role in photoreceptor apoptosis. During retinal degeneration, activated microglia expressed Sn. The temporal relationship between photoreceptor apoptosis and microglial response suggests that microglia are not responsible for the initial wave of photoreceptor death, and this was corroborated by the absence of iNOS and nitrotyrosine. Expression of Sn may indicate blood-retinal barrier breakdown, which has immune implications for subretinal gene therapeutic strategies (Hughes et al. 2003).

16.3.7 Lessons from Animal Experiments

Sn is expressed at high levels on discrete subsets of tissue macrophages, particularly those found in secondary lymphoid tissues (Munday et al. 1999). High expression is also seen in chronic inflammatory diseases such as rheumatoid arthritis (Hartnell et al. 2001), atherosclerosis (Gijbels et al. 1999) and models of inherited demyelinating diseases of the nervous system (Ip et al. 2007). Sn-deficient mice exhibit changes in B- and T-cell populations suggesting that sialoadhesin regulates cells of immune system rather than influencing steady-state hematopoiesis (Oetke et al. 2006). In experimental autoimmune uveoretinitis (EAU), different macrophage surface markers are expressed during different stages of EAU. Sn expression occurs at peak and later stages of the disease, not during initiation of EAU Jiang et al. (1999). In Sn-deficient (Sn-KO) mice model, EAU was reduced in severity in the initial stages. Furthermore, activated T cells from the draining lymph nodes of Sn-KO mice secreted lower levels of IFN-γ. It suggested that Sn plays a role in "fine tuning" of the immune response to autoantigens by modulating T cell priming (Avichezer et al. 2000; Jiang et al. 2006).

Role in Renal Disease: Accumulation of Sn^+ macrophages is a marker of disease progression versus remission in rat mesangial proliferative nephritis. Sn^+ macrophages were localized in areas of focal glomerular and interstitial damage. Accumulation of Sn^+ macrophage subset in the kidney correlated with proteinuria and histologic damage. It suggests that Sn^+ macrophages may play an important role in progressive renal disease (Ikezumi et al. 2005). IL-11, a cytokine with anti-inflammatory activity reduced the number of Sn^+ macrophages in nephrotoxic nephritis rats and markedly reduced glomerular injury and macrophage Sn expression, but without an alteration of macrophage

numbers, suggesting that IL-11 may be acting in part to reduce macrophage activation (Lai et al. 2001).

Sialoadhesin Deficiency and Myelin Degeneration: Mouse mutants heterozygously deficient for the myelin component P0 mimic some forms of inherited neuropathies and offer future treatment strategies for inherited demyelinating neuropathies in humans. During search of possible role of macrophage-restricted Sn in the pathogenesis of inherited demyelination in $P0^{+/-}$ mice, it was found that most peripheral nerve macrophages express Sn in the mutants. Myelin mutants devoid of Sn showd reduced numbers of $CD8^+$ T lymphocytes and macrophages in peripheral nerves and less severe demyelination, resulting in improved nerve conduction properties (Kobsar et al. 2006). By cross-breeding these mutants with RAG-1-deficient mice lacking mature lymphocytes, pathogenetic impact of the $CD8^+$ cells was demonstrated. Ip et al. (2007) investigated the pathogenetic impact of $CD11b^+$ macrophages by cross-breeding the myelin mutants mice deficient for Sn. In the wild-type mice, Sn is barely detectable on $CD11b^+$ cells, whereas in the myelin mutants, almost all $CD11b^+$ cells expressed Sn. In the double mutants, upregulation of $CD8^+$ T-cells and $CD11b^+$ macrophages was reduced and pathological alterations were ameliorated. These results suggest that in a genetically caused myelin disorder of CNS, macrophages expressing Sn partially mediate pathogenesis. These results have substantial impact on treatment strategies for leukodystrophic disorders and some forms of multiple sclerosis.

16.3.8 Interactions with Pathogens

Candida albicans yeast cells specifically adhere to mouse macrophages in the splenic marginal zone and in lymph node subcapsular and medullary sinuses. These macrophages express Sn that binds erythrocytes, but binding of yeast cells is not mediated by Sn (Han et al. 1994). *Trypanosoma cruzi* is a parasite with large amounts of sialic acid residues exposed at its surface that seems to be involved in macrophages infection. Some macrophages, present in *T. cruzi* infected tissues, expresses Sn. Sn was induced in mice peritoneal macrophages by homologous serum (HS) cultivation. Desialylation reduced the association of parasites to HS cultured macrophages indicating importance of Sn. Sn positive macrophages seem to be important in the initial trypomastigote infection and in the establishment of Chagas disease (Monteiro et al. 2005).

Internalization of Porcine Arterivirus: Porcine Sn mediates internalization of the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) in alveolar macrophages (Vanderheijden et al. 2003). α2-3- and α2-6-linked sialic acids on the virion are important for PRRSV infection of porcine alveolar macrophages (PAM). It suggested that pSn is a sialic acid binding lectin which interacts with sialic acid on PRRS virion and essential for PRRSV infection of PAM (Delputte and Nauwynck 2004, 2006; Genini et al. 2008). The p210 protein involved in infection of PAM showed sequence identities ranging from 56% to 91% with mouse Sn. The full p210 cDNA sequence (5,193 bp) shared 69 and 78% amino acid identity, respectively, with mouse and human sialoadhesins. Results show that sialoadhesin is involved in the entry process of PRRSV in PAM (Vanderheijden et al. 2003). Study of attachment kinetics of PRRSV to macrophages revealed that early attachment is mediated mainly via an interaction with heparan sulphate, followed by a gradual interaction with Sn. By using wild-type CHO and CHO deficient in heparan sulphate expression, it was shown that heparan sulphate alone is sufficient to mediate PRRSV attachment, but not entry, and that heparin sulphate is not necessary for Sn to function as a PRRSV internalization receptor, but enhances the interaction of the virus with Sn (Delputte et al. 2005).

In addition to its role in cell adhesion, Sn has also been shown to facilitate pathogen interactions. For example, Sn can promote macrophage uptake of sialylated strains of *Neisseria meningitidis* (Jones et al. 2003) and functions in endocytosis of the macrophage/monocyte-tropic PRRSV (porcine reproductive and respiratory syndrome virus) (Delputte et al. 2005) (Fig. 16.4) and internalization is thought to be triggered via interactions between Sn and sialylated N-linked glycans present on the four structural viral glycoproteins GP_2–GP_5 of PRRSV (Vanderheijden et al. 2003; Delputte and Nauwynck 2004). HIV-1 has also been shown to interact with Sn (Rempel et al. 2008). During acute period of HIV-1 infection, IFNγ is produced by NK cells and T-cells, and IFNα released by pDCs (plasmacytoid dendritic cells) as part of antiviral response may lead to induction of Sn on monocytes. This in turn binds to the virus in a sialic acid-dependent manner and may permit *trans*-infection of permissive cells and the delivery and distribution of HIV-1 to target cells. This suggests that Sn may be involved in both capture of free virus through sialylated glyconjugates on target cells such as T-cells, and enhancing the binding of HIV-1 gp120 to its cognate receptors (Rempel et al. 2008).

16.4 CD22 (Siglec-2)

16.4.1 Characterization and Gene Organization

The human CD22 gene is expressed specifically in B lymphocytes and likely has an important function in cell-cell interactions. Sequence analysis of a full-length B cell cDNA clone revealed an open reading frame of 2,541 bases coding for a predicted protein of 847 amino acids with a molecular mass of 95 kDa. The B lymphocytes-CAM cDNA is nearly identical to a cDNA clone for CD22, with the exception of an additional 531 bases in the coding region of BL-CAM. BL-CAM has a predicted transmembrane spanning region and a 140-amino acid intracytoplasmic domain. This protein had significant homology with three homotypic cell adhesion proteins: carcinoembryonic antigen (29% identity over 460 amino acids), MAG, and neural cell adhesion molecule (NCAM) (21.5% over 274 amino acids). BL-CAM mRNA expression was increased after B cell activation with *S. aureus* Cowan strain 1 and phorbol myristate acetate, but not by various cytokines. An antisense BL-CAM RNA probe revealed expression in B cell-rich areas in tonsil and lymph node, although the most striking hybridization was in the germinal centers (Wilson et al. 1992).

Genomic Structure: A full length human CD22 cDNA clone, spread over 22 kb of DNA, is composed of 15 exons. The first exon contains the major transcriptional start sites. The translation initiation codon is located in exon 3, which also encodes a portion of signal peptide. Exons 4–10 encode the seven Ig domains of CD22, exon 11 encodes the transmembrane domain, exons 12–15 encode the intracytoplasmic domain of CD22, and exon 15 also contains the 3' untranslated region. A minor form of CD22 mRNA results from splicing of exon 5 to exon 8, skipping exons 6 and 7. TheCD22 gene is located within the band region q13.1 of chromosome 19. Two closely clustered major transcription start sites and several minor start sites were mapped by primer extension. Similarly to many other lymphoid-specific genes, the CD22 promoter lacks an obvious TATA box. Approximately 4 kb of DNA 5' of the transcription start sites were sequenced and found to contain multiple Alu elements. Potential binding sites for the transcriptional factors NF-kB, AP-1, and Oct-2 are located within 300 bp 5′ of the major transcription start sites. A 400-bp fragment (bp −339 through +71) of CD22 promoter region was found to be active in both B and T cells (Wilson et al. 1991, 1993).

Gene Variations in Autoimmune Diseases: Investigations of the pathogenic role of autoantibodies in rheumatic diseases, studies suggest a more central role of B cells in the maintenance of the disease beyond just being precursors of (auto) antibody-producing plasma cells. Detailed analyses have implicated a number of surface molecules and subsequent downstream signaling pathways in regulation of events induced by BCR engagement. After screening of CD22 coding region, seven non-synonymous and four synonymous substitutions were found associated with rheumatic diseases in Japanese patients with systemic lupus erythematosus (SLE), and patients with rheumatoid arthritis (RA). In addition, single base substitutions were found in two introns flanking exon-intron junctions. Among these variations, Q152E substitution within the second extracellular domain was observed with a marginally higher frequency in patients with SLE (3/68, 4.4%) than in healthy individuals (1/207, 0.5%), although this difference was no longer significant after correction for number of comparisons. No significant association was observed between any of the variations and RA (Hatta et al. 1999).

Activating and inhibitory receptors have been implicated both in human systemic sclerosis (SSc) and tight-skin mouse, a model for SSc. A SNP in CD22 were genotyped in Japanese patients with SSc. At c.2304 C > A SNP coding for a synonymous substitution in exon 13, A/A genotype was observed in six patients with SSc (4.8%) but none in the controls. All six patients with A/A genotype belonged to lcSSc subgroup (7.6%). Surface expression level of CD22 tended to be lower in B cells from the patients with A/A genotype as compared with C/A or C/C genotype (17% decrease). Taken together with observation on CD19 polymorphism, the expression level of CD22 was suggested to play a causative role in a proportion of patients with lcSSc (Hitomi et al. 2007).

16.4.2 Functional Characteristics

CD22/Siglec-2 is a B cell–specific glycoprotein expressed in the cytosol of pre- and pro-B cells, and on the plasma membrane of mature B cells (Cyster and Goodnow 1997; Law et al. 1994, 1996; Tedder et al. 1997). The predominant form of cell surface CD22 (CD22ß) is a 140-kDa type I transmembrane protein. The CD22 associates with B cell receptor (BCR) both physically and functionally (Leprince et al. 1993; Peaker and Neuberger 1993) and negatively regulates BCR signaling (Doody et al. 1995). Six tyrosine residues are located in the cytosolic portion of CD22, and BCR stimulation can induce phosphorylation on some of them. CD22-deficient mice generally show a hyperreactive B cell phenotype (Nitschke et al. 1997; O'Keefe et al. 1996; Otipoby et al. 1996), affirming that the primary function of CD22 is to dampen BCR signaling.

Studies suggest that cell surface CD22 undergoes constitutive endocytosis and degradation (Chan et al. 1998; Shan and Press 1995). Because CD22 expression level on B cells is significantly reduced in ST6Gal-I knockout mouse, where

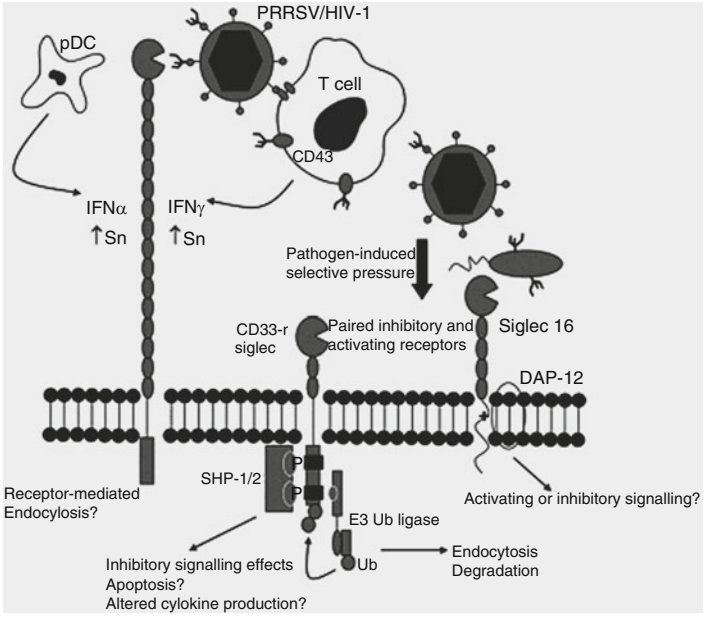

Fig. 16.4 Sn is a highly extended siglec which mediates cell–cell and cell-matrix interactions. Sn expression is induced by IFNs produced by pDCs and T-cells, and it acts both as an endocytic receptor for PRRSV as well as a potential mediator of the *trans*-infection of T-cells by HIV-1. Sialylated pathogens may have driven the evolution of siglecs such as siglec-14 and -16 into DAP-12-associated activating receptors which are paired with inhibitory counterparts such as siglec-5 and -11. Tyrosyl phosphorylation, ubiquitination and internalization are mechanisms by which siglecs transmit their inhibitory and activating signals in response to ligand binding (*Ub* ubiquitin) (Adapted by permission from Crocker and Redelinghuys (2008) © Biochemical Society)

CD22 ligand formation is abolished (Hennet et al. 1998), it is possible that cis binding sialic acid ligands might help maintain optimal CD22 levels on the cell surface by restricting its rate of endocytosis. Zhang and Varki (2004), while examining antibody-mediated endocytosis of CD22, an issue that is important in anti-CD22-based immunotherapy of B cell leukemias and lymphomas (Tuscano et al. 2003), suggested a mechanism how sialic acid-binding and sIgM ligation affect these processes. An approach for simultaneous biotinylation and cross-linking showed that CD22 associates with CD45 and sIgM, possibly involving cell surface multimers of CD22. Sialic acid removal or mutation of a CD22 arginine residue required for sialic acid recognition did not affect these associations even in human:mouse heterologous systems, indicating that they are primarily determined by evolutionarily conserved protein–protein interactions. Thus masking of the sialic acid-binding site of CD22 involves many cell surface sialoglycoproteins, without requiring specific ligand(s) and/or is mediated by secondary interactions with sialic acids on CD45 and sIgM (Zhang and Varki 2004).

N-Linked Glycosylation Site in Ligand Recognition: Site-specific mutagenesis of potential N-linked glycosylation sites on CD22 showed that mutation of a single potential N-linked glycosylation site in first Ig-domain of CD22 completely abrogates ligand recognition. Interestingly, this site is characterized by the sequence NCT, where the cysteine is thought to be involved in an intrachain disulfide bond. Site-directed mutagenesis of similar NC(T/S) motifs in the first or second Ig domains of MAG, and Sn did not disrupt their ability to mediate sialic acid binding. In contrast, mutation of a NCS motif in first Ig domain of CD33 (Siglec-3) unmasked its sialic acid binding activity. Thus, a single N-linked glycosylation site located at a similar position in the CD22 and CD33 glycoproteins is critical for regulating ligand recognition by both receptors (Nath et al. 1995; Sgroi et al. 1996).

16.4.3 Ligands of CD22

As in other members of the family, the extracellular N-terminal Ig domain of CD22 binds to glycan ligands containing sialic acid, which are highly expressed on B-cell glycoproteins, in a highly linkage specific manner. Human and mouse CD22 bind selectively to 2-6-linked sialic acids (Engel et al. 1993; Kelm et al. 1994; Powell et al. 1995; Powell and Varki 1994; Sgroi et al. 1996). The amino-terminal Ig-like V-set domain is critical in this binding (Engel et al. 1995), requiring a conserved arginine residue that likely forms a salt bridge with the carboxylate

groups of sialic acid ligand (Van der Merwe et al. 1996). Like most of the other Siglecs, CD22 is natively bound to sialylated cis ligands on the same cell surface (Razi and Varki 1998, 1999). This "masking" effect is abolished by sialidase treatment, and a small amount of "unmasking" has been found on activated human B cells (Razi and Varki 1998). Desialylation of in vivo macrophage sialylconjugates enhances Sn-mediated lectin activity. Receptor sialylation of soluble Sn inhibits its binding to Jurkat cell ligands, and that charge-dependent repulsion alone cannot explain this inhibition. Moreover, the inhibitory effect of sialic acid is partially dependent on the presence of an intact exocyclic side chain. Sialylation of siglecs by specific glycosyltransferases may be a common mechanism by which siglec-mediated adhesion is regulated (Barnes et al. 1999). Thus, different studies showed that cis binding of sialic acid by CD22 is required for its optimal function as an inhibitory regulator of BCR (Jin et al. 2002; Kelm et al. 2002). Notably, CD45, surface IgM and other glycoproteins that bind to CD22 in vitro do not appear to be important cis ligands of CD22 in situ. Instead, CD22 seems to recognize glycans of neighboring CD22 molecules as cis ligands, forming homomultimeric complexes (Han et al. 2005).

The physiological cis ligands for CD22 are not well defined (Tedder et al. 1997). Several molecules that carry 2-6-linked sialic acids, for example, cell surface IgM (sIgM) or CD45, as well as circulating glycoproteins such as IgM and haptoglobin have been suggested as candidate ligands (Hanasaki et al. 1995a; Sgroi et al. 1996). Studies hypothesize that associations of CD22 with sIgM and CD45 are mediated by CD22 recognition of their sialic acid residues (Collins et al. 2002; Cyster and Goodnow 1997). Support for this hypothesis comes from the finding that B cells from mice deficient in sialylated CD22 ligands show constitutive unmasking of CD22 and altered sIgM signaling responses (Hennet et al. 1998). However, there is as yet no direct proof for a role of sialic acids in forming or maintaining specific interactions of other proteins with CD22. It was shown that although a low-density CD22-Fc column selectively interacted with sIgM and haptoglobin from blood plasma, a high-density column could bind most of the 2–6 sialylated glycoproteins in the sample (Hanasaki et al. 1995a, b). Mild detergent extracts detected very limited (1–2%) interactions of CD22 with sIgM (Law et al. 1994, 1996; Leprince et al. 1993; Peaker and Neuberger 1993).

Binding to CD45 and Synthetic Oligosaccharide: One prominent ligand for CD22 is the highly glycosylated leukocyte surface protein CD45, which carries α2-6-linked sialic acid on N-glycans. In situ desialylation and resialylation of immobilized CD45-thy, mouse CD22 binds to the sialoglycoconjugate NeuGc α2-6Gal β1-4GlcNAc on CD45-thy N-glycans. Evidences indicate that cell adhesion molecules (CAMs) of Ig family use GFCC'C β-sheet of membrane-distal V-set domains that bind structurally different ligands. Such surface is favored for cell-cell recognition (van der Merwe et al. 1996). Using surface plasmon resonance, thermodynamic analysis of CD22 binding to native CD45 showed a low affinity (K_D = 130 μM at 25 º C) with very fast kinetics. Binding reaction was enthalpically driven at physiological temperatures, as found in most lectin-carbohydrate interactions. Since CD22 binds preferably to CD45, even though many cell surface proteins carry α2-6-linked sialic acid, comparison of affinities of CD22 to CD45, with other sialoglyco-conjugates carrying α2-6-linked sialic acid as CD4, and to a synthetic sialoglyco-conjugate, did not differ significantly. This suggested that CD22 binds preferentially to CD45 not because the latter presents higher affinity ligands but because it carries multiple copies of CD45 (Bakker et al. 2002).

Surface 9-O-Acetylation and Recognition Processes: O-Acetylation of 9-hydroxyl group of sialic acids has been suggested to modify various recognition phenomena involving these molecules. The extent of 9-O-acetylation of surface sialic acids on murine erythroleukemia (MEL) cells can be modified by various manipulations, including differentiation, nocodazole treatment, and 9-O-acetyl esterase treatment (Shi et al. 1996a). Induced differentiation of MEL cells causes resistance to lysis, and this correlates directly with extent of decrease in 9-O-acetylation. A similar resistance to alternative pathway lysis can be obtained by selective enzymatic removal of 9-O-acetyl groups from sialic acids. Thus, a 9-O-acetyl group added to the side chain of cell surface sialic acids may abrogate its normal function in restricting alternative pathway activation. MEL cells are also known to have cell surface ligands for Sn and CD22. Sialoadhesin (but not CD22) binding is selectively enhanced by differentiation-induced loss of cell surface 9-O-acetylation and by direct enzymatic removal of ester groups. Since Sn is expressed on some macrophages in vivo, it was reasoned and proved that tissue homing of MEL cells might be affected by O-acetylation. In particular, de-O-acetylation caused significant increase in homing to the liver and spleen. These results indicate that cell surface 9-O-acetylation can affect a variety of biological recognition phenomena and provide a system for further exploration of molecular mechanisms involved (Shi et al. 1996b).

Sialylated Multivalent Antigens Inhibit B Cell Activation: Although antigens can display CD22 ligands, the receptor is known to bind to α2-6-sialylated glycan as a specific ligand on the cell surface. Kimura et al. (2007) proposed that α2-6-sialylated and 6-GlcNAc-sulfated

determinant serve as a preferred ligand for CD22. The α2-6-sialylated 6-sulfo-LacNAc determinant serves as an endogenous ligand for human CD22 that suggests the possibility that 6-GlcNAc sulfation as well as α2-6-sialylation may regulate CD22/Siglec-2 functions in humans.

A ligand for CD22 was identified on human T cells as a low molecular mass isoform of leukocyte common Ag, CD45RO. Murine and human sequences overall have 62% identity, which includes 18 of 20 extracellular cysteines and six of six cytoplasmic tyrosines. BHK cells transfected with mCD22 cDNA specifically adhere to resting and activated T lymphocytes and in addition bound activated, but not resting, B cells. The propinquity of CD22 and cell-surface glycoprotein ligands has led to the conclusion that the inhibitory properties of the receptor are due to cis interactions. Courtney et al. (2009) examined the functional consequences of trans interactions by employing sialylated multivalent antigens that can engage both CD22 and the BCR. Exposure of B cells to sialylated antigens results in the inhibition of key steps in BCR signaling. These results reveal that antigens bearing CD22 ligands are powerful suppressors of B cell activation. The ability of sialylated antigens to inhibit BCR signaling through trans CD22 interactions reveals a previously unrecognized role for the Siglec-family of receptors as modulators of immune signaling.

Loss of N-Glycolylneuraminic Acid in Human Evolution: The common sialic acids of mammalian cells are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Humans are exception, because of a mutation in cytidine-50-monophosphate (CMP)-sialic acidhydroxylase, which occurred after our ancestor, great apes. The resulting loss of Neu5Gc and increase in Neu5Ac in humans altered the biology of the siglecs, which recognize sialic acids. Human siglec-1 (Sn) strongly prefers Neu5Ac over Neu5Gc. Thus, humans have a higher density of siglec-1 ligands than great apes. Siglec-1-positive macrophages in humans are found primarily in perifollicular zone, whereas in chimpanzees they also occur in the marginal zone and surrounding the periarteriolar lymphocyte sheaths. Although only a subset of chimpanzee macrophages expresses siglec- 1, most human macrophages are positive. A known evolutionary difference is the strong preference of mouse siglec-2 (CD22) for Neu5Gc, contrasting with human siglec-2, which binds Neu5Ac equally well. In fact, siglec-2 had evolved a higher degree of recognition flexibility before Neu5Gc was lost in humans. Human siglec-3 (CD33) and siglec-6 (obesity-binding protein 1) also recognize both Neu5Ac and Neu5Gc, and siglec-5 may have some preference for Neu5Gc. Others showed that siglec-4a (MAG) prefers Neu5Ac over Neu5Gc. In fact, siglec-2 had evolved a higher degree of recognition flexibility before Neu5Gc was lost in humans. Thus, the human loss of Neu5Gc may alter biological processes involving siglec-1, and possibly, siglec-4a or -5 (Brinkman-Van der Linden et al. 2000).

16.4.4 Regulation of CD22

IFNα, a potent antiviral cytokine and immune modulator, induces Sn expression in monocytes which normally do not express the receptor and also to increase Sn expression in macrophages (York et al. 2007). IFNγ produced by activated T-cells and NK cells has also been shown to induce Sn expression on monocytes (Hartnell et al. ; Rempel et al. 2008). The induction of Sn expression in cells of the monocyte–macrophage lineage by IFNs may play a role in the potentiation of inflammatory diseases including rheumatoid arthritis (where Sn serves as a restricted inflammatory marker for tissue macrophages), systemic sclerosis (York et al. 2007), SLE (systemic lupus erythematosus) (Biesen et al. 2008) and proliferative glomerulonephritis (Ikezumi et al. 2005).

Inhibitory receptors are involved in negatively regulating B cell immune response and in preventing autoimmunity. The IL-4 reduces expression of CD22, FcγRII (CD32), CD72, and paired Ig-like receptor (PIR)-B on activated B cells at m RNA and protein level. This reduced expression is dependent on continuous exposure to IL-4 and is mediated through Stat6 (Rudge et al. 2002). Furthermore, treatment of SLE patients with glucocorticoids resulted in a strong decrease in Sn^+ monocytes, suggesting that Sn may be a useful biomarker for disease monitoring in response to therapeutic treatments. High expression is seen in chronic inflammatory diseases such as rheumatoid arthritis (Hartnell et al. 2001), atherosclerosis (Gijbels et al. 1999) and models of inherited demyelinating diseases of the nervous system (Ip et al. 2007). Moreover, a correlation between the frequency of circulating Sn^+ monocytes and the titres of anti-dsDNA (double-stranded DNA) auto-antibodies indicates that the expression of Sn closely parallels extent of SLE disease (Biesen et al. 2008).

Mucins isolated from colon cancer cells and bovine submaxillary mucins bound to CD22 cDNA transfectants and a human B cell line, Daudi cell. Results suggest that in the tumor-bearing state a portion of the mucins in the bloodstream was taken up by the spleen and ligated to CD22 expressed on splenic B cells, which may have led to down-regulation of signal transduction (Toda et al. 2008).

16.4.5 Functions of CD22

B cells express two members of the Siglec family, CD22 (Siglec-2) and Siglec-G, both of which have been shown to inhibit B-cell signaling. Interaction with antigen-presenting accessory cells is thought to be an important step in B-cell activation, and the B-cell receptor CD22, which is coordinately expressed with surface immunoglobulin, has been proposed to participate in the antigen response. Stamenkovic and Seed (1990) showed that CD22, a structure related to MAG (a NCAM), mediates monocyte and erythrocyte adhesion. Like CD2, the CD22 may facilitate antigen recognition by promoting antigen-nonspecific contacts with accessory cells.

Regulation of B Cell Development and B Cell Signaling by CD22: Engagement of CD22 with a mAb (HB22.23) results in rapid CD22 tyrosine phosphorylation and in increased association of CD22 with p53/56lyn kinase, p85 phosphatidyl inositol-3 kinase, and p72syk kinase. Synthetic peptides that span various regions of CD22 showed that these kinases associated with a tyrosine-phosphorylated peptide, which spans tyrosine amino acid residues 822 and 842, and implicate this as an important region in mediating CD22 signal transduction. Engagement of CD22 with HB22.23 was sufficient to stimulate normal B cell proliferation and indicates CD22 as a B lymphocyte signaling molecule (Tuscano et al. 1996). CD22 is an inhibitory co-receptor of BCR-mediated signaling and binds specifically to glycan ligands containing α2,6-linked sialic acids. B cells deficient in enzyme (ST6Gal I) that forms the CD22 ligand (α2,6-linked sialic acids) show suppressed BCR signaling. Mice deficient in receptor/ligand pair (double mutant) in both ST6GalI-deficient and ST6GalI x CD22 showed normal B cell development, but an impaired marginal zone B cell population in the spleen. Both types of mutant mice also showed a reduced population of bone marrow recirculating B cells, a defect previously detected in $CD22^{-/-}$ mice. This suggests a direct involvement of CD22 and its ligands α2,6-linked sialic acid in a homing process of recirculating B cells to the bone marrow. Interestingly, defective B cell Ca^{2+} signaling and proliferation of $ST6Gal^{-/-}$ mice was rescued in ST6GalI x CD22-deficient mice. These studies suggest an important function for CD22-ligand interaction in regulating BCR signal and microdomain localization (Collins et al. 2006b; Ghosh et al. 2006). It has been observed that CD22 recruits the tyrosine phosphatase SHP-1 ITIMs and inhibits BCR-induced Ca^{2+} signaling on normal B cells. CD22 interacts specifically with ligands carrying α2-6-linked sialic acids. Interaction with these ligands in cis regulates the association of CD22 with BCR and thereby modulates the inhibitory function of CD22. Interaction of CD22 to ligands in trans can regulate both B-cell migration as well as BCR signaling threshold (Nitschke 2009).

Siglec-G is a recently identified protein with an inhibitory function restricted to a B-cell subset, the B1 cells. Siglec-G inhibits Ca^{2+} signaling specifically in these cells. In addition, it controls the cellular expansion and antibody secretion of B1 cells. Nitschke (2009) indicated that both Siglecs CD22 and Siglec-G modulate BCR signaling on different B-cell populations in a mutually exclusive fashion.

Recognition of Pathogens and Endocytosis: Antibody ligation of siglec proteins initiates their endocytosis, suggesting that endocytic activity is also a general property of this subfamily (Biedermann et al. 2007; Jones et al. 2003; Nguyen et al. 2006; Walter et al. 2005; Zhang et al. 2006). Over 20 pathogenic microorganisms express sialic acid-containing glycans on their surface (Crocker 2005). Demonstration of the binding or uptake of several sialylated pathogens, including *Neisseria meningitidis*, *Trypanosoma cruzi*, *Campylobacter jejuni*, and group B *Streptococcus*, by Sn and Siglec-5, -7, and -9 has suggested various roles of these siglec proteins in the immune responses to these organisms (Avril et al. 2006; Crocker 2005; Delputte et al. 2005; Jones et al. 2003; Monteiro et al. 2005). Since mechanisms of pathogen entry and engulfment are increasingly recognized to involve the host cells' endocytic machinery, the endocytic functions of the siglec proteins are also relevant to their roles in pathogen recognition and uptake.

16.4.6 Signaling Pathway of Human CD22 and Siglec-F in Murine

16.4.6.1 Many siglec proteins contain one or more ITIMs

Many siglec proteins contain one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs), (I/L/V)XYXX (L/V), suggesting that they play important roles as inhibitory receptors of cell signaling (Crocker 2005; Varki and Angata 2006), as exemplified by CD22, which is well documented as a regulator of BCR signaling. Upon antigen binding to the BCR, the ITIMs of CD22 are quickly tyrosine phosphorylated and recruit protein tyrosine phosphatase SHP-1, which dephosphorylates the BCR and dampens the B-cell response, setting a threshold for B-cell activation (Crocker et al. 2007; Tedder et al. 2005).

CD22 is also known to undergo endocytosis following ligation by anti-CD22 antibody (Jones et al. 2003) or high-affinity multivalent-sialoside ligands (Collins et al. 2006a). Sn and most CD33-related siglec proteins are expressed on cells of the innate immune system, including monocytes, macrophages, neutrophils, eosinophils, and dendritic cells (Crocker 2005; Lock et al. 2004; Nguyen et al. 2006; Zhang et al. 2004). Like CD22, ligation of CD33-related

siglec proteins (CD33 and Siglec-5, -7, and -9) also induces recruitment of SHP-1 via phosphorylated ITIMs (c/r Avril et al. 2005).

Murine Siglec-F is predominately expressed on eosinophils (Zhang et al. 2004). Eosinophils are best known for their role in allergic diseases. Siglec-F null mice revealed that Siglec-F is a negative regulator of eosinophil response to allergens (Zhang et al. 2007). Eosinophils also contribute to an immune response against foreign pathogens, including binding, engulfment, and killing of microbes (Galioto et al. 2006; Inoue et al. 2005). Tateno et al. (2007) investigated the endocytic pathways of CD22 (Siglec-2) and mouse eosinophil Siglec-F that are expressed on cells of the innate immune system. Though both the Siglecs required intact cytoplasmic ITIM motifs, they differed in molecular mechanisms in endocytic pathways, suggesting that these two siglec proteins evolved distinctly (Tateno et al. 2007).

During endocytosis following ligation by anti-CD22 antibody (Jones et al. 2003) or high-affinity multivalent-sialoside ligands (Collins et al. 2006a), the tyrosine-based ITIMs of CD22 also fit the sorting signal YXXØ (where Ø is a hydrophobic residue) for association with the adaptor complex 2 (AP2), which directs recruitment of receptors into clathrin-coated pits (Bonifacino and Traub 2003). John et al. reported that CD22 associates with the AP50 subunit of AP2 through these tyrosine-based motifs and that they are required for endocytosis (John et al. 2003). Consistent with this observation, CD22 is predominantly localized in clathrin-rich domains (Collins et al. 2006b; Grewal et al. 2006). Antigen ligation of the BCR results in mobilization of the BCR to "activation rafts," which subsequently fuse with clathrin domains prior to endocytosis (Stoddart et al. 2002; Stoddart et al. 2005). Since CD22 is specifically excluded from activation rafts (Pierce 2002), the negative regulatory effect of CD22 on BCR signaling has been proposed to occur following its movement to clathrin domains (Collins et al. 2006b), linking the endocytic activity of CD22 to its role in regulation of BCR signaling.

16.4.6.2 PTKs, PTPs and PLCγ1 in signal transduction in B cell activation

CD22 interacts specifically with ligands carrying α2-6-linked sialic acids. Interaction with these ligands in cis regulates the association of CD22 with the BCR and thereby modulates the inhibitory function of CD22. Interaction of CD22 to ligands in trans can regulate both B-cell migration as well as the BCR signaling threshold (Nitschke 2009).

Cross-linking BCR elicits early signal transduction events, including activation of protein tyrosine kinases, phosphorylation of receptor components, activation of phospholipase C-γ (PLC-γ), and increase in intracellular Ca^{2+}. Cross-linking of BCR leads to rapid translocation of cytosolic protein tyrosine phosphatase (PTP)1 C to the particulate fraction, where it became associated with CD22. The association of PTP-1 C with CD22 was mediated by the NH2-terminal SH2 domain of PTP-1 C. Complexes of either CD22/PTP-1 C/Syk/PLC-γ could be isolated from B cells stimulated by BCR engagement or a mixture of hydrogen peroxidase and sodium orthovanadate, respectively. The binding of PLC-γ1 and Syk to tyrosyl-phosphorylated CD22 was mediated by NH2-terminal SH2 domain of PLC-γ1 and the COOH-terminal SH2 domain of Syk, respectively. Results suggest that tyrosyl-phosphorylated CD22 may down-regulate the activity of this complex by dephosphorylation of CD22, Syk, and/or PLC-γ1. Transient expression of CD22 and a null mutant of PTP-1 C (PTP-1CM) in COS cells resulted in an increase in tyrosyl phosphorylation of CD22 and its interaction with PTP-1CM. By contrast, CD22 was not tyrosyl phosphorylated or associated with PTP-1CM in presence of wild-type PTP-1 C. These results suggest that tyrosyl-phosphorylated CD22 may be a substrate for PTP-1 C regulates tyrosyl phosphorylation of CD22.

Binding Site for SH-2-Containing Protein-Tyrosine Phosphatase-1 in CD22: CD22 contains ITIMs in the cytoplasmic region and recruits SHP-1 to the phosphorylated ITIMs upon ligation of BCR, thereby negatively regulating BCR signaling. Among three identified ITIMs, two ITIMs containing tyrosine residues at position 843 (Tyr^{843}) and 863 (Tyr^{863}), respectively, are required for CD22 to recruit SHP-1 and regulate BCR signaling upon BCR ligation by anti-Ig Ab, indicating that CD22 has the SHP-1-binding domain at the region containing (Tyr^{843}) and (Tyr^{863}). Further, the CD22 mutant in which both Tyr^{843} and Tyr^{863} are replaced by phenylalanine (CD22F5/6) recruits SHP-1 and regulates BCR signaling upon stimulation with antigen but not anti-Ig Ab. This suggests that CD22 contains another SHP-1 binding domain that is specifically activated upon stimulation with antigen. Both of the flanking sequences of Tyr^{783} and Tyr^{817} fit the consensus sequence of ITIM, and the CD22F5/6 mutant requires these tyrosine residues for SHP-1 binding and BCR regulation. Thus, these ITIMs constitute a novel conditional SHP-1-binding site of CD22 that is activated upon BCR ligation by antigen but not by anti-Ig Ab (Zhu et al. 2008).

16.4.6.3 Endocytic mechanisms of CD22 and Siglec-F in cell signaling

Tateno et al. (2007) investigated the endocytic pathways of CD22 and mouse eosinophil Siglec-F that are expressed on cells of the innate immune system. Siglec-F showed efficient endocytosis of anti-Siglec antibody, and *Neisseria meningitidis* bearing sialylated glycans. Like CD22, endocytosis was dependent on its cytoplasmic ITIM and ITIM-like

motifs. While endocytosis of CD22 was mediated by a clathrin-dependent mechanism and was sorted to early endosome and recycling compartments, Siglec-F endocytosis was directed to lysosomal compartments and was mediated by a mechanism that was independent of clathrin and dynamin. Like CD22, Siglec-F mediated endocytosis of anti-Siglec-F and sialoside ligands, a function requiring intact tyrosine-based motifs. In contrast, however, Siglec-F endocytosis was clathrin and dynamin independent, required ADP ribosylation factor 6, and trafficked to lysosomes. Comparative results suggest that these two siglec proteins have evolved distinct endocytic mechanisms consistent with roles in cell signaling and innate immunity (Tateno et al. 2007).

16.4.7 CD22 as Target for Therapy

The restricted expression of several siglecs to one or a few cell types makes them attractive targets for cell-directed therapies. The anti-CD33 (also known as Siglec-3) antibody gemtuzumab (Mylotarg) is approved for the treatment of acute myeloid leukemia, and antibodies targeting CD22 (Siglec-2) are currently in clinical trials for treatment of B cell non-Hodgkins lymphomas and autoimmune diseases. Because siglecs are endocytic receptors, they are well suited for a 'Trojan horse' strategy, whereby therapeutic agents conjugated to an antibody, or multimeric glycan ligand, bind to the siglec and are efficiently carried into the cell. Although the rapid internalization of unmodified siglec antibodies reduces their utility for induction of antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity, antibody binding of Siglec-8, Siglec-9 and CD22 has been demonstrated to induce apoptosis of eosinophils, neutrophils and depletion of B cells, respectively. Properties of siglecs that make them attractive for cell-targeted therapies have been reviewed (Kreitman 2006; O'Reilly and Paulson 2009). Anti-CD22 antibodies are theoretically good candidates alone and in combination with other drugs in the treatment of B cell malignancies. Strategies include targeting B-cell surface markers such as CD22, as well as blocking B-cell-activating factors or their receptors. Refinement of existing immunotoxins and development of new immunotoxins are underway to improve the treatment of cancer (Kreitman 2006). CMC-544 (inotuzumab ozogamicin), a CD22-specific cytotoxic immunoconjugate of calicheamicin is intended for treatment of B-lymphoid malignancies. CMC-544 targets CD22 expressed by B-lymphoid malignancies. CMC-544 comprises a humanized IgG4 anti-CD22 mAb, G5/44, covalently linked to CalichDMH via an acid-labile linker. CMC-544 inhibited in vitro growth of acute lymphoblastic leukemia (ALL) cell lines more potently than that of Ramos B-lymphoma cells. In nude mice with established sc xenografts of REH ALL, CMC-544 caused dose-dependent inhibition of xenograft growth producing complete tumor regression and cures in tumor-bearing mice after treatment of conjugated calicheamicin. The anti-leukemia activity of CMC-544 supports clinical evaluation of CMC-544 for the treatment of CD22+ leukemia (Dijoseph et al. 2004, 2007).

B cells play an important role in the pathogenesis of many autoimmune diseases. Different approaches targeting B cell compartment are under investigation. Selective modulation of B cells has been achieved using a humanized mAb against CD22. The antibody (epratuzumab), originally developed for treatment of non-Hodgkin's lymphoma, was found to be effective, with a very good safety profile. Studies have demonstrated the efficacy and safety of epratuzumab in several autoimmune diseases, including systemic lupus erythematosus and primary SjÃ gren's syndrome (Steinfeld and Youinou 2006). Anti-CD22/cal mAb therapy resulted in early and prolonged B-cell depletion and delayed disease in pre-diabetic mice. Importantly, when new-onset hyperglycemic mice were treated with anti-CD22/cal mAb, 100% of B-cell-depleted mice became normoglycemic by 2 days, and 70% of them maintained a state of long-term normoglycemia. Targeting CD22 depletes and reprograms B-cells and reverses autoimmune diabetes, thereby providing a blueprint for development of novel therapies to cure autoimmune diabetes (Fiorina et al. 2008).

Newer methods are in progress to generate multivalent antibodies. The dock and lock (DNL) method is a new technology for generating multivalent antibodies. Rossi et al. (2009) reported characterizations of 20–22 and 22–20, a pair of humanized hexavalent anti-CD20/22 bispecific antibodies (bsAbs) derived from veltuzumab (v-mab) and epratuzumab (e-mab). Each bsAb translocates both CD22 and CD20 into lipid rafts, induces apoptosis and growth inhibition without second-antibody cross-linking, and is significantly more potent in killing lymphoma cells in vitro than their parental antibodies. Results suggest multiple advantages of hexavalent anti-CD20/22 bsAbs over the individual parental antibodies and suggest that these may represent a new class of cancer therapeutics (Rossi et al. 2009).

16.5 Siglec-4 [Myelin-Associated Glycoprotein, (MAG)]

16.5.1 MAG and Myelin Formation

Several glia-associated cell surface molecules have been implicated in myelin formation in CNS and peripheral nervous system (PNS). In PNS, the major peripheral myelin protein PO and the peripheral myelin protein (PMP) 22 are

involved in spiral formation as reflected by retarded myelin formation in mice deficient for the respective molecules. The involvement of myelin-associated glycoprotein (MAG) in this process was detected in mice deficient in both PO and MAG, suggesting that PO can replace MAG during the formation of spiraling loops. For the maintenance of the association of Schwann cell and myelin with its ensheathed axon, the myelin components PO, MAG, and Connexin 32 are crucial. In CNS, recognition of oligodendrocytes and axons and the formation of spiraling loops is mediated by MAG (Martini and Schachner 1997).

16.5.2 Characteristics of MAG

MAG, membrane glycoprotein of 100 kDa, is expressed abundantly early in the myelination process, indicating an important role for MAG in initial stages of myelination. Reports suggest that CNS nerves may be coaxed into full functional regeneration in a clinical setting. However, only a small number of axons regenerate in these studies, in part because of other inhibitory proteins associated with myelin. One candidate for such a protein is myelin-associated glycoprotein (MAG). The MAG is a 100-kD type I transmembrane integral membrane glycoprotein, which is a member of Siglec family. It makes up ~1% of CNS and ~0.1% of PNS myelin proteins (Trapp 1990). The MAG is localized in periaxonal Schwann cell and oligodendroglial membranes of myelin sheaths where it functions in interactions between myelin-forming cells (both oligodendrocytes and Schwann cells) and the axolemma in PNS and CNS.

MAG contains five Ig-like domains and belongs to the Siglec subgroup of Ig superfamily and shares significant homology with neural cell adhesion molecule (N-CAM) (Salzer et al. 1987). MAG is expressed as two developmentally regulated isoforms with different cytoplasmic domains that may activate different signal transduction pathways in myelin-forming cells. MAG contains a carbohydrate epitope shared with other glycoconjugates that is a target antigen in autoimmune peripheral neuropathy associated with IgM γ-pathy and has been implicated in a dying back oligodendrogliopathy in multiple sclerosis (Quarles 2007).

16.5.3 MAG Isoforms

Myelin-associated Glycoprotein can be obtained from adult mouse brain from detergent-lysates of a crude membrane fraction as a 96–100 kDa form (detergent solubilized MAG or L-MAG), and from 100,000 g supernatants of homogenates as a 90–96 kDa form (soluble MAG or S-MAG). Both molecular forms bind to heparin in hypo- and isotonic buffers. Soluble MAG binds to several collagens (type G, I, II, III, IV, V, VI, IX) with a K_D of 5.7×10^{-8} M for collagen type IX and 2.0×10^{-7} for collagen type IV. The MAG is localized in basal lamina and interstitial collagens of the sciatic nerve in situ (Fahrig et al. 1987). It is heavily glycosylated containing 30% carbohydrate by weight. Of the 9 MAG sequons 7 were glycosylated and 1 was partially glycosylated at Asn106. Asn332, which was not recovered in the glycopeptide fractions and one was probably not glycosylated. All MAG glycosylated sequons might bear the L2/HNK-1 epitope (Burger et al. 1993). Two isoforms of the MAG are the result of alternative splicing of the primary MAG transcript. The small (S-MAG) and large (L-MAG) isoforms are identical in their extracellular and transmembrane domains but are distinct at their C-terminal ends. Early in the myelination process expression of L-MAG predominates, whereas S-MAG accumulates in later stages (Inuzuka et al. 1991; Pedraza et al. 1991).

The functions of two MAG isoforms, which differ only in their cytoplasmic domains, are not well known. In rat and mouse, expression of the two forms of mRNA is developmentally regulated; the mRNA without exon 12 portion is expressed mainly in the actively myelinating stage of development. In quaking mouse, the mRNA without a 45-nucleotide exon portion was scarcely expressed throughout development (Fujita et al. 1989). Fujita et al. (1989) determined the structures of three forms of mouse MAG mRNAs. Two forms of mRNAs were reported to be different by alternate inclusion of exon 2 and 12 in rat brain. One of the three forms of clones appeared to be mRNA, which lacked both the exon 2 and 12 portions, although others were identical splicing patterns to those of rat. Northern blot analysis using specific probes to mRNAs with or without the exon 2 portion in normal and quaking mouse confirmed that the splicing of exon 2 and 12 occurred independently (Fujita et al. 1989; Sato et al. 1989).

On human locus MAG is assigned to chromosome 19 and the mouse locus to chromosome 7. Since the region of mouse chromosome 7-known to contain several other genes that are homologous to genes on human chromosome 19-also carries the quivering (qv) locus; the possibility that a mutation in the MAG gene could be responsible for this neurological disorder. While MAG-specific DNA restriction fragments, mRNA, and protein from qv/qv mice were apparently normal in size and abundance, the possibility was not ruled out that qv could be caused by a point mutation in the MAG gene (Barton et al. 1987). The human MAG sequence provides an open reading frame of 1,878 nt encoding a peptide of 626 amino acids with a molecular mass of 69.1 kD. It is 89% homologous to nt sequence to the large isoform of rat MAG, with 95% homology in the amino acid sequence. It contains 9 potential glycosylation sites, one more than in rat, and shares other key features with rat MAG, including 5 Ig-like regions of internal homology, an RGD sequence, and

potential phosphorylation sites. Its structure appears to be highly conserved in evolution, possibly suggesting a close interdependence between its structure and function. The human gene is located on the proximal long arm of chromosome 19 (19q12–q13.2) (Spagnol et al. 1989). In transgenic mouse line that specifically expresses GFP-tagged S-MAG, Erb et al. (2006) reported differential expression pattern and spatial distribution of L- and S-MAG during development as well as in the adult central and peripheral nervous system. In peripheral nerves, where S-MAG is the sole isoform, S-MAG concentrated in different ring-like structures such as periaxonal and abaxonal rings, and discs spanning through the compact myelin sheath perpendicular to the axon. This provides a new insight in the subcellular distribution of MAG isoforms for the understanding of their specific functions in myelin formation.

Interestingly, cytoplasmic region unique to L-MAG contains a tyrosine phosphorylation site, suggesting a role in the regulation of MAG function (Edwards et al. 1988; Jaramillo et al. 1994). MAG is tyrosine-phosphorylated in the developing brain. The major tyrosine phosphorylation at residue 620 interacts specifically with the SH2 domains of phospholipase C (PLCγ). This domain may represent a protein binding motif that can be regulated by tyrosine phosphorylation. MAG also specifically bound the Fyn tyrosine kinase, suggesting that MAG serves as a docking protein that allows the interaction between different signaling molecules (Jaramillo et al. 1994). Gene-targeted mutant mice express a truncated form of L-MAG isoform, eliminating the unique portion of its cytoplasmic domain, but they continue to express S-MAG. Similar to the total MAG knockouts, these animals do not express an overt clinical phenotype. CNS myelin of L-MAG mutant mice displays most of the pathological abnormalities reported for the total MAG knockouts. In contrast to the null MAG mutants, however, PNS axons and myelin of older L-MAG mutant animals do not degenerate, indicating that S-MAG is sufficient to maintain PNS integrity. These observations demonstrate a differential role of L-MAG isoform in CNS and PNS myelin (Fujita et al. 1998).

16.5.4 Ligands of MAG: Glycan Specificity of MAG

Using oligosaccharides with modifications in the sialic acid, galactose or N-acetylglucosamine moieties, it was demonstrated that both MAG and Sn bind with high preference to α2,3-linked sialic acid and interact at least with the three terminal monosaccharide units. An additional sialic acid at position six of the third-terminal monosaccharide unit enhances binding to MAG, whereas it does not influence binding to Sn significantly. The hydroxy groups at positions 8 and 9 are required for binding to both proteins. Surprisingly, MAG binds 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid significantly better than N-acetylneuraminic acid, whereas Sn prefers the latter structure. Thus the interactions of MAG and Sn are mainly with sialic acid and that additional contacts with the subterminal galactose and N-acetylglucosamine residues also contribute to the binding strength, although to a lesser degree (Strenge et al. 1998).

Neuronal Ligands: The carbohydrate binding specificities of three sialoadhesins were compared by measuring lectin-transfected COS cell adhesion to natural and synthetic gangliosides. The neural sialoadhesins, MAG and Schwann cell myelin protein (SMP) had similar and stringent binding specificities. Each required an α2,3-linked sialic acid on the terminal galactose of a neutral saccharide core, and they shared the following rank-order potency of binding: GQ1bα >> GD1a = GT1b >> GM3 = GM4 >> GM1, GD1b, GD3, GQ1b (nonbinders). In contrast, sialoadhesin had less specificity, binding to gangliosides that bear either terminal α2,3- or α2,8-linked sialic acids with the following rank-order potency of binding: GQ1bα > GD1a = GD1b = GT1b = GM3 = GM4 > GD3 = GQ1b >> GM1 (nonbinder). Binding of MAG, SMP, and sialoadhesin was abrogated by chemical modification of either the sialic acid carboxylic acid group or glycerol side chain on a target ganglioside. These results are consistent with sialoadhesin binding to one face of the sialic acid moiety, whereas MAG (and SMP) may have more complex binding sites or may bind sialic acids only in the context of more restricted oligosaccharide conformations (Collins et al. 1997).

Gangliosides, the most abundant sialylated glycoconjugates in brain, may be the functional neuronal ligands for MAG. Cells engineered to express MAG on their surface adhered specifically to gangliosides bearing an α2,3-linked N-acetylneuraminic acid on a terminal galactose, with the following relative potency: GQ1b α >> GD1a, GT1b >> GM3, GM4 (GM1, GD1b, GD3, and GQ1b did not support adhesion). MAG binding was abrogated by modification of the carboxylic acid, any hydroxyl, or the N-acetyl group of the ganglioside's N-acetylneuraminic acid moiety. Related Ig superfamily members either failed to bind gangliosides (CD22) or bound with less stringent specificity (sialoadhesin), whereas a modified form of MAG (bearing three of its five extra-cellular Ig-like domains) bound only GQ1b α. Enzymatic removal of sialic acids from the surface of intact nerve cells altered their functional interaction with myelin. These data are consistent with a role for gangliosides in MAG-neuron interactions (Schnaar et al. 1998).

MAG binds with high affinity and specificity to two major brain gangliosides, GD1a and GT1b, that are

expressed prominently on axons and that bear the MAG-binding terminal sequence NeuAc1-3Galß1-3GalNAc (Collins et al. 1997; Yang et al. 1996). Mice lacking a key enzyme involved in ganglioside biosynthesis, UDP-N-acetyl-D-galactosamine:GM3/GD3 N-acetyl-D-galactosaminyltransferase (EC 2.4.1.92), do not express the NeuAc1-3Galß1-3GalNAc terminus, and display axon degeneration and dysmyelination similar to Mag-null mice (Sheikh et al. 1999), as well as progressive motor behavioral deficits (Chiavegatto et al. 2000). Furthermore, nerve cells from these mice are less responsive to MAG as an inhibitor of neurite outgrowth (Vyas et al. 2002). These and other studies (Vinson et al. 2001; Yamashita et al. 2002) implicate complex brain gangliosides, particularly GD1a and GT1b, as functional MAG ligands.

MAG expression was also decreased in mice lacking complex brain gangliosides (Sheikh et al. 1999). Mice with a disrupted Galgt1 gene lack UDP-GalNAc:GM3/GD3 N-acetylgalactosaminyltransferase (GM2/GD2 synthase) and fail to express complex brain gangliosides, including GD1a and GT1b, instead expressing a comparable amount of the simpler gangliosides GM3, GD3, and O-acetyl-GD3. Sun et al. (2004) indicated that the maintenance of MAG protein levels depends on the presence of complex gangliosides, perhaps due to enhanced stability when MAG on myelin binds to its complementary ligands, GD1a and GT1b, on the apposing axon surface. Data support the conclusion that MAG interaction with complex brain gangliosides markedly affects its steady-state expression.

16.5.5 Functions of MAG

MAG is a bifunctional molecule, which has been implicated in the formation and maintenance of myelin sheaths. CNS myelin formation is delayed in MAG gene mutant mice (Montag et al. 1994; Bartsch et al. 1997). Moreover, oligodendrocytic cytoplasmic collars of mature CNS myelin are frequently missing or reduced, whereas controversy persists with regard to the effect of MAG deficiencies on periaxonal spacing (see Bartsch 1996). Compact myelin of MAG mutants also contains an increased presence of cytoplasmic loops of oligodendrocytes. In contrast, peripheral nervous system (PNS) myelin formation proceeds normally in MAG-deficient animals. Older mutants, however, display PNS axonal and myelin degeneration with the presence of superfluous Schwann cell processes, indicating that MAG plays a critical role in maintaining PNS integrity (Fruttiger et al. 1995).

The axon is dependent on signals from myelin, specifically MAG, for its cytoarchitecture, structure, and long-term stability (Bjartmar et al. 1999). MAG appears to function both as a ligand for an axonal receptor that is needed for the maintenance of myelinated axons and as a receptor for an axonal signal that promotes the differentiation, maintenance and survival of oligodendrocytes. Its function in the maintenance of myelinated axons may be related to its role as one of the white matter inhibitors of neurite outgrowth acting through a receptor complex involving Nogo receptor and/or gangliosides containing 2,3-linked sialic acid. Genetic ablation of MAG results in reduced axon caliber, reduced axon neurofilament spacing and phosphorylation, and progressive axon degeneration (Fruttiger et al. 1995; Li et al. 1994; Montag et al. 1994; Yin et al. 1998). These observations led to conclusion that MAG is an important signaling molecule in myelin–axon interactions and is required for optimal long-term axon stability (Schachner and Bartsch 2000).

MAG Inhibits Neurite Outgrowth in Cell Culture: MAG and Nogo are potent inhibitors of neurite outgrowth from a variety of neurons, and they have been identified as possible components of CNS myelin that prevents axonal regeneration in the adult vertebrate CNS. Nogo, MAG, and -oligodendrocyte-myelin glycoprotein act on neurons through 75 receptor (p75) (Wang et al. 2002a*)* in complex with Nogo receptor (Mimura et al. 2006). Although also found in PNS myelin and abundant in the CNS, MAG inhibits neurite outgrowth in cell culture, and its role in regeneration is controversial (David et al. 1995; Bartsch 1996). It is present in a neurite outgrowth-inhibitory fraction of CNS myelin (David et al. 1995; Bartsch 1996; McKerracher et al. 1994). MAG causes growth cone collapse and inhibits neurite outgrowth from various vertebrate neuronal cell types, including retinal ganglion cells (RGCs) (Li et al. 1996; McKerracher et al. 1994; Mukhopadhyay et al. 1994; Song et al. 1998). However, the inhibitory activity of CNS myelin from MAG-deficient transgenic mice was not significantly diminished, and axon regeneration in CNS of these MAG knock-out mice was improved only slightly (David et al. 1995) or not at all (Bartsch et al. 1995). In addition, MAG inhibits axon regeneration after injury (Li et al. 1996; McKerracher et al. 1994; Mukhopadhyay et al. 1994). Along with Nogo, oligodendrocyte myelin glycoprotein, and chondroitin sulfate proteoglycans, MAG also contributes to CNS an environment that is highly inhibitory for nerve regeneration (Sandvig et al. 2004; Wang et al. 2002b). It has been suggested that MAG prevents axonal regeneration in lesioned nervous tissue. MAG is now well known as one of several white matter

inhibitors of neurite outgrowth in vitro and axonal regeneration in vivo. MAG knockout mice revealed that MAG is not essential for the initiation of myelination; but, it plays an important role in maintaining a stable interaction between axons and myelin (Filbin 1995). By acute inactivation of MAG in situ in chick retina-optic nerve cultures, Wong et al. (2003) suggested that the acute loss of MAG function can promote significant axon growth across a site of CNS nerve damage.

In contrast to these studies, $MAG^{-/-}$ mice cross-bred with $C57BL/Wld^{S}$ showed improved PNS nerve regeneration in vivo (Schafer et al. 1996). The loss of inhibitory MAG activity may be compensated by expression of other myelin inhibitory proteins (depending on the genetic background). Analysis of optic nerves from mutant mice revealed that MAG is functionally involved in the recognition of axons by oligodendrocytes and in the morphological maturation of myelin sheaths. However, results did not support a role of MAG as a potent inhibitor of axonal regeneration in the adult mammalian CNS (Bartsch 1996). The MAG has been implicated in the formation and maintenance of myelin. Although the analysis of MAG null mutants confirmed this view, the phenotype of this mutant is surprisingly subtle. In the CNS of MAG-deficient mice, initiation of myelination, formation of morphologically intact myelin sheaths and to a minor extent, integrity of myelin is affected. In PNS, in comparison, only maintenance of myelin was impaired. Observations also suggest that other molecules performing similar functions as MAG might compensate, at least partially, for the absence of MAG in the null mutant (Schachner and Bartsch 2000; Tang et al. 2001). Thus, there is still no clear role for MAG in inhibiting nerve regeneration in the CNS in situ. Additional research is needed to determine if receptors and signaling systems are similar to those responsible for MAG inhibition of neurite outgrowth (Quarles 2009).

MAG Induces Intramembrane Proteolysis of p75 Neurotrophin Receptor to Inhibit Neurite Outgrowth: The three known inhibitors of axonal regeneration present in myelin—MAG, Nogo, and OMgp—all interact with the same receptor complex to effect inhibition via PKC-dependent activation of the small GTPase Rho. The activation of RhoA and Rho-kinase is reported to be an essential part of signaling mechanism of MAG. Data suggest the important roles of collapsing response mediator protein-2 (CRMP-2) and microtubules in the inhibition of the axon regeneration by the myelin-derived inhibitors. The transducing component of this receptor complex is the p75 neurotrophin receptor. It was shown that MAG binding to cerebellar neurons induces α- and then γ-secretase proteolytic cleavage of p75, in a protein kinase C-dependent manner, and that this cleavage is necessary for both activation of Rho and inhibition of neurite outgrowth (Domeniconi et al. 2005).

16.5.6 MAG in Demyelinating Disorders

The early loss of MAG in the development of multiple sclerosis plaques in comparison to other myelin proteins suggests that it plays a key role in the pathogenesis of this disease. The selective loss of MAG may relate to the high susceptibility of human MAG to cleavage by a Ca^{2+}-activated neutral protease. Human MAG also contains a highly immunogenic carbohydrate determinant that is also expressed on other neural glycoconjugates and is the antigen recognized by many human IgM paraproteins that occur in patients with peripheral neuropathy.

Association of the MAG Locus with Schizophrenia in Chinese Population: Neurotransmitter-based hypotheses have so far led to only moderate success in predicting new pathogenetic findings in etiology of schizophrenia. On the other hand, the more recent oligodendroglia hypotheses of this disorder have been supported by an increasing body of evidence. The expression MAG gene has been shown to be significantly lower in schizophrenia patient groups compared to control groups. Such an effect might be a result of genetic variations of the MAG gene. Genotyping of four markers within the MAG locus in 413 trios sample of the Han Chinese using allele-specific PCR demonstrated that MAG might play a role in genetic susceptibility to schizophrenia (Yang et al. 2005). In order to further assess the role of MAG in schizophrenia, Wan et al. (2005) examined four single nucleotide polymorphisms (SNPs), namely rs2301600, rs3746248, rs720309 and rs720308, of this gene in Chinese schizophrenic patients and healthy controls. The distribution of rs720309 T/A genotypes showed a strong association with schizophrenia. A haplotype constructed of rs720309-rs720308 also revealed a significant association with schizophrenia. The finding of a possible association between the MAG locus and schizophrenia is in agreement with the hypotheses of oligodendrltic and myelination dysfunction in the Chinese Han population (Wan et al. 2005).

Loss of MAG Reflects Hypoxia-Like White Matter Damage in Brain Diseases: Destruction of myelin and oligodendrocytes leading to the formation of large demyelinated plaques is the hallmark of multiple sclerosis (MS) pathology. In a subset of MS patients termed pattern III, actively demyelinating lesions show preferential loss of MAG and apoptotic-like oligodendrocyte destruction, whereas other myelin proteins remain well preserved. MAG is located in the most distal periaxonal oligodendrocyte processes and primary "dying back" oligodendrogliopathy may be the initial step of myelin degeneration in pattern III lesions. In addition to a subset of MS cases, a similar pattern of demyelination was found in some cases of virus encephalitis as

well as in all lesions of acute white matter stroke. Brain white matter lesions presenting with MAG loss and apoptotic-like oligodendrocyte destruction, irrespective of their primary disease cause, revealed a prominent nuclear expression of hypoxia inducible factor-1α in various cell types, including oligodendrocytes. Data suggest that a hypoxia-like tissue injury may play a pathogenetic role in a subset of inflammatory demyelinating brain lesions (Aboul-Enein et al. 2003).

Shear Stress Alters the Expression of MAG: Reports revealed that Schwann cells undergo concurrent proliferation and apoptosis after a chronic nerve injury that is independent of axonal pathology. Gupta et al (2005) postulated that this response may be triggered directly by mechanical stimuli. Immunochemical analysis showed that the Schwann cells are positive for S-100, MAG, and MBP in greater than 99% of the experimental cells. Stimulated cells also revealed an increased rate of proliferation by as much as 100%. The mRNA expression of MAG and MBP was down-regulated by 21% and 18%, respectively, in experimental cells, while protein was down-regulated by 29% and 35%, respectively. This study provides information regarding Schwann cell direct response to physical stimulus, which is not secondary to an axonal injury (Gupta et al. 2005).

16.5.7 Inhibitors of Regeneration of Myelin

The lack of axonal growth after injury in the adult CNS is due to several factors including the formation of a glial scar, the absence of neurotrophic factors, the presence of growth-inhibitory molecules associated with myelin and the intrinsic growth-state of the neurons. The proteolytic fragment of MAG (dMAG), consisting of entire extracellular domain, is readily released from myelin and is found in vivo. Three inhibitors of axonal growth have been identified in myelin: MAG, Nogo-A, and Oligodendrocyte-Myelin glycoprotein (OMgp). MAG inhibits axonal regeneration by high affinity interaction ($K_d = 8$ nM) with the Nogo66 receptor (NgR) and activation of a p75 neurotrophin receptor (p75NTR)-mediated signaling pathway. Two myelin inhibitors, MAG and Nogo, both transmembrane proteins, have been identified. MAG, a sialic acid binding protein and a component of myelin, is a potent inhibitor of neurite outgrowth from a variety of neurons both in vitro and in vivo. The MAG's sialic acid binding site is distinct from its neurite inhibitory activity. Alone, sialic acid-dependent binding of MAG to neurons is insufficient to affect inhibition of axonal growth. Thus, while soluble MAG-Fc (MAG extracellular domain fused to Fc), a truncated form of MAG-Fc missing Ig-domains 4 and 5, MAG(d1-3)-Fc, and another sialic acid binding protein, sialoadhesin, each bind to neurons in a sialic acid- dependent manner, only full-length MAG-Fc inhibits neurite outgrowth. Results indicated that a second site must exist on MAG which elicits this response. Consistent with this model, mutation of Arg^{118} in MAG to either alanine or aspartate abolishes its sialic acid-dependent binding. However, when expressed at the surface of either CHO or Schwann cells, Arg^{118}-mutated MAG retains the ability to inhibit axonal outgrowth. Hence, MAG has two recognition sites for neurons, the sialic acid binding site at $Arg^{118}8$ and a distinct inhibition site which is absent from the first three Ig domains (Tang et al. 1997).

Tang et al. (2001) showed that a soluble MAG-Fc, when secreted from CHO cells in a collagen gel inhibits/deflects neurite outgrowth from P6 dorsal root ganglion (DRG) neurons. Using the same assay system, results showed that factors secreted from damaged white matter inhibit axonal regeneration and that the majority of inhibitory activity could be accounted for by the proteolytic fragment of MAG (dMAG). Thus, released dMAG is likely to play an important role in preventing regeneration, immediately after injury before the glial scar forms (Tang et al. 2001).

16.5.8 Axonal Regeneration by Overcoming Inhibitory Activity of MAG

The interaction of MAG and its neuronal receptors mediates bidirectional signaling between neurons and oligodendrocytes. When cultured on MAG-expressing cells, dorsal root ganglia neurons (DRG) older than post-natal day 4 (PND4) extend neuritis 50% shorter on average than when cultured on control cells. In contrast, MAG promotes neurite outgrowth from DRG neurons from animals younger than PND4. The response switch, which is also seen in retinal ganglia (RGC) and Raphe nucleus neurons, is concomitant with a developmental decrease in the endogenous neuronal cAMP levels. The artificially increasing cAMP levels in older neurons can alter their growth-state and induce axonal growth in the presence of myelin-associated inhibitors (Domeniconi and Filbin 2005).

Glycan Inhibitors of MAG Enhance Axon Outgrowth In Vitro: MAG is one of several endogenous axon regeneration inhibitors that limit recovery from central nervous system injury and disease. Molecules that block such inhibitors may enhance axon regeneration and functional recovery. Blixt et al. (2003) evaluated ten known human siglecs and their murine orthologs for their specificity for more than 25

synthetic sialosides. Among these siglecs, Siglec-4 binds with 500–10,000-fold higher affinity to a series of mono- and di-sialylated derivatives of the O-linked T-antigen (Galβ (1–3)-GalNAc(α)OThr) as compared with α-methyl-NeuAc.

Potent monovalent sialoside inhibitors of MAG have been identified (Blixt et al. 2003). The potent of these were tested for their ability to reverse MAG-mediated inhibition of axon outgrowth from rat cerebellar granule neurons in vitro. It was found that monovalent sialoglycans enhance axon regeneration in proportion to their MAG binding affinities. The most potent glycoside was disialyl T antigen (NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc-R), followed by 3-sialyl T antigen (NeuAcα2-3Galβ1-3GalNAc-R), structures expressed on O-linked glycoproteins as well as on gangliosides. Blocking gangliosides reversed MAG inhibition. But blocking O-linked glycoprotein sialylation with benzyl-α-GalNAc had no effect. The ability to reverse MAG inhibition with monovalent glycosides suggests further exploration of glycans as blockers of MAG-mediated axon outgrowth inhibition (Vyas et al. 2005).

Immunization with r-Nogo-66/MAG Promotes Axon Regeneration: Immunization with myelin is able to promote robust regeneration of corticospinal tract fibers in adult mice. The effectiveness of such immunization with myelin was compared to that of a combination of two axon growth inhibitors in myelin, Nogo-66 (the 66-amino-acid inhibitory region of Nogo-A) and MAG in SJL/J mice, a strain that is susceptible to autoimmune experimental allergic encephalomyelitis (EAE). None of the immunized mice showed EAE. Long-distance axon regeneration and sprouting of the corticospinal tract was observed in myelin and Nogo-66/MAG immunized mice. Thus, this work shows that axon growth inhibitors in myelin can be selectively blocked using this immunization approach to promote long-distance axon regeneration in the spinal cord (Sicotte et al. 2003). The finding that an anti-MAG monoclonal antibody not only possesses the ability to neutralise the inhibitory effect of MAG on neurons but also directly protects oligodendrocytes from glutamate-mediated oxidative stress-induced cell death. Administration of anti-MAG antibody (centrally and systemically) starting 1 h after middle cerebral artery occlusion in the rat significantly reduced lesion volume at 7 days. This neuroprotection was associated with a robust improvement in motor function compared with animals receiving control IgG1, highlighting the potential for the use of anti-MAG antibodies as therapeutic agents for the treatment of stroke (Irving et al. 2005).

16.5.9 Fish Siglec-4

The combination of microarray technology with the zebrafish model system can provide useful information on how genes are coordinated in a genetic network to control zebrafish embryogenesis and can help to identify novel genes that are important for organogenesis (Lo et al. 2003). To understand the evolution of siglecs, in particular the origin of this family, Lehmann et al. (2004) investigated the occurrence of corresponding genes in bony fish. Interestingly, only unambiguous orthologs of mammalian siglec-4, a cell adhesion molecule expressed exclusively in the nervous system, could be identified in the genomes of fugu and zebrafish, whereas no obvious orthologs of the other mammalian siglecs were found. As in mammals, fish siglec-4 expression is restricted to nervous tissues. Expressed as recombinant protein, fish siglec-4 binds to sialic acids with a specificity similar to the mammalian orthologs. Relatively low sequence similarities in the cytoplasmic tail as well as an additional splice variant found in fish siglec-4 suggest alternative signaling pathways compared to mammalian species. Observations suggest that this siglec occurs at least in the nervous system of all vertebrates (Lehmann et al. 2004).

16.6 Siglec-15

Siglec-15 is a type-I transmembrane protein consisting of: two Ig-like domains, a transmembrane domain containing a lysine residue, and a short cytoplasmic tail. Siglec-15 is expressed on the cells of immune system, can recognize sialylated ligands. The extracellular domain of Siglec-15 preferentially recognizes the Neu5Acα2–6GalNAcα– structure. Siglec-15 associates with the activating adaptor proteins DNAX activation protein (DAP)12 and DAP10 via its lysine residue in the transmembrane domain, implying that it functions as an activating signaling molecule. Siglec-15 is another human Siglec identified to have an activating signaling potential. However, unlike Siglec-14, it does not have an inhibitory counterpart. Orthologs of Siglec-15 are present not only in mammals but also in other branches of vertebrates (fish); in contrast, no other known Siglec expressed in the immune system has been conserved throughout vertebrate evolution. Probably Siglec-15 plays a conserved, regulatory role in the immune system of vertebrates (Angata et al. 2007).

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CD33 (Siglec 3) and CD33-Related Siglecs

17

G.S. Gupta

Sialic-acid-binding immunoglobulin-like lectins (Siglecs) bind sialic acids in different linkages in a wide variety of glycoconjugates. These membrane receptors are expressed in a highly specific manner, predominantly within haematopoietic system. Activating and inhibitory receptors act in concert to regulate cellular activation. Second sub-set of rapidly evolving siglecs is designated the CD33-related siglecs. In humans, these include CD33 and siglecs-5, -6, -7 (7/p75/AIRM1), -8, -9, 10, -11, -14 and -16, whereas, in mice, they comprise murine CD33 and siglec-E, -F, -G and -H. CD33 is a myeloid-specific inhibitory receptor, which along with CD33-related Siglecs represent a distinct subgroup that is undergoing rapid evolution. The structural features of CD33-related Siglecs and the frequent presence of conserved cytoplasmic signaling motifs point to their roles in regulating leukocyte functions that are important during inflammatory and immune responses. McMillan and Crocker (2008) reviewed ligand binding preferences and described the functional roles of CD33-related Siglecs in the immune system and the potential for targeting novel therapeutics against these surface receptors. Siglec-4 belonging to subset 1 of sialoadhesin family is discussed in Chap. 16.

17.1 Human CD33 (Siglec-3)

17.1.1 Human CD33 (Siglec-3): A Myeloid-specific Inhibitotry Receptor

Human CD33 (Siglec-3) is a 67-kDa transmembrane lectin-like glycoprotein that contains one V-set and one C2-set Ig-like domain (Freeman et al. 1995). In addition to two Ig domains, it contains a transmembrane region and a cytoplasmic tail that has two potential ITIM sequences (Fig. 17.1). CD33 is a marker of myeloid progenitor cells, mature myeloid cells, and most myeloid leukemias (see Fig. 16.2, Chap. 16). Although its biologic function remains unknown, it functions as a sialic acid-specific lectin and a cell adhesion molecule. The CD33 binds sialic acid residues in N- and O-glycans on cell surfaces. CD33 is also a serine/threonine phosphoprotein, containing at least 2 sites of serine phosphorylation in its cytoplasmic domain, catalyzed by protein kinase C (PKC) Many of the Siglecs have been reported to be tyrosine phosphorylated in the cytosolic tails under specific stimulation conditions.

CD33 is an and inhibitory receptor that acts in concert to regulate cellular activation. Inhibitory receptors are characterized by the presence of a characteristic sequence known as an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tail. Phosphorylated ITIM serves as docking sites for the SH2-containing phosphatases which then inhibit signal transduction. The proximal ITIM is necessary and sufficient for SHP-1 binding which is mediated by the amino-terminal SH2 domain. Treatment of SHP-1 with a phosphopeptide representing the proximal CD33 ITIM results in increased SHP-1 enzymatic activity. CD33 exerts an inhibitory effect on tyrosine phosphorylation and Ca^{2+} mobilization (Taylor et al. 1999) when co-engaged with activating FcγRI receptor. Thus, CD33 is an inhibitory receptor that may regulate FcγRI signal transduction. Engagement of CD33 on chronic and acute myeloid leukemias (AML) inhibits the proliferation of these cells and activates a process leading to apoptotic cell death on AML cells (Vitale et al. 2001). Phosphorylation could be augmented in presence of IL-3, erythropoietin, or GM-CS factor, in a cytokine-dependent cell line, TF-1. The phosphorylation of CD33 is cross-regulated with its lectin activity. Although this is one example of serine/threonine phosphorylation in the subfamily of CD33-related Siglecs, some of the other members also have putative sites in their cytoplasmic tails (Grobe and Powell 2002).

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17.2 CD33-Related Siglecs (CD33-rSiglecs)

17.2.1 CD33-Related Siglecs Family

Within Siglec family there exists a subgroup of molecules which bear a very high degree of homology with CD33/Siglec-3, and has thus been designated Siglec-3-like subgroup of Siglecs. The CD33-related siglecs show complex recognition patterns for sialylated glycans. The CD33 and CD33-related type 1 transmembrane proteins are thought to exert their functions through glycan recognition (Crocker et al. 2007; Varki and Angata 2006). This subset of Siglec proteins shows relatively a high sequence similarity to CD33 and many members have been shown to associate with protein tyrosine phosphatase SHP-1 via ITIM. These include CD33/Siglec-3, Siglec-5, Siglec-6/OB-BP1, Siglec-7/AIRM1, Siglec-8, Siglec-9, Siglec-10, Siglec-11, Siglec-14, Siglec-16, and a Siglec-like molecule (Siglec-L1) in humans, as well as five confirmed or putative Siglec proteins in mice (CD33, Siglec-E, Siglec-F, Siglec-G, and Siglec-H) and shares 50–99% sequence identity. This group is rapidly evolving, and has poorly conserved binding specificities and domain structures (Crocker et al. 2007; Crocker and Redelinghuys 2008; Varki and Angata 2006). Many CD33-rSiglecs are expressed on cells involved in innate immunity. For example, hSiglec-7/AIRM-1 is expressed on NK cells and monocytes (Nicoll et al. 1999, 2003); hSiglec-8 is expressed on eosinophils (Floyd et al. 2000), and mSiglec-F is expressed on immature cells of myeloid lineage (Angata et al. 2001a).

17.2.1.1 Differential Expression of Siglecs on Mononuclear Phagocytes and DCs

CD33-rSiglecs are expressed mostly in hematopoietic and immune systems with a highly cell type-specific expression pattern; for example, CD33 is found mainly on immature and mature myeloid cells (Crocker et al. 2007; Varki and Angata 2006; Crocker and Varki 2001a, b). Monocytes cultured to differentiate into macrophages using either GM-CSF or M-CSF retain the expression of siglec-3, -5, -7, -9 and -10 and their levels remain unaffected following stimulation with LPS. In comparison, monocyte-derived dendritic cells down-modulated siglec-7 and -9 following maturation with LPS. Plasmacytoid DCs in human blood expressed siglec-5 only. On monocytes, siglec-5 was shown to mediate rapid uptake of anti-siglec-5 (Fab)2 fragments into early endosomes. This suggests, in addition to inhibitory signaling, a potential role in endocytosis for siglec-5 and the other CD33-related siglecs. Studies show that siglecs are differentially expressed on mononuclear phagocytes and DCs and that some can be modulated by stimuli that promote maturation and differentiation (Lock et al. 2004). In order to characterize the spectrum of expression of other CD33rSiglecs on bone marrow precursors and AML cells, Nguyen et al. (2006a) demonstrated that Siglecs-3, -5, -6, -7, and -9 are expressed on subsets of normal bone marrow precursors, including promonocytes and myelocytes. Furthermore, most AML (but not ALL) cells express these Siglecs.

17.2.2 CD33-rSiglec Structures

The structurally CD33-rSiglecs are characterized by one N-terminal V-set Ig domain mediating sialic acid binding, followed by a variable number of C2-set Ig domains (1–16), ranging from 4 (in Siglec-10 and -11) to 1 (in CD33) (Crocker et al. 2007; Crocker 2002; Crocker 2005; Varki and Angata 2006) and a transmembrane domain, followed by a short cytoplasmic tail. Each siglec exhibits distinct and varied specificity for sialoside sequences on glycoprotein and glycolipid glycans that are expressed on the same cell (in *cis*) or on adjacent cells (in *trans*) (Crocker et al. 2007). Siglec-3-rSiglecs are characterized by their sequence similarity in first two Ig-like domains. The cytoplasmic domains of CD22 and most CD33-related siglecs contain ITIM and ITIM-like motifs involved in regulation of cell signaling. Several other siglecs (Siglecs-14-16 and murine Siglec-H) have no tyrosine motifs, but contain a positively charged trans-membrane spanning region. A charged residue permits association with the adapter protein DAP12 (12 kDa DNAX-activating protein), which bears a cytoplasmic ITAM that imparts both positive and negative signals (Crocker et al. 2007; Crocker and Redelinghuys 2008) and another putative signaling motif situated nearby (Crocker and Varki 2001a, b; Crocker 2002; Ravetch and Lanier 2000) (Fig. 17.1).

Paired Receptors: Immune cell surface receptors sharing similar sequences and having counteracting signaling properties are called “paired receptors.” Known paired receptors belong to two classes of molecular families, namely, the Ig superfamily and the C-type lectin family (Long 1999). These receptor pairs (or receptor families) include the killer cell Ig-like receptors (KIR) of primates (Vilches and Parham 2002), the leukocyte Ig-like receptors (LILR) of primates (Martin et al. 2002), the paired Ig-like receptors (PIR) of rodents (Kubagawa et al. 1997), the myeloid-associated Ig-like receptors (MAIR) of rodents (Yotsumoto et al. 2003), the Ly49 family of rodents (Yokoyama and Plougastel 2003), and the CD94/NKG2 family of both primates and rodents (Gunturi et al. 2004). Although Ly49 and CD94/NKG2 families belong to C-type lectin family, none of them has been unequivocally shown to recognize glycan ligands (Angata et al. 2006). Rather, their ligands have been shown to be MHC class I proteins. The functional significance of the presence of paired inhibitory

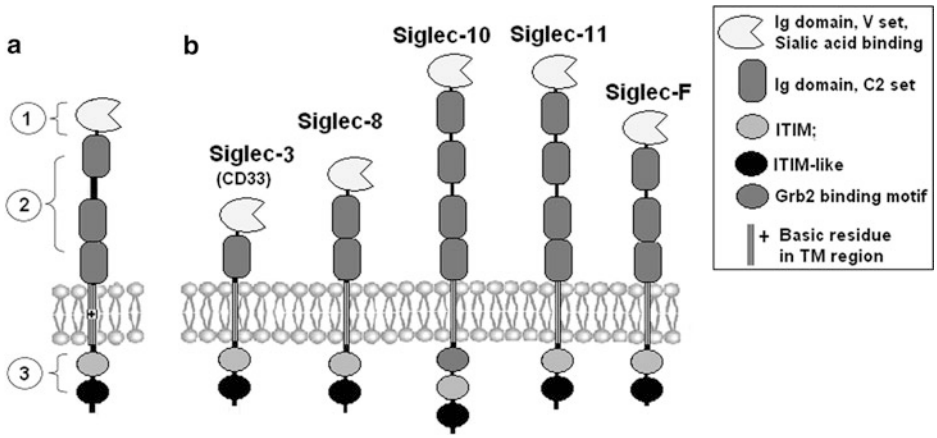

Fig. 17.1 (**a**) *Common structural features of siglecs:* The N-terminal 'V-set' Ig domain (1) contains a conserved arginine residue that confers sialic acid-binding ability. This domain is followed by a variable number (1–16) of 'C2-set' Ig domains (2). In the cytosolic domain, most siglecs contain some combination of tyrosine motifs, including ITIM, ITIM-like, Grb2-binding, and Fyn kinase sites (3). Siglecs-14, -15, and -16 contain a positively charged residue in the transmembrane spanning region (4) that enables association with the ITAM-bearing adaptor protein, DAP-12. It is speculated that these may have evolved to counteract ITIM-bearing siglecs (Crocker et al. 2007). With 99% sequence identity in the two first N-terminal Ig domains, Siglecs-5 and -14 are believed to be such paired receptors (O'Reilly and Paulson 2009). (**b**). Structural characteristics of human Siglec-3 (CD33), human Siglec-8, 10, 11 and mouse Siglec-F. For the cytoplasmic portions of each Siglec, the light gray and black circles represent ITIM and ITIM-like motifs, respectively. Siglec-6, -7, -8 and -9 have same number of Ig domain C2 set (Varki and Crocker 2009). With 99% sequence identity in the two first N-terminal Ig domains, Siglecs-5 and -14 are believed to be such paired receptors (O'Reilly and Paulson 2009)

and activating receptors remains elusive, but it is proposed that these paired receptors are involved in fine-tuning of immune responses (Lanier 2001) . Human Siglec-5, encoded by *SIGLEC5* gene, has four extracellular Ig-like domains and a cytosolic inhibitory motif. Human Siglec-14 has three Ig-like domains, encoded by the *SIGLEC14* gene, adjacent to *SIGLEC5*. Human Siglec-14 has almost complete sequence identity with human Siglec-5 at the first two Ig-like domains, shows a glycan binding preference similar to that of human Siglec-5, and associates with the activating adapter protein DAP12. Thus, Siglec-14 and Siglec-5 appear to be the first glycan binding paired receptors. Near-complete sequence identity of the amino-terminal part of human Siglec-14 and Siglec-5 indicates partial gene conversion between *SIGLEC14* and *SIGLEC5*. *SIGLEC14* and *SIGLEC5* in other primates also show evidence of gene conversions within each lineage. Evidently, balancing the interactions between Siglec-14, Siglec-5 and their common ligand(s) had selective advantage during the course of evolution. The "essential arginine" critical for sialic acid recognition in both Siglec-14 and Siglec-5 is present in humans but mutated in almost all great ape alleles (Angata et al. 2006b).

Activatory and Inhibitory Signals: Regulation of responses by cells in hemopoietic and immune systems depends on a balance between activatory and inhibitory signals. Their relative strengths set an appropriate activation threshold that helps fine-tune the response. CD33-rSiglecs have been shown to regulate negatively the cells, which express them (Mingari et al. 2001; Ulyanova et al. 2001; Nutku et al. 2005; von Gunten et al. 2005). Ab-mediated cross-linking of CD33rSiglecs leads to negative regulation of cellular activities, such as cell death or reduced cell proliferation (Von Gunten et al. 2005). Inhibitory signals are typically initiated by receptors containing one or more cytoplasmic ITIMs (Ravetch and Lanier 2000). ITIM in the intracytoplasmic domain of inhibitory receptors acts as a regulatory molecule to inhibit activation. Among 11 human CD33-related siglecs, most of them contain a membrane proximal ITIM and a membrane distal ITIM-like motif in their cytoplasmic tails. Where studied, CD33-rSiglecs can become tyrosine phosphorylated and recruit SHP-1 and SHP-2 after treatment of cells with pervanadate, a protein-tyrosine phosphatase inhibitor (Avril et al. 2004; Ikehara et al. 2004; Taylor et al. 1999; Ulyanova et al. 2001). In addition, CD33 and Siglecs-7 and -9 have been shown to exhibit enhanced siglec-dependent adhesion after tyrosine mutation of proximal ITIM (Taylor et al. 1999); antibody-mediated cross-linking of some CD33-rSiglecs results in inhibition of cell proliferation and function, and/or induction of apoptosis (Nutku et al. 2003; von Gunten et al. 2005). While these in vitro results suggest that CD33-rSiglecs are inhibitory signaling molecules that dampen immune-cell functions, in vivo proof is lacking. Anti-Siglec antibodies also tend to induce rapid endocytic clearing of cognate Siglec from cell surfaces (Nguyen et al. 2005, 2006a), complicating interpretation of the observed effects. The CD33-rSiglecs contain not only a cytoplasmic ITIM but

also an immunoreceptor tyrosine-based switch–like motif (ITSM). While ligand-induced clustering of inhibitory receptors results in tyrosine phosphorylation of these ITIMs and recruitment of Src homology 2 (SH2) containing phosphatases (SHP-1/2) and inositol phosphatase (SHIP) (Malbec et al. 1998), the ITSM has been shown to switch between binding to signaling lymphocyte activated molecule (SLAM) associated protein (SAP) and EAT-2 or between SAP and SHP-2 in other receptors (Howie et al. 2002).

The consensus sequence for ITIMs is (V/I/L)XYXX (L/V), where X is any amino acid. A bioinformatics study by Staub et al. (2004) showed that the human proteome contains ~ 109 membrane proteins with cytoplasmic ITIMs, and many of these have been shown already to function as inhibitory receptors. Generally, when ITIM-containing receptors are engaged, they can become tyrosine-phosphorylated and then transmit inhibitory signals by binding and activating Src homology-2 domain (SH2)-containing tyrosine phosphatases (SHP1 and SHP2) and/or the SH2-containing inositol polyphosphate 5'-phosphatase (SHIP). In the case of CD33, several studies have demonstrated that SHP1 and SHP2, but not SHIP, are recruited and activated once the ITIMs are phosphorylated (Taylor et al. 1999).

ITAM Motif: Recent discoveries of Siglecs that associate with the activating adaptor molecule DAP12, which has an ITAM, have shed light on a subgroup of Siglecs that have an activating signaling potential (Angata et al. 2006; Blasius et al. 2006). The presence of both activating and inhibitory members within a family is a feature that is also found in other immunity-related cell-surface receptor families such as killer cell Ig-like receptors of primates (Vilches and Parham 2002), leukocyte Ig-like receptors of primates (Martin et al. 2002), and the Ly49 family of rodents (Yokoyama and Plougastel 2003). Although the functional significance of the presence of counteracting members within a family is not fully understood, it has been proposed that such members act cooperatively to fine-tune cellular responses (Lanier 2001), or that the activating members function to counteract pathogens that exploit their inhibitory counterparts (Arase and Lanier 2004).

17.2.3 Organization of CD33-rSiglec Genes on Chromosome 19q13.4

Genes of Siglec-3-like subgroup of Siglecs were mapped to a region of ~ 500 kb of chromosome 19q (cytological band 19q13.3–13.4) near the gene clusters containing KIR and LILR genes, suggesting their origin through repeated gene duplications (Crocker and Varki 2001a,b). The detailed map of the locus contains 8 Siglec genes and one Siglec-like gene (Siglec-L) and at least 13 Siglec-like pseudogenes (Angata et al. 2001b; Angata et al. 2004). Members of this subfamily exhibit two patterns of organization of the signal peptide, which is followed by one V-set domain (except for the long form of the siglecL1 gene). Exons containing the C2-set domains are all comparable in size and are separated by linker exons. The transmembrane domain is encoded for by a separate exon of almost the same size in all genes. The total number of exons differs according to the number of C2-set Ig domains, but intron phases are identical. The cytoplasmic domain is always encoded by two exons. Yousef et al. (2002) further identified two new Siglec pseudogenes in this locus, and analyzed their tissue expression pattern and their structural features. Although a few Siglecs are well-conserved throughout vertebrate evolution and show similar binding preference regardless of species of origin, CD33-related subfamily of Siglecs show marked inter-species differences in repertoire, sequence, and binding preference. Hayakawa et al. (2005) demonstrated human-specific gene conversion between *SIGLEC11* and the adjacent pseudogene *SIGLECP16*, resulting in human-specific expression of Siglec-11 in brain microglia (Hayakawa et al. 2005).

17.2.3.1 Loss of Siglec Expression on T Lymphocytes During Human Evolution

Human T cells give much stronger proliferative responses to specific activation via TCR than those from chimpanzees, our closest relatives. Among human immune cells, T lymphocytes are a striking exception, expressing little to none of these CD33-related Siglecs. In sharp contrast, T lymphocytes from chimpanzees as well as from "great apes" (bonobos, gorillas, and orangutans) express several CD33-related Siglecs on their surfaces. This suggests that human-specific loss of T cell Siglec expression occurred after our last common ancestor with great apes, potentially resulting in an evolutionary difference with regard to inhibitory signaling. This was confirmed by studying Siglec-5, which is prominently expressed on chimpanzee lymphocytes, including CD4 T cells. The human-specific loss of T cell Siglec expression associated with T cell hyperactivity may help explain the strikingly disparate prevalence and severity of T cell-mediated diseases such as AIDS and chronic active hepatitis between humans and chimpanzees (Nguyen et al. 2006b).

17.3 Siglec-5 (CD170)

Human Siglec-5, also known as CD170, encoded by *SIGLEC5* gene, has four extracellular Ig-like domains and a cytosolic inhibitory motif. Siglec-5 also known as obesity binding protein-2 (OB-BP2) shows no similarity to leptin receptor (Ob-R). Angata et al. (2006) discovered human Siglec-14 with three Ig-like domains, encoded by the

SIGLEC14 gene, adjacent to *SIGLEC5*. Human Siglec-14 has almost complete sequence identity with human Siglec-5 at the first two Ig-like domains, shows a glycan binding preference similar to that of human Siglec-5, and associates with the activating adapter protein DAP12. Thus, Siglec-14 and Siglec-5 appear to be first among glycan binding paired receptors.

17.3.1 Characterization

Siglec-5, a human CD33-related siglec, is expressed on granulocytes and monocytes (Cornish et al. 1998), as well as on plasmacytoid dendritic cells, monocyte-derived dendritic cells, and macrophages (Lock et al. 2004). A full-length cDNA encoding siglec-5 predicted that siglec-5 contains four extracellular Ig-like domains, the N-terminal two of which are 57% identical to the corresponding region of CD33. The cytoplasmic tail is also related to that of CD33, containing two tyrosine residues embodied in ITIM-like motifs. The siglec-5 gene was shown to map to chromosome 19q13.41-43, closely linked to the CD33 gene. Siglec-5 may be involved in cell-cell interactions. However, siglec-5 was found to have an expression pattern distinct from that of CD33, being present at relatively high levels on neutrophils but absent from leukemic cell lines representing early stages of myelomonocytic differentiation. Siglec-5 exists as a disulfide-linked dimer of approximately 140 kD (Cornish et al. 1998).

Angata et al. (2001a, 2004) identified *SIGLEC5**, a genomic segment adjacent to *SIGLEC5* gene in the Siglec gene cluster that showed extreme sequence identity with a part of *SIGLEC5* (Angata et al. 2004; Angata et al. 2001a). However, it was not clear if this genomic segment was a part of an active genetic element. It was evident that the genomic region downstream of *SIGLEC5** lacked sequences similar to the exon containing ITIM typically found in CD33rSiglecs, suggesting a possibility that the Siglec protein encoded by *SIGLEC5**, if any, may have a different signaling potential from that of Siglec-5, or any other human CD33rSiglecs. Patel et al. (1999) reported cloning of a leptin-binding protein of IgSF (OB-BP1/Siglec-6) and a cross-hybridizing clone (OB-BP2) that is identical to Siglec-5. Human tissues showed OB-BP2/Siglec-5 mRNA in peripheral blood leukocytes, lung, spleen, and placenta. Angata et al. (2006) proposed that Siglec-5 and Siglec-14 are paired inhibitory and activating receptors. It was also demonstrated that *SIGLEC14* and *SIGLEC5* have undergone concerted evolution via gene conversion in multiple primate species. Based on these results, the evolutionary dynamics behind the birth of Siglec-14 and its concerted evolution with Siglec-5 was suggested.

17.3.2 Siglec-5: An Inhibitory Receptor

Siglec-5 can recruit SHP-1 and SHP-2 after tyrosine phosphorylation and mediate inhibitory signaling, as measured by calcium flux and serotonin release after co-ligation with the activatory FcγRI. Surprisingly, however, mutagenesis studies showed that inhibition of serotonin release could still occur efficiently after a double tyrosine to alanine substitution, whereas suppression of Siglec-5-dependent adhesion required an intact tyrosine residue at the membrane proximal ITIM. A potential mechanism for tyrosine phosphorylation-independent inhibitory signaling was provided by results of in vitro phosphatase assays, which demonstrated low level activation of SHP-1 by the Siglec-5 cytoplasmic tail in the absence of tyrosine phosphorylation (Avril et al. 2005). Based on these findings, Siglec-5 can be classified as an inhibitory receptor with the potential to mediate SHP-1 and/or SHP-2-dependent signaling in the absence of tyrosine phosphorylation (Avril et al. 2005; Angata et al. 2006).

Siglec-5 was not expressed at significant levels by CD34+ progenitors either from bone marrow or mobilized peripheral blood. Siglec-5 expression remained absent or very low on cultured CD34+ cells, unlike CD33, which was present on almost all CD34+ cells by day 4. However, analysis of blasts from patients with AML revealed aberrant expression of Siglec-5 with CD34 in 50% of patients with CD34+ AML; 61% of AML cases were positive for Siglec-5 with an increased frequency in the French-American-British subtypes M3-5. All 13 acute lymphoblastic leukaemic (ALL) samples tested, were Siglec-5 negative (Virgo et al. 2003).

17.3.3 Siglec-5-Mediated Sialoglycan Recognition

The crystal structure of two N-terminal extracellular domains of human Siglec-5 and its complexes with two sialylated carbohydrates was determined. The native structure revealed an unusual conformation of the CC' ligand specificity loop and a unique interdomain disulfide bond (Fig. 17.2). The α(2,3)- and α(2,6)-sialyllactose complexed structures showed a conserved Sia recognition motif that involves both Arg124 and a portion of the G-strand in the V-set domain forming β-sheet-like hydrogen bonds with the glycerol side chain of the Sia. Only few protein contacts to the subterminal sugars are observed and mediated by the highly variable GG' linker and CC' loop. Structural observations provided mechanistic insights into linkage-dependent Siglec carbohydrate recognition and suggested that Siglec-5 and other CD33-related Siglec receptors are more promiscuous in sialoglycan recognition than previously understood (Zhuravleva et al. 2008)

Fig. 17.2 Ribbon diagram showing the native Siglec-5 structure with V-domain in magenta and C2-domain in blue. The disulfides are depicted with sticks. BC and C′D loops and the specificity-determining GG′ and CC′ regions are depicted in orange, cyan, green and yellow, respectively (Adapted by permission from Zhuravleva et al. 2008 © Elsevier)

17.4 Siglec-6

17.4.1 Cloning and Gene Organization of Siglec-6 (OB-BP1)

Siglec-6 was cloned independently by two groups: in one instance from a human placental library (Takei et al. 1997) and in another as a leptin-binding protein (Patel et al. 1999). While Takei et al. (1997) noted an alternately spliced form of obesity binding protein-1 (OB-BP1) (CD33L2) that is predicted to encode a soluble form of protein, Siglec-6 shows strong expression in human placental trophoblast and also at variable levels on human B cells (Patel et al. 1999). Placental trophoblast expression of Siglec-6 is a human-specific phenomenon, which is associated with presence of Siglec-6 ligands on cells of placental and endometrial (uterine epithelial) origin. There is a relationship between the tempo of Siglec-6 expression and the onset and progression of labor. Given the presence of inhibitory signaling motifs on the cytosolic tail of Siglec-6 and its leptin-binding ability, one can speculate about mechanistic connections to the unusually prolonged nature of the human birth process. Human siglec-6 (OB-BP1), like siglec-3 (CD33) recognize both Neu5Ac and Neu5Gc. Siglec-6 shows significant binding to sialyl-Tn (Neu5Acα2-6-GalNAc), a tumor marker associated with poor prognosis. Siglec-6 is an exception among siglecs in not requiring the glycerol side chain of sialic acid for recognition.

The complete cDNA contains an open reading frame of 1,326 nt encoding 442 amino acids and belongs to the IgSF. It is composed of three Ig-like domains; its high degree of similarity to CD33 (70% identity) in the first and second of these domains implies that the placenta-specific gene product is likely to be associated with cell-cell interaction. Northern-blot analysis revealed transcripts of four distinct sizes, 7.5 kb, 5.0 kb, 4.1 kb, and 2.0 kb, specifically in placenta. Consequently, this gene was termed CD33-antigen-like. An alternatively-spliced transcript encoding a 342-amino-acid peptide which lacked the transmembrane region and the cytoplasmic tail was also isolated. It was localized to human chromosome 19q13.3, where the CD33 gene is also located (Takei et al. 1997).

The leptin-binding protein of IgSF (OB-BP1/Siglec-6) along with OB-BP2/Siglec-5, and CD33/Siglec-3 constitute a unique related subgroup with a high level of overall amino acid identity. The cytoplasmic domains are not as highly conserved, but display motifs which are putative sites of tyrosine phosphorylation, including an ITIM and a motif found in SLAM and SLAM-like proteins. Human tissues showed high levels of OB-BP1 mRNA in placenta and moderate expression in spleen, peripheral blood leukocytes, and small intestine. OB-BP2/Siglec-5 mRNA was detected in peripheral blood leukocytes, lung, spleen, and placenta. OB-BP1 showed high expression in placental cyto- and syncytiotrophoblasts. While OB-BP1 exhibited tight binding ($K_D = 91$ nM), the other two showed weak binding with K_D values in 1–2 μM range. OB-BP1 specifically bound Neu5Acα2–6GalNAcα (sialyl-Tn) allowing its formal designation as Siglec-6. The OB-BP1/Siglec-6 as a Siglec may mediate cell-cell recognition events by interacting with sialylated glycoprotein ligands expressed on specific cell populations (Patel et al. 1999). OB-BP1 and 2 display no similarity to leptin receptor (Ob-R). In surface plasmon resonance studies, OB-BP1 bound leptin with a moderate affinity, while OB-BP2 and CD33 bound with low affinities. The three exhibited binding kinetics with relatively slow on and off rates, which differed significantly from typical receptor-cytokine kinetics in which both on and off rates are fast (Patel et al. 1999). If leptin is an endogenous ligand of OB-BP1, role in leptin physiology could be speculated. Patel et al. (1999) hypothesized that OB-BP1 regulates circulating levels of leptin or acts as a leptin carrier in blood via B cells.

17.4.2 Siglec-6 (OB-BP1) and Reproductive Functions

Siglec-6/OB-BP1 is expressed on immune cells of both humans and the closely related great apes. Placental trophoblast expression is human-specific, with little or no expression in ape placentae. Human placenta also expresses natural ligands for Siglec-6 (a mixture of glycoproteins carrying cognate sialylated targets), in areas adjacent to Siglec-6 expression. Ligands were also found in uterine endometrium and on cell lines of trophoblastic or endometrial origin. Thus, Siglec-6 was recruited to placental expression during human evolution, presumably to interact with sialylated ligands for specific negative signaling functions and/or to regulate leptin availability. The control of human labor is poorly understood, but involves multiple cues, including placental signaling. Human birthing is also prolonged in comparison to that in our closest evolutionary relatives, the great apes. Siglec-6 levels are generally low in placentae from elective surgical deliveries without known labor and the highest following completion of labor. It is speculated that the negative signaling potential of Siglec-6 was recruited to human-specific placental expression, to slow the tempo of human birth process. The leptin-binding ability of Siglec-6 is also consistent with this hypothesis, as leptin-deficient mice have increased parturition times (Brinkman-Van der Linden et al. 2007).

Preeclampsia (PE), which affects 4–8% of human pregnancies, causes significant maternal and neonatal morbidity and mortality. Within the basal plate, placental cytotrophoblasts (CTBs) encode proteins associated with PE and molecules like Siglec-6 and pappalysin-2, localized to invasive CTBs and syncytiotrophoblasts. Alterations in Siglec-6 have been reported in PE. Siglec-6 placental expression is unique to human, as is spontaneous PE (Winn et al. 2009).

17.5 Siglec-7 (p75/AIRM1)

17.5.1 Characterization

Siglec-7, the adhesion inhibitory receptor molecule 1 (p75/AIRM1), is a 75-kD surface glycoprotein that displays homology with the myeloid cell antigen CD33. In lymphoid cells, p75/AIRM1 is confined to NK cells and mediates inhibition of their cytolytic activity. p75/AIRM1 is also expressed by cells of the myelomonocytic cell lineage, in which it appears at a later stage as compared with CD33 (Vitale et al. 2001). The p75/AIRM1 gene is located on human chromosome 19 and encodes a member of the sialoadhesin family characterized by three Ig-like extracellular domains (one NH_2-terminal V-type and two C2-type) and a classical ITIM in the cytoplasmic portion. The highest amino acid sequence similarity has been found with the myeloid-specific CD33 molecule and the placental CD33L1 protein. Similar to other sialoadhesin molecules, p75/AIRM1 appears to mediate sialic acid-dependent ligand recognition (Nicoll et al. 1999; Angata and Varki 2000b).

Sugar-binding specificity of Siglec-7 expressed on CHO cells was characterized. Glyco-probes carrying unique oligosaccharide structures such as GD3 (NeuAc α2,8NeuAc α2,3Gal β 1,4Glc) and LSTb (Gal β1,3[NeuAc α 2,6]GlcNAc β1,3Gal β1,4Glc) oligosaccharides bound to Siglec-7 better than those carrying LSTc (NeuAc α2,6Gal β1,4GlcNAc β1,3Gal β1,4Glc) or GD1a (NeuAc α 2,3Gal β1,3GalNAc β1,4[NeuAc α2,3]Gal β1,4Glc) oligosaccharides. In contrast, Siglec-9, which is 84% identical to Siglec-7, did not bind to the GD3 and LSTb probes but did bind to the LSTc and GD1a probes. Substitution of a small region, Asn70-Lys75, of Siglec-7 with the equivalent region of Siglec-9 resulted in loss of Siglec-7-like binding specificity and acquisition of Siglec-9-like binding properties. In comparison, a Siglec-9-based chimera, which contains Asn70-Lys75 with additional amino acids derived from Siglec-7, exhibited Siglec-7-like specificity. These results with molecular modeling suggest that the C-C' loop in the sugar-binding domain plays a major role in determining the binding specificities of Siglecs-7 and -9 (Yamaji et al. 2002).

17.5.2 Cytoplasmic Domain of Siglec-7 (p75/AIRM1)

The cytoplasmic domain of Siglec-7 contains two signaling motifs: a membrane-proximal ITIM (Ile435-Gln-Tyr-Ala-Pro-Leu440) and a membrane-distal motif (Asn458-Glu-Tyr-Ser-Glu-Ile463). Upon pervanadate (PV) treatment, Siglec-7 recruited the protein tyrosine phosphatases Src homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) and SHP-2 less efficiently than did other inhibitory receptors such as Siglec-9. Alignment of the amino acid sequences of the two Siglecs revealed only three amino acids difference in these motifs. These amino acids appeared to affect not only phosphatase recruitment but also the subsequent attenuation of Syk phosphorylation (Yamaji et al. 2005).

Siglec-7 shows a preference for α(2,8)-disialylated ligands and provides a structural template for studying the key interactions that drive this selectivity. The crystal

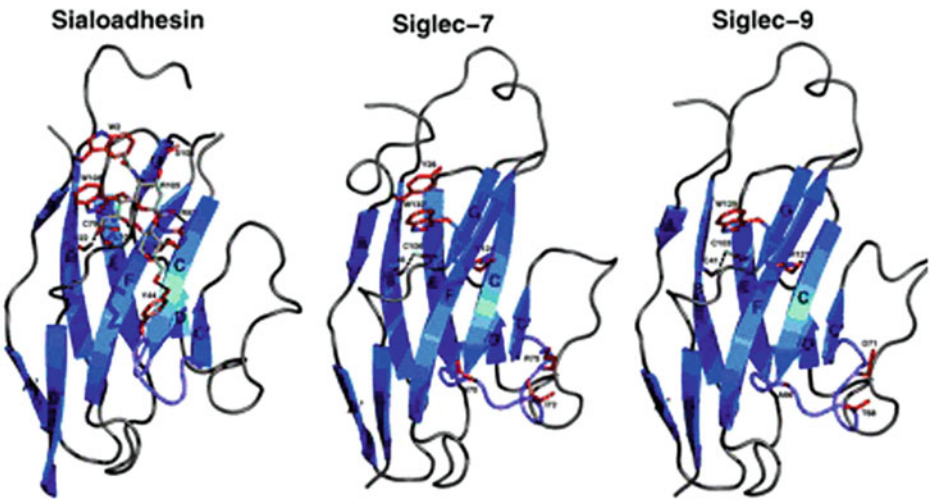

Fig. 17.3 *Comparison of sialoadhesin, Siglec-7, and Siglec-9 structures*. The crystal structures of sialoadhesin in complex with sialyllactose, the crystal structure of Siglec-7, and a model for Siglec-9 are shown in two representations. The ackbones are shown as *ribbons* with the side chains surrounding the ligand-binding site shown as *sticks* with *orange* carbons. The C-C' loop is *highlighted* in *magenta*. The two pyranose sugars from the sialyllactose ligand are shown as *sticks* with *green* carbons. Hydrogen bonds with the protein are shown as *black dotted lines*. For sialoadhesin, the two pyranose sugars of the ligand are shown in a *sticks* representation (Adapted by permission from Alphey et al. 2003 © The American Society for Biochemisty and Molecular Biology)

structure of the co-crystallized Siglec-7 with a synthetic oligosaccharide corresponding to the α(2,8)-disialylated ganglioside GT1b offers a first glimpse into how this important family of lectins binds the structurally diverse gangliosides. The complex structure revealed that the C-C' loop, a region implicated in previous studies as driving siglec specificity, undergoes a dramatic conformational shift, allowing it to interact with the underlying neutral glycan core of the ganglioside. Studies showed that binding of the ganglioside is driven by extensive hydrophobic contacts together with key polar interactions and that the binding site structure is complementary to preferred solution conformations of GT1b (Attrill et al. 2006a).

17.5.3 Crystallographic Analysis

The expression, crystallization and preliminary X-ray characterization of the Ig-V like domain of p75/AIRM1 have been reported. X-ray data were collected from a single crystal at 100 K at 1.45 A resolution showed that the crystal belongs to a primitive monoclinic space group, with unit-cell parameters a = 32.65, b = 49.72, c = 39.79 A, α = γ = 90, β = 113. The systematic absences indicate that the space group is P2(1). Assuming one molecule per asymmetric unit, V(M) (Matthews coefficient) was calculated to be 1.879 A^3 Da^{-1} and the solvent content was estimated to be 32.01%. The structure belongs to a different space group than Siglec-7 structure and was obtained using a bacterial expression system. The structure unveils the fine structural requirements adopted by a natural killer cell inhibitory receptor of the Siglec family in target-cell recognition and binding (Dimasi et al. 2004).

Alphey et al. (2003) described the high resolution structures of the N-terminal V-set Ig-like domain of Siglec-7 in two crystal forms. The latter crystal form reveals the full structure of this domain and allows us to speculate on the differential ligand binding properties displayed by members of the Siglec family. A fully ordered N-linked glycan is observed, tethered by tight interactions with symmetry-related protein molecules in the crystal. Comparison of the structure with that of sialoadhesin and a model of Siglec-9 shows that the unique preference of Siglec-7 for α(2,8)-linked disialic acid is likely to reside in the C-C' loop, which is variable in the Siglec family (Fig. 17.3). In the Siglec-7 structure, the ligand-binding pocket is occupied by a loop of a symmetry-related molecule, mimicking the interactions with sialic acid (Alphey et al. 2003).

17.5.4 Interactions of Siglec-7

Avril et al. (2006) examined the interaction of 10 siglecs with lipooligosaccharides (LOS) purified from four different *C. jejuni* isolates expressing GM1-like, GD1a-like, GD3-like, and GT1a-like oligosaccharides. Of all siglecs examined, only Siglec-7 exhibited specific, sialic acid-dependent interactions with *C. jejuni* LOS. Binding of Siglec-7 was also observed with

intact bacteria expressing these LOS structures. Specific binding of HS:19($GM1^+$ $GT1a^+$) bacteria was demonstrated with Siglec-7 expressed on transfected CHO cells and with peripheral blood leukocytes, among which HS:19($GM1^+$ $GT1a^+$) bacteria bound selectively to both NK cells and monocytes which naturally express Siglec-7. These results raise the possibility that, in addition to their role in generating autoimmune antibody responses, *C. jejuni* LOS could interact with Siglec-7 expressed by leukocytes, modulate the host-pathogen interaction, and contribute to the clinical outcome and the development of secondary complications such as Guillain-Barre syndrome (Avril et al. 2006).

In addition to their ability to recognize sialic acid residues, Siglec-7 and Siglec-9 display two conserved tyrosine-based motifs in their cytoplasmic region similar to those found in inhibitory receptors of the immune system. Siglecs-7 and -9 are able to inhibit the FcεRI-mediated serotonin release from RBL cells following co-crosslinking. In addition, under these conditions or after pervanadate treatment, Siglecs-7 and -9 associate with the Src homology region 2 domain-containing phosphatases (SHP), SHP-1 and SHP-2. Site-directed mutagenesis showed that the membrane-proximal tyrosine motif is essential for the inhibitory function of both Siglec-7 and -9, and is also required for tyrosine phosphory lation and recruitment of SHP-1 and SHP-2 phosphatases. The mutation of the membrane-proximal motif increased the sialic acid binding activity of Siglecs-7 and -9, suggesting the possibility that "inside-out" signaling may occur to regulate ligand binding (Angata et al. 2006). The crystal structure of siglec-7 in complex with a sialylated ligand, the ganglioside analogue DSLc4 [α(2,3)/α(2,6) disialyl lactotetraosyl 2-(trimethylsilyl)ethyl], allows for a detailed description of the binding site, required for structure-guided inhibitor design. Mutagenesis and binding assays demonstrated the key structural role for Lys131, a residue that changes conformation upon sialic acid binding. Differences between the binding sites of siglec family members were then exploited using α-methyl Neu5Ac (N-acetylneuraminic acid) as a basic scaffold. A co-crystal of siglec-7 in complex with the sialoside inhibitor, oxamido-Neu5Ac [methyl α-9-(amino-oxalyl-amino)-9-deoxy-Neu5Ac] and inhibition data for the sialosides gives clear leads for future inhibitor design (Attrill et al. 2006b).

17.5.5 Functions of Siglec-7

Negative Regulation of T Cell Receptor Signaling: Siglec-7 and Siglec-9 are capable of modulating T cell receptor (TCR) signaling in Jurkat T cells stably and transiently transfected with Siglec-7 or Siglec-9. Following either pervanadate stimulation or TCR engagement, both Siglecs exhibited increased tyrosine phosphorylation and recruitment of SHP-1. There was also a corresponding decreased transcriptional activity of nuclear factor of activated T cells (NFAT) as determined using a luciferase reporter gene. Like all siglecs, Siglec-7 and -9 recognize sialic acid-containing glycans of glycoproteins and glycolipids as ligands. Mutation of the conserved Arg in the ligand binding site of Siglec-7 (Arg^{124}) or Siglec-9 (Arg^{120}) resulted in reduced inhibitory function in the NFAT/luciferase transcription assay, suggesting that ligand binding is required for optimal inhibition of TCR signaling. The combined results demonstrate that both Siglec-7 and Siglec-9 are capable of negative regulation of TCR signaling and that ligand binding is required for optimal activity (Ikehara et al. 2004).

SOCS3 Targets Siglec 7 for Proteasomal Degradation: SOCS3 (Suppressor of Cytokine Signaling 3) is up-regulated during inflammation and competes with SHP-1/2 for binding to ITIM-like motifs on various cytokine receptors resulting in inhibition of signaling. Orr et al. (2007a) showed that SOCS3 binds the phosphorylated ITIM of Siglec 7 and targets it for proteasomal-mediated degradation suggesting that the Siglec 7 receptor is a SOCS target. In addition, SOCS3 expression blocks Siglec 7 mediated inhibition of cytokine-induced proliferation. It seems that SOCS target degrades simultaneously with the SOCS protein and that inhibitory receptors may be degraded in this way. This may be a mechanism by which the inflammatory response is potentiated during infection (Orr et al. 2007a).

17.6 Siglec-8

17.6.1 Characteristics and Cellular Specificity

Siglec-8 (SAF-2) is selectively expressed on human eosinophils, basophils, and mast cells, where it regulates their function and survival (Kikly et al. 2000; Floyd et al. 2000). SAF-2, identified by Kikly et al. (2000) was homologous with CD33 and siglec-5. SAF-2 is a 431-amino acid protein composed of 3 Ig domains with a 358-amino acid extracellular domain and a 47-amino acid tail. SAF-2 is useful in the detection and/or modulation of allergic cells (Bochner 2009; Aizawa et al. 2003). Siglec-8 was also found to be expressed on human mast cells and to a weak but consistent degree on human basophils (Kikly et al. 2000). Siglec-8 exists in 2 isoforms with identical extracellular and transmembrane sequences. A splice variant of Siglec-8, termed Siglec-8L, which contains an identical extracellular domain but a longer cytoplasmic tail possessing two ITIMs, was discovered from human genomic DNA (Bochner 2009; Foussias et al. 2001; Yousef et al. 2002). One isoform (Siglec-8) has a short cytoplasmic tail with no

known signaling sequences, while the other, Siglec-8 long form (Siglec-8L) has a longer cytoplasmic tail containing 2 -tyrosine-based signaling motifs (Foussias et al. 2000; Munday et al. 2001). The cytoplasmic region of Siglec-8L contains one consensus ITIM and a signaling lymphocyte activation molecule (SLAM)–like motif, suggesting that Siglec-8L may possess signal transduction activity (Crocker and Varki 2001a, b; Foussias et al. 2000; Munday et al. 2001). The highest levels of homology are found between Siglec-8 and Siglec-3 (49%), Siglec-5 (42%), and Siglec-7 (68%), with virtually all homology due to similarities in the extracellular and transmembrane regions. Siglec-8 is expressed not only on the surface of eosinophils but also on basophils and mast cells (Kikly et al. 2000), and the existence of both the Siglec-8 and Siglec-8L isoforms was verified in human eosinophils, basophils, and mast cells (Aizawa et al. 2002; Bochner 2009). Antibody cross-linking of Siglec-8 on human eosinophils induces caspase-dependent apoptosis in vitro (Nutku et al. 2003). Like Siglec-3 and Siglec-7 (p75/AIRM-1), ligation of Siglec-8 inhibits eosinophil survival. Cross-linking Siglec-8 with antibodies rapidly generated caspase-3–like activity and reduced eosinophil viability through induction of apoptosis. Siglec-8 activation may provide a useful therapeutic approach to reduce numbers of eosinophils in disease states where these cells are important (Nutku et al. 2008).

The siglec-8 gene mapped on chromosome 19q13.33-41, ~330 kb down-stream of Siglec-9 gene, is closely linked to genes encoding CD33, siglec-5, siglec-6, and siglec-7. It mediates sialic acid-dependent binding to human erythrocytes and to soluble sialoglycoconjugates (Floyd et al. 2000). Both Siglec-8 and Siglec8-L comprise of seven exons, of which the first five are identical, followed by marked differences in exon usage and mRNA splicing. The 499 amino acid protein encoded by the Siglec8-L open reading frame has a molecular weight of 54 kDa (Foussias et al. 2000) (Fig. 17.1).

17.6.2 Ligands for Siglec-8

Red blood cell rosettes are formed in presence of Siglec-8, and neuraminidase treatment alters rosette formation (Kikly et al. 2000; Floyd et al. 2000). Specific structures shown to bind Siglec-8 include forms of sialic acid that are linked α2–3 or α2–6 to Galβ1–4GlcNAc (Floyd et al. 2000). The binding of Siglec-8 is sialic acid-dependent. A Siglec-8-Ig chimeric protein revealed that of 172 glycan structures, ~40 structures were sialylated. Among these, avid binding was detected to a single defined glycan, NeuAcα2–3(6-*O*-sulfo)Galβ1–4[Fucα1–3]GlcNAc, also referred to as 6' sulphated sialyl Lewis X (6′-sulfo-sLe^x). Notably, neither unsulfated sLe^x (NeuAcα2–3Galβ1–4[Fucα1–3]GlcNAc), a known ligand for E-, P- and L-selectin nor an isomer with the sulfate on the 6-position of the GlcNAc residue (6-sulfo-sLe^x, NeuAcα2–3Galβ1–4[Fucα1–3](6-*O*-sulfo)GlcNAc) supported detectable binding. Whereas surfaces derivatized with sLe^x and 6-sulfo-sLe^x failed to support detectable Siglec-8 binding, 6′-sulfo-sLe^x supported significant binding with a K_D of 2.3 μM. Siglec-8 binds preferentially to the sLe^x structure bearing an additional sulfate ester on the galactose 6-hydroxyl (Bochner et al. 2005; Bochner 2009). Bochner et al. (2005) indicated that Siglec-8 is a highly specific lectin, binding preferentially to the sLe^x structure bearing an additional sulfate ester on the galactose 6-hydroxyl, namely NeuAcα2–3(6-*O*-sulfo)Galβ1–4[Fucα1–3]GlcNAc, also referred to in the literature as 6′-sulfo-sLe^x, which is a structure closely related to 6-sulfo-sLe^x, a candidate ligand for L-selectin. However, Blixt et al. (2003) suggested that Siglec-Ig chimeras showed a wide range of binding patterns with no clear binding preference for Siglec-8. Thus, Siglec-8 requires the presence of the sulphate on the sixth position of the galactose residue (Bochner 2009), though, the exact biochemical identity of natural tissue ligands for Siglec-8 remains unknown. Based on the occurrence of sulphated sLex-like structures made by airway cells, bronchial (and perhaps mononuclear cell) mucins have been suggested as candidate ligands for Siglec-F and Siglec-8 (Bochner 2009; Zhang et al. 2007).

17.6.3 Functions in Apoptosis

Recent work suggests that Siglecs are also empowered to transmit death signals, at least in myeloid cells. Strikingly, death induction by Siglecs is enhanced when cells are exposed to proinflammatory survival cytokines. Based on these insights, von Gunten and Simon (2006) hypothesized that at least some members of the Siglec family regulate immune responses via the activation of caspase-dependent and caspase-independent cell death pathways. Siglec-8 cross-linking with antibodies rapidly generated caspase-3-like activity and reduced eosinophil viability through induction of apoptosis. Siglec-8 crosslinking on eosinophils increased dissipation of mitochondrial membrane potential upstream of caspase activation. Inhibitors of mitochondrial respiratory chain components completely inhibited apoptosis. Additional experiments demonstrated that ROS was also essential for Siglec-8-mediated apoptosis and preceded Siglec-8-mediated mitochondrial dissipation (Nutku et al. 2003; 2005).

Based on structural homology alone, there was no clear mouse ortholog of Siglec-8. Therefore, using different strategies, it is now clear that Siglec-G is expressed on B lymphocytes (Hoffmann et al. 2007) while Siglec-F is most prominently expressed by mouse eosinophils (Angata et al. 2001a; Zhang et al. 2004; Tateno et al. 2005). This was somewhat unexpected based on sequence homology alone, because Siglec-F more closely resembles human Siglec-5,

which is not expressed by human eosinophils (Aizawa et al. 2003). Siglec-F is not expressed on mouse mast cells and instead is expressed on a wider range of cells including alveolar macrophages and at very low levels on T cells and neutrophils, none of which express Siglec-8 in human; and unlike Siglec-8, surface levels of Siglec-F on eosinophils and other cells increase during allergic inflammatory responses (Bochner 2009). Therefore, because of its preferential expression on eosinophils and preference for binding the same ligand, Siglec-F and Siglec-8 are best thought of as functionally convergent paralogs.

17.6.4 Siglec-8 in Alzheimer's Disease

Recent progress in pattern recognition receptors of monocytes and macrophages has revealed that Siglec family of receptors is an important recognition receptor for sialylated glycoproteins and glycolipids. Studies revealed that microglial cells contain only one type of Siglec receptors, Siglec-11, which mediates immunosuppressive signals and thus inhibits the function of other microglial pattern recognition receptors, such as TLRs, NLRs, and RAGE receptors. Salminen and Kaarniranta (2009) reviewed and indicated that aggregating amyloid plaques are masked in AD by sialylated glycoproteins and gangliosides. Sialylation and glycosylation of plaques, mimicking the cell surface glycocalyx, can activate the immunosuppressive Siglec-11 receptors, as well as hiding the neuritic plaques, allowing them to evade the immune surveillance of microglial cells. This kind of immune evasion can prevent the microglial cleansing process of aggregating amyloid plaques in AD.

17.7 Siglec-9

17.7.1 Characterization and Phylogenetic Analysis

The cDNA of human Siglec-9 encodes a type 1 transmembrane protein with three extracellular immunoglobulin-like domains and a cytosolic tail containing two tyrosines, one within a typical ITIM. The N-terminal V-set Ig domain has most amino acid residues typical of Siglecs. Expression of the full-length cDNA in COS cells induces sialic-acid dependent erythrocyte binding. A recombinant soluble form of the extracellular domain binds to α2-3 and α2-6-linked sialic acids. The carboxyl group and side chain of sialic acid are essential for recognition, and mutation of a critical arginine residue in domain 1 abrogates binding. The underlying glycan structure also affects binding, with Galβ1-4Glc[NAc] being preferred. Siglec-9 shows closest homology to Siglec-7 and both belong to a Siglec-3/CD33-related subset of Siglecs (with Siglecs-5, -6, and -8). The Siglec-9 gene is on chromosome 19q13.3-13.4, in a cluster with all Siglec-3/CD33-related Siglec genes, suggesting their origin by gene duplications. A homology search of the *Drosophila melanogaster* and *C.elegans* genomes suggests that Siglec expression may be limited to animals of deuterostome lineage, coincident with the appearance of the genes of the sialic acid biosynthetic pathway (Angata and Varki 2000a).

A full-length cDNA encoding Siglec-9, isolated from a dibutyryl cAMP-treated HL-60 cell cDNA library, is predicted to contain three extracellular Ig-like domains that comprise an N-terminal V-set domain and two C2-set domains, a transmembrane region and a cytoplasmic tail containing two putative tyrosine-based signaling motifs. Overall, Siglec-9 is approximately 80% identical in amino acid sequence to Siglec-7, suggesting that the genes encoding these two proteins arose relatively recently by gene duplication. Siglec-9 was found to be expressed by monocytes, neutrophils, and $CD16^+$, $CD56^-$ cells. Weaker expression was observed on app. 50% of B cells and NK cells and minor subsets of $CD8^+$ T cells and $CD4^+$ T cells (Zhang et al. 2000).

17.7.2 Functions of Siglec-9

17.7.2.1 Siglec-9 Transduces Apoptotic and Nonapoptotic Death Signals

In normal neutrophils, Siglec-9 ligation induces apoptosis. The increased Siglec-9-mediated death was mimicked in vitro by proinflammatory cytokines, such as GM-CSF, IFN-α, and IFN-γ, and was demonstrated to be caspase independent. Study suggested that apoptotic (ROS- and caspase-dependent) and nonapoptotic (ROS-dependent) death pathways are initiated in neutrophils via Siglec-9. The new insights have important implications for the pathogenesis, diagnosis, and treatment of inflammatory diseases such as sepsis and rheumatoid arthritis (von Gunten et al. 2005). Siglec-9, present on neutrophils is activated by intravenous (iv) administration of Ig during immuno-therapy, resulting in caspase-dependent and caspase-independent forms of cell death, resulting in neutropenia that is sometimes seen in association with iv-Ig therapy Neutrophil death was mediated by naturally occurring anti-Siglec-9 autoantibodies present in iv-Ig. Anti- Siglec-9 autoantibody-depleted iv-Ig failed to induce this caspase-independent neutrophil death. Results explain the cause of neutropenia that is seen in association with iv Ig therapy (von Gunten et al. 2006).

17.7.2.2 Bacterial Pathogen Lower Siglec-9 Innate Immune Response

Human neutrophil Siglec-9 recognizes host Sias as ""self,"" including *in cis* interactions with Sias on the neutrophil's own surface, thereby dampening unwanted neutrophil reactivity. Neutrophils presented with immobilized multimerized Sia α2-3Galβ1-4GlcNAc units engage them *in trans* via Siglec-9. The sialylated capsular polysaccharide of group B *Streptococcus* (GBS) also presents terminal Sia α2-3Galβ1-4GlcNAc units, and similarly engages neutrophil Siglec-9, dampening neutrophil responses in a Sia- and Siglec-9–dependent manner. GBS can impair neutrophil defense functions by coopting a host inhibitory receptor via sialoglycan molecular mimicry, a novel mechanism of bacterial immune evasion (Carlin et al. 2009). Experiments using a pan-caspase-inhibitor provided evidence for caspase-independent neutrophil death in Siglec-9 responders upon Siglec-9 ligation. Septic shock patients exhibit different ex vivo death responses of blood neutrophils after Siglec-9 ligation early in shock (von Gunten et al. 2009).

17.7.2.3 Siglec-9 Enhances IL-10 Production in Macrophages

Siglec-9 modulates cytokine production in macrophage cell line RAW264. Overexpression of siglec-9 in macrophage cell lines inhibited production of pro-inflammatory cytokines such as TNFα and enhanced the production of IL-10 in an ITIM-dependent manner in response to Toll-like receptor signaling (Ando et al. 2008). Cells expressing Siglec-9 produced low levels of TNF-α upon stimulation with LPS, peptidoglycan, unmethylated CpG DNA, and double-stranded RNA. On the other hand, IL-10 production was strongly enhanced in Siglec-9-expressing cells. Similar activity was also demonstrated by Siglec-5. However, the up-regulation of IL-10 as well as the down-regulation of TNF-α was abrogated when two tyrosine residues in the cytoplasmic tail of Siglec-9 were mutated to phenylalanine (Ando et al. 2008).

17.7.2.4 Siglec-9 is Expressed on Subsets of Acute Myeloid Leukemia Cells

Like Siglec-3, Siglec-9 is also expressed on subsets of AML and may provide additional therapeutic targets in the future (Biedermann et al. 2007). Siglec-9 was absent from normal bone marrow myeloid progenitors but present on monocytic precursors. Using primary AML cells or transfected rat basophilic leukemia cells, Siglec-9 mediated rapid endocytosis of anti-Siglec-9 mAb. Siglec-9 is not only a useful marker for certain subsets of AML, but also presents as a potential therapeutic target (Biedermann et al. 2007).

17.8 Siglec-10, -11, -12, and -16

17.8.1 Siglec-10

The full-length-Siglec-10 cDNA encodes a type 1 transmembrane protein containing four extracellular Ig-like domains, a transmembrane region, and a cytoplasmic tail with two ITIMs. The N-terminal V-set Ig domain has most of the amino acid residues typical of the Siglecs. Siglec-10 shows the closest homology to Siglec-5 and Siglec-3/CD33 and mapped to the same region, on chromosome 19q13.3. Various cells and cell lines including monocytes and dendritic cells express Siglec-10. Siglec-10 is an immune system-restricted membrane-bound protein that is highly expressed in peripheral blood leukocytes, spleen, and liver. The expressed protein was able to mediate sialic acid-dependent binding to human erythrocytes and soluble sialoglycoconjugates. Siglec-10 was detected on subsets of human leucocytes including eosinophils, monocytes and a minor population of NK-like cells. The molecular properties and expression pattern suggest that Siglec-10 may function as an inhibitory receptor within the innate immune system (Li et al. 2001; Munday et al. 2001). Genomic sequence of siglec-10 is localized within the cluster of genes on chromosome 19q13.3-4 that encodes other siglec family members. The extracellular domain of siglec-10 was capable of binding to peripheral blood leukocytes. The cytoplasmic tail of siglec-10 contains four tyrosines, two of which are embedded in ITIM-signaling motifs (Y^{597} and Y^{667}) and are likely involved in intracellular signaling. The ability of tyrosine kinases to phosphorylate the cytoplasmic tyrosines was evaluated by kinase assay using wild-type siglec-10 cytoplasmic domain and Y—>F mutants. The majority of the phosphorylation could be attributed to Y^{597} and Y^{667}. Further experiments with cell extracts suggest that SHP-1 interacts with Y^{667} and SHP-2 interacts with Y^{667} in addition to another tyrosine. Therefore siglec-10, as CD33, may be characterized as an inhibitory receptor (Whitney et al. 2001). A splice variant of Siglec-10, called Siglec-10Sv3 expresses in T- and B-cells. Moreover, another splicing form of Siglec-10, named Siglec-10Sv4, was also identified. One common characteristic of all Siglec-10 splice forms (except for Siglec-10Sv2) is their cytoplasmic tail with two ITIMs and one CD150-like sequence (Kitzig et al. 2002).

17.8.2 Siglec-11

As with others in this subgroup, the cytosolic domain of Siglec-11 is phosphorylated at tyrosine residue(s) upon

pervanadate treatment of cells and then recruits the protein-tyrosine phosphatases SHP-1 and SHP-2. However, Siglec-11 has several novel features relative to the other CD33/Siglec-3-related Siglecs. First, it binds specifically to α2-8-linked sialic acids. Second, unlike other CD33/Siglec-3-related Siglecs, Siglec-11 was not found on peripheral blood leukocytes. Instead, it was expressed on macrophages in various tissues, such as liver Kupffer cells. Third, it was also expressed on brain microglia, thus becoming the second Siglec to be found in the nervous system. Fourth, whereas the Siglec-11 gene is on human chromosome 19, it lies outside the previously described CD33/Siglec-3-related Siglec cluster on this chromosome. Fifth, analyses of genome data bases indicate that Siglec-11 has no mouse ortholog and that it is likely to be the last canonical human Siglec to be reported. Finally, although Siglec-11 showed marked sequence similarity to human Siglec-10 in its extracellular domain, the cytosolic tail appeard only distantly related. Analysis of genomic regions surrounding the Siglec-11 gene suggests that it is actually a chimeric molecule that arose from relatively recent gene duplication and recombination events, involving the extracellular domain of a closely related ancestral Siglec gene (which subsequently became a pseudogene) and a transmembrane and cytosolic tail derived from another ancestral Siglec (Angata et al. 2002b). Human Siglec-11 was expressed by tissue macrophages, including brain microglia (Angata et al. 2004). Comparisons with the chimpanzee genome indicate that human Siglec-11 emerged through human-specific gene conversion by an adjacent pseudogene. Conversion involved 5 cent untranslated sequences and the Sia-recognition domain. This human protein shows reduced binding relative to the ancestral form but recognizes oligosialic acids, which are enriched in the brain. Siglec-11 is expressed in human but not in chimpanzee brain microglia. Further studies will determine if this event was related to the evolution of Homo (Hayakawa et al. 2005).

17.8.3 Siglec 12

Analyses of genomic *SIGLEC* sequences across humans, chimpanzees, baboons, rats, and mice showed that CD33rSiglecs are evolving rapidly. This is particularly pronounced in the Sia-recognizing V-set domain, suggesting that this domain is under the greatest selection pressure. The human ortholog of Siglec-12 (formerly Siglec-L1) has an Arg Cys (R122C) substitution resulting in a protein unable to bind Sias (Angata et al. 2001). This protein is referred to as Siglec-XII in humans and differentially named as Siglec-12 in primates, where the Sia-binding arginine is present. The gene in both cases is referred to as *SIGLEC12*. Reversing this mutation *in vitro* restored Sia binding. The R122C mutation of the Siglec-XII protein is fixed in the human population, i.e. it occurred prior to the origin of modern humans. Additional mutations have since completely inactivated the *SIGLEC12* gene in some but not all humans. The chimpanzee Siglec-12 is fully functional and preferentially recognizes N-glycolylneuraminic acid, which is a common sialic acid in great apes and other mammals. Reintroducing the ancestral arginine into the human molecule regenerates the same properties. Thus, the single base pair mutation that replaced the arginine on human Siglec-L1 is likely to be evolutionarily related to the previously reported loss of N-glycolylneuraminic acid expression in the human lineage. The human genome contains several Siglec-like pseudogenes that have independent mutations that would have replaced the arginine residue required for optimal sialic acid recognition during human evolution (Angata et al, 2001). Unlike other CD33-rSiglecs that are primarily found on immune cells, Siglec-XII protein is expressed on some macrophages and also on various epithelial cell surfaces in humans and chimpanzees. *SIGLEC12* gene also expresses on certain human prostate epithelial carcinomas and carcinoma cell lines. This expression correlates with the presence of the non-frame-shifted, intact *SIGLEC12* allele. Polymorphic expression of Siglec-XII in humans thus has implications for prostate cancer biology and therapeutics (Mitra et al. 2011)

17.8.3.1 Siglec-like Gene (SLG)/S2V

Foussias et al. (2001) identified the complete genomic structure of Siglec-like gene (SLG), a putative member of Siglec-3-like subgroup of Siglecs, as well as two alternative splice variants. The SLG gene is localized 32.9 kb downstream of Siglec-8 on chromosome 19q13.4. The putative SLG-S and SLG-L proteins, of 477 and 595 amino acid residues, respectively, show extensive homology to many members of the Siglec-3-like subgroup. This homology is conserved in the extracellular Ig-like domains, as well as in the cytoplasmic tyrosine-based motifs. However, SLG-L protein contains two N-terminal V-set Ig-like domains, as opposed to SLG-S and other Siglec-3-like subgroup members which contain only one such domain. The SLG-S is highly expressed in spleen, small intestine and adrenal gland, while SLG-L exhibits high levels of expression in spleen, small intestine, and bone marrow (Foussias et al. 2001).

The Siglec-like gene which comprises of 11 exons, with 10 intervening introns, is localized 278 kb telomeric to Siglec-9 and 35 kb centromeric to Siglec-8 and on chromosome 19q13.4. The coding region consists of 2,094 bp, and

encodes for a putative 76.6 kDa protein. All Siglec-conserved structural features, including V-set domain, three C-set domains, transmembrane domain, ITIM and SLAM motifs, were found in this Siglec-like gene. Also, it has the conserved amino acids essential for sialic acid binding (Yousef et al. 2002). The cDNA of S2V Siglec encodes a type 1 transmembrane protein with four extracellular Ig-like domains and a cytoplasmic tail bearing a typical ITIM and an ITIM-like motif. A unique feature of S2V is the presence of two V-set Ig-like domains responsible for the binding to sialic acid, whereas all other known siglecs possess only one. S2V is predominantly expressed in macrophage. S2V is involved in the negative regulation of signaling in macrophage by functioning as an inhibitory receptor (Yu et al. 2001a).

17.8.4 Siglec-16

The lineages of *SIGLEC11* genes in human, rodent, dog, cow and non-human primates have undergone dynamic gene duplication and conversion, forming a potential inhibitory (Siglec-11)/activating (Siglec-16) receptor pair in chimpanzee and humans. A cDNA encoding human Siglec-16, classed as a pseudogene in databases (*SIGLECP16*), is expressed in various cell lines and tissues. A polymorphism screen for the two alleles (wild type and four-base pair deletion) of *SIGLEC16* found their frequencies to be 50% amongst the UK population. A search for donor sequences for Siglec-16 revealed a subfamily of activating Siglec with charged transmembrane domains predicted to associate with ITAM-encoding adaptor proteins (Cao et al. 2008). Using antisera specific to the cytoplasmic tail of Siglec-16, Cao et al. (2008) identified Siglec-16 expression in $CD14^+$ tissue macrophages and in normal human brain, cancerous oesophagus and lung. Although, most CD33rSiglecs have immune receptor tyrosine-based inhibitory motifs and signal negatively, novel DAP-12-coupled 'activating' CD33rSiglecs have been identified, such as siglec-14 and siglec-16, which are paired with the inhibitory receptors, siglec-5 and siglec-11, respectively (Cao and Crocker 2011). Siglec-16 was expressed at cell surface in the presence of DAP12, but not the FcRγ chain.

17.9 Mouse Siglecs

17.9.1 Evolution of Mouse and Human CD33-rSiglec Gene Clusters

Unlike most human Siglec-3 (hSiglec-3)-related Siglecs with promiscuous linkage specificity, mouse Siglec (mSiglec-F) shows a strong preference for α2–3-linked sialic acids. It is predominantly expressed in immature cells of the myelomonocytic lineage and in a subset of CD11b (Mac-1)-positive cells in some tissues. A comprehensive comparison of Siglecs between human and mouse genomes suggests that mouse genome contains eight Siglec genes, whereas the human genome contains 14 Siglec genes and a Siglec-like gene. Although a one-to-one orthologous correspondence between human and mouse Siglecs 1, 2, and 4 is confirmed, the Siglec-3-related Siglecs showed marked differences between human and mouse. Angata et al. (2001b) identified only four Siglec genes and two pseudogenes in the mouse chromosome 7 region syntenic to the Siglec-3-related gene cluster on human chromosome 19, which, in contrast, contains seven Siglec genes, a Siglec-like gene, and thirteen pseudogenes. Although analysis of gene maps and exon structures allows tentative assignments of mouse-human Siglec ortholog pairs, the possibility of unequal genetic recombination makes the assignments inconclusive. Current information suggests that mSiglec-F is likely a hSiglec-5 ortholog. The previously reported mSiglec-3/CD33 and mSiglec-E/MIS are likely orthologs of hSiglec-3 and hSiglec-9, respectively. The other Siglec-3-like gene in the cluster (mSiglec-G) is probably a hSiglec-10 ortholog. Another mouse gene (mSiglec-H), without an apparent human ortholog, lies outside of the cluster. Thus, although some duplications of Siglec-3-related genes predated separation of the primate and rodent lineages (about 80–100 million years ago), this gene cluster underwent extensive duplications in the primate lineage thereafter (Angata et al. 2001b).

17.9.2 Mouse CD33/Siglec-3

A cDNA for the putative mouse ortholog of hCD33 was cloned in 1994 (Tchilian et al. 1994). Similarities within the extracellular domain between hCD33 and mouse CD33 (mCD33; 62% identity in amino acid sequence) (Tchilian et al. 1994) and similar gene structure and chromosomal position relative to adjacent genes (Angata et al. 2001b) warrant its designation as the mouse ortholog of hCD33. However, the lack of sequence similarity in the cytosolic domains and difficulties in resolving phylogenetic relationships among the related Siglec molecules in humans and mice have raised questions about its functional equivalence to hCD33 (Angata et al. 2000, 2001a).

Differences with Human CD33: Although 16 human siglec proteins have been discovered, the mouse siglec family has fewer members (Yu et al. 2001b; Ulyanova et al. 2001): sialoadhesin, MAG, SMP, CD22, and CD33. Structurally, sialoadhesin, MAG, SMP, and CD22 are very similar in mice and humans. In contrast, human and mouse CD33, although

somewhat similar in their extracellular domains, are strikingly different in their transmembrane and cytoplasmic regions. Human CD33 contains two ITIM sequences in the cytoplasmic tail, whereas mouse CD33 does not. Characterization of several ITIM-containing human siglecs implies that there must be murine ITIM-containing siglecs. Ulyanova et al. (2001) described the molecular cloning and characterization of a murine ITIM-containing myeloid cell-restricted inhibitory siglec, MIS. None of the two Siglec-3-related Siglecs in mouse, i.e. mSiglec-3/CD33 (Tchilian et al. 1994) and mSiglec-E/MIS (Yu et al. 2001b) shows a clear one-to-one orthologous correspondence to hSiglecs (Angata and Varki 2000; Ulyanova et al. 2001). Although it is likely that these mSiglec-3-related Siglecs, as well as one reported by Dehal et al. (2001) are present in a similar gene cluster in mouse chromosome 7 (Dehal et al. 2001), there have been few reports of a comprehensive analysis of Siglec-3-related mouse Siglecs. Like hCD33, mCD33 is expressed on myeloid precursors in the bone marrow, albeit mostly in the more mature stages of the granulocytic lineage. Moreover, unlike hCD33, mCD33 in peripheral blood is primarily expressed on granulocytes. Also, unlike hCD33, mCD33 did not bind to α2-3- or α2-6-linked sialic acids on lactosamine units. Instead, it showed distinctive sialic acid-dependent binding only to the short O-linked glycans of certain mucins and weak binding to the sialyl-Tn epitope. Results indicate substantial species differences in CD33 expression patterns and ligand recognition and suggest functional degeneracy between mCD33 and the other CD33-related Siglec proteins expressed on cells of the myeloid lineage (Brinkman-Van der Linden et al. 2003).

17.9.3 Siglec-3-Related Siglecs in Mice

17.9.3.1 mSiglec-E

The mouse siglec E (mSiglec-E)- cDNA encodes a protein of 467 amino acids that contains three extracellular Ig-like domains, a transmembrane region and a cytoplasmic tail bearing two ITIMs. mSiglec-E is highly expressed in mouse spleen, which is rich in leucocytes. The ITIMs of mSiglec-E can recruit SHP-1 and SHP-2, two inhibitory regulators of immunoreceptor signal transduction. mSiglec-E, through cell-cell interactions, is probably involveed in haematopoietic cells and the immune system as an inhibitory receptor. In comparison with the known members, mSiglec-E exhibits a high degree of sequence similarity to both human siglec-7 and siglec-9. The gene encoding mSiglec-E is localized in the same chromosome as that encoding mouse CD33. Phylogenetically, though Yu et al. (2001a,b) did not show clear relation between mouse mSiglec-E with any human siglecs, Angata et al. (2001b) suggested that mSiglec-E is likely an ortholog of hSiglec-9.

17.9.3.2 mSiglec-F

Siglec-F is a CD33-rSiglec prominently expressed on mature circulating mouse eosinophils, and on some myeloid precursors in bone marrow (Zhang et al. 2004; Angata et al. 2001a). It has a binding preference for α2-3–linked Sias (Angata et al. 2001), with the best-known ligand being 6'sulfo-sialyl-Le^X (Tateno et al. 2005). Of interest, this structure is also the preferred ligand for human Siglec-8- (Bochner et al. 2005), a molecule also specifically expressed on human eosinophils (Floyd et al. 2000; Kikly et al. 2000). Although mouse Siglec-F is not the true ortholog of human Siglec-8 (Angata et al. 2001a), their marked similarities in expression patterns and ligand preferences suggest that they play equivalent roles.

The elevated eosinophil count in allergic conditions is well known as is a critical role for $CD4^+$ Th2 cells in regulating allergic inflammatory responses involving eosinophils. Zhang et al. (2007) investigated the biologic roles of Siglec-F in Siglec-F–null mice in an induced lung allergic response model. This model also mimics some other features of bronchial asthma in humans, such as IgE-mediated mast-cell activation and degranulation, airway inflammation and hyperreactivity, $CD4^+$ T-cell infiltration and cytokine production, goblet-cell hyperplasia, and mucus overproduction. Data with WT mice using this model suggested a negative feedback loop involving Siglec-F in controlling eosinophilic responses, a hypothesis confirmed by studies of Siglec-F–null mice. Zhang et al. (2007) studied in vivo functions of Siglec-F, expressed on mouse eosinophils, which are prominent in allergic processes. Induction of allergic lung inflammation in mice caused up-regulation of Siglec-F on blood and bone marrow eosinophils, accompanied by newly induced expression on some $CD4^+$ cells, as well as quantitative up-regulation of endogenous Siglec-F ligands in the lung tissue and airways. With the ITIM in the cytosolic tail of Siglec-F, Zhang et al. (2007) suggested a negative feedback loop, controlling allergic responses of eosinophils and helper T cells, via Siglec-F and Siglec-F ligands. Siglec-F–null mice, allergen-challenged null mice showed increased lung eosinophil infiltration, enhanced bone marrow and blood eosinophilia, delayed resolution of lung eosinophilia, and reduced peribronchial-cell apoptosis. Data supporting the proposed negative feedback role for Siglec-F, represent the in vivo demonstration of biologic functions for any CD33rSiglec, and predict a role for human Siglec-8 (the isofunctional paralog of mouse Siglec-F) in regulating the pathogenesis of human eosinophil-mediated disorders. This demonstration suggests an in vivo biologic role for a CD33rSiglec, and also potential role for CD33rSiglecs in regulating T-cell induction of eosinophilic responses. To search for a mouse Siglec (mSiglec) ortholog of Siglec-8 and other mouse Siglec paralogs, Aizawa et al. (2003) searched public database with cDNA sequences of human

Siglec-5 to -10 and identified two novel mSiglecs. One has significant sequence identity to human Siglec-5 and is a splice variant of mSiglec-F. The other has greatest sequence identity to human Siglec-10 (mSiglec-G). Both mSiglecs have extracellular Ig-like domains and intracellular tyrosine-based motifs. A expression of mSiglec-5 (or -F), -10, and -E mRNA was detected in purified mouse eosinophils, but analyses suggested that mSiglec-10 is probably most relevant to mouse eosinophils.

Mouse Siglec-F and Human Siglec-8 are Functionally Convergent Paralogs: Unlike most human hSiglec-3rSiglecs with promiscuous linkage specificity, mSiglec-F shows a strong preference for α2-3-linked sialic acids. It is predominantly expressed in immature cells of the myelomonocytic lineage and in a subset of CD11b (Mac-1)-positive cells in some tissues. The sialoside sequence 6'-sulfo-sLe^x (Neu5Ac α2-3 [6-SO4]Galβ1-4[Fuc α1-3]GlcNAc) is a preferred ligand for Siglec-F. The lectin activity of Siglec-F on mouse eosinophils was "masked" by endogenous cis ligands and could be unmasked by treatment with sialidase. Unmasked Siglec-F mediated mouse eosinophil binding and adhesion to multivalent 6'-sulfo-sLe^x structure. Although there is no clear-cut human ortholog of Siglec-F, Siglec-8 is encoded by a paralogous gene that is expressed selectively by human eosinophils and has been found to recognize 6'-sulfo-sLe^x. It seems that mouse Siglec-F and human Siglec-8 have undergone functional convergence during evolution and implicate a role for the interaction of these siglecs with their preferred 6'-sulfo-sLe^x ligand in eosinophil biology (Tateno et al. 2005).

Siglec-F showed efficient endocytosis of anti-Siglec antibody, and *Neisseria meningitidis* bearing sialylated glycans. Like CD22, endocytosis was dependent on ITIM and ITIM-like motifs. While endocytosis of CD22 was mediated by a clathrin-dependent mechanism and was sorted to early endosome and recycling compartments, Siglec-F endocytosis was directed to lysosomal compartments and was mediated by a mechanism that was independent of clathrin and dynamin. Like CD22, Siglec-F mediated endocytosis of anti-Siglec-F and sialoside ligands, a function requiring intact tyrosine-based motifs. In contrast, however, Siglec-F endocytosis was clathrin and dynamin independent, required ADP ribosylation factor 6, and trafficed to lysosomes (Tateno et al. 2007).

17.9.3.3 mSiglec-G

It is now well accepted that the innate immune system recognizes both damage (or danger)- and pathogen-associated molecular patterns (DAMP and PAMP, respectively) through pattern recognition receptors, such as Toll-like receptors (TLR) and/or Nod-like receptors (NLR). Less clear are whether and how the response to PAMP and DAMP are regulated differentially. The answers may reveal whether the primary goal of the immune system is to defend against infections or to alert the host of tissue injuries. As demonstrated the host response to DAMP is controlled by a DAMP-CD24-Siglec axis. CD24-Siglec G/10 pathway plays a key role in discriminating between DAMPs and PAMPs (Liu et al. 2009). Siglec-G is a recently identified protein with an inhibitory function restricted to a B-cell subset, the B1 cells and is an ortholog of Siglec-10. Siglec-G inhibits Ca2+ signaling specifically in these cells. In addition, it controls the cellular expansion and antibody secretion of B1 cell population (Hoffmann et al. 2007). Thus, both Siglecs, Siglec-G/Siglec-10 and CD22 modulate BCR signaling on different B-cell populations in a mutually exclusive fashion (Nitschke 2009).

17.9.3.4 mSiglec-H as an Endocytic Receptor

Siglec-H is a murine CD33-related endocytic receptor that lacks intrinsic tyrosine-based signaling motifs. Although Siglec-H has the typical structural features required for sialic acid binding, no evidence for carbohydrate recognition has been obtained. Siglec-H is expressed specifically on plasmacytoid dendritic cell (pDC) precursors in bone marrow, spleen, blood, and lymph nodes. It is also expressed in a subset of marginal zone macrophages in spleen and in medullary macrophages in lymph nodes. Siglec-H functions as an endocytic receptor and mediates efficient internalization of anti-Siglec-H Abs. Immunizing mice with ovalbumin-conjugated anti-Siglec-H Ab in presence of CpG generated antigen-specific CD8 T cells in vivo. Targeting Siglec-H may therefore be a useful way of delivering antigens to pDC precursors for cross-presentation (Zhang et al. 2006). Siglec-H depends on DAP12 for surface expression; and cross-linking with anti-siglec-H antibodies can selectively inhibit interferon-α production by pDCs following TLR9 (Toll-like receptor 9) ligation.

17.9.3.5 Myeloid Inhibitory Siglec in Mice

Myeloid inhibitory siglec (MIS) belongs to the family of sialic acid-binding Ig-like lectins. The full-length MIS cDNA from murine bone marrow cells is predicted to contain an extracellular region comprising three Ig-like domains (V-set amino-terminal domain followed by two C-set domains), a transmembrane domain and a cytoplasmic tail with two ITIM-like sequences. The closest relative of MIS in the siglec family is human siglec 8. Extracellular regions of these two siglecs share 47% identity at the amino acid level. Southern blot analysis suggests the presence of one MIS gene. MIS is expressed in the spleen, liver, heart, kidney, lung and testis tissues. Several isoforms of MIS protein exist due to the alternative splicing. In a human promonocyte cell line, MIS was able to bind Src homology 2-containing protein-tyrosine phosphatases, SHP-1 and SHP-2. This binding was mediated by the membrane-proximal ITIM of MIS. Moreover, MIS

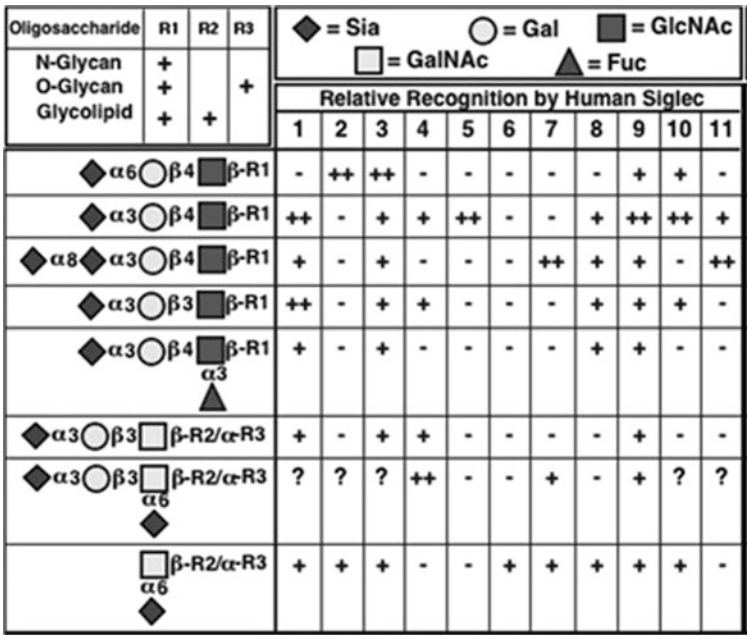

Fig. 17.4 *Glycan-binding specificities of human Siglecs.* With a few exceptions (CD22 and MAG), binding specificities of human Siglecs have varied significantly. In addition to assay formats and glycan linker issues, the density and arrangement of the ligands studied could be responsible for this variation. The most commonly reported specificities for the most commonly studied sialylated glycans are shown. Relative binding within studies of each Siglec is indicated as ++, strong binding; +, detectable binding; and –, very weak or undetectable binding. Not shown is the recently reported strong-binding preference of hSiglec-8 and mSiglec-F for 6′-sulfated-sialyl-Lewis x (sLex) and of hSiglec-9 for 6-sulfated-sLex. See text for the discussion (Reprinted with permission from Varki and Angata 2006 © Oxford University Press)

exerted an inhibitory effect on FcγRI receptor-induced calcium mobilization (Ulyanova et al. 2001).

17.10 Glycoconjugate Binding Specificities of Siglecs

Siglecs exhibit specificities for both the linkage and the nature of sialic acids in N-glycans, O-glycans and glycolipids. Many Siglecs recognize α2–3- and α2–6-linked sialic acids (Freeman et al. 1995; Brinkman-Van der Linden and Varki 2000), whereas others bind to other sialylated structures (Fig. 17.4). For example, Siglec-1 has been shown to bind the highly glycosylated surface protein CD43 (van den Berg et al. 2001), the epithelial mucin MUC-1 (Nath et al. 1999), and sialylated LPS of *Neisseria meningitidis* (Nm) (Jones et al. 2003). The Nm LPS sialylation can lead to increased bacterial susceptibility to phagocytic uptake, a phenomenon in direct contrast to earlier reported protective effects of LPS sialylation (Jones et al. 2003). Earlier studies of siglec specificities focused on α2-3- and α2-6-sialyllactos(amin)es and on one or two of the siglecs at a time. The binding of siglec-1, siglec-3 (CD33), siglec-4a (MAG), and siglec-5 to α2-3 sialyllactosamine is affected markedly by the presence of an α1-3-linked fucose. Studies revealed that: (1) while siglecs may not interfere with selectin-mediated recognition, fucosylation could negatively regulate siglec binding; (2) In contrast to earlier studies, siglec-3 prefers α2-6-sialyllactose; (3) siglec-5 binds α2-8-linked sialic acid, making it least specific for linkage recognition; (4) siglecs-2 (CD22), -3, -5, and -6 (obesity-binding protein-1) showed significant binding to sialyl-Tn (Neu5Acα2-6-GalNAc), a tumor marker associated with poor prognosis; (5) siglec-6 is an exception among siglecs in not requiring the glycerol side chain of sialic acid for recognition; (6) all siglecs require the carboxyl group of sialic acid for binding; (7) the presentation of sialyl-Tn epitope and/or more extended structures that include this motif may be important for optimal recognition by siglecs (Brinkman-Van der Linden and Varki 2001). Among a panel of glycans tested, Siglec-3 showed enhanced binding to a multivalent form of sialyl-Tn (NeuAcα2–6GalNAc) disaccharides (Brinkman-Van der Linden et al. 2003).

To determine if differences in glycan composition of siglec-5, siglec-7 and siglec-8 that may modify their function, Freeman et al. (2001) characterized N-linked oligosaccharide distribution in these three glycoproteins. The glycan pools from siglec-5 and siglec-7 contained a larger proportion of sialylated and core-fucosylated biantennary, triantennary and tetra-antennary oligosaccharides, whereas carbohydrate mixture released from siglec-8 is noticeably less sialylated and is

more abundant in 'high-mannose'-type glycans. Moreover, it was found that, in contrast to CD22 and CD33, mutating the conserved potentially N-linked glycosylation site in first domain has no effect on binding mediated by siglec-5 or siglec-7 (Freeman et al. 2001). Using recombinant chimeric soluble receptors, siglec-transfected cell lines and macrophages from wild-type and Sn-deficient mice, it was observed that sialylated but not non-sialylated variants of either genetic background were specifically recognized by Sn and siglec-5, whereas other siglecs examined were ineffective. In addition, macrophages expressing Sn, as well as transfectants expressing Sn or siglec-5, bound and phagocytosed sialylated bacteria in a siglec- and sialic acid-dependent manner.

Most mammalian cell surfaces display two major sialic acids (Sias), N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Humans lack Neu5Gc due to a mutation in CMP-Neu5Ac hydroxylase, which occurred after evolutionary divergence from great apes. Sonnenburg et al. (2004) described an apparent consequence of human Neu5Gc loss: domain-specific functional adaptation of Siglec-9. While recombinant human Siglec-9 showed recognition of both Neu5Ac and Neu5Gc, in striking contrast, chimpanzee and gorilla Siglec-9 strongly preferred binding Neu5Gc. Results also indicated that endogenous Sias (rather than surface Sias of bacterial pathogens) are the functional ligands of CD33rSiglecs and suggested that the endogenous Sia landscape is the major factor directing evolution of CD33rSiglec binding specificity. Findings suggested ongoing adaptive evolution specific to the Sia-binding domain, possibly of an episodic nature. Such domain-specific divergences should also be considered in upcoming comparisons of human and chimpanzee genomes (Sonnenburg et al. 2004; Varki and Crocker 2009). Recent work has also shown that engagement of neutrophil-expressed siglec-9 by certain strains of sialylated Group B streptococci can suppress killing responses, thereby providing experimental support for pathogen exploitation of host CD33rSiglecs (Cao and Crocker 2011).

Siglec-Mediated Cell Adhesion to Gangliosides: Binding specificities of MAG, SMP, and sialoadhesin were compared by measuring siglec-mediated cell adhesion to immobilized gangliosides. The α-series gangliosides displayed enhanced potency for MAG- and SMP-mediated cell adhesion, whereas sialoadhesin-mediated adhesion was comparable with α-series and non-α-series gangliosides. GD1α derivatives with modified sialic acids (7-, 8-, or 9-deoxy) or sulfate (instead of sialic acid) at the III(6)-position supported adhesion comparable with that of GD1α. A novel GT1aα analog with sulfates at two internal sites of sialylation (NeuAcα 2,3Galβ1,4GalNAc-6-sulfateβ1, 4Gal3-sulfateβ1, 4Glcβ1,1'ceramide) was the most potent siglec-binding structure. Compiled reports indicated that MAG and SMP display an extended structural specificity with a requirement for a terminal α2, 3-linked NeuAc and great enhancement by nearby precisely spaced anionic charges (Collins et al. 1999). Studies on the interaction of CD33 related-siglecs-5,-7,-8,-9,-10 with gangliosides GT1b, GQ1b, GD3, GM2, GM3 and GD1a revealed that Siglec-5 bound preferentially to GQ1b, but weakly to GT1b, whereas siglec-10 interacted only with GT1b ganglioside. Siglec-7 and siglec-9 displayed binding to gangliosides GD3, GQ1b and GT1b bearing a disialoside motif, though siglec-7 was more potent; besides, siglec-9 interacted also with GM3. Siglec-8 demonstrated low affinity to the gangliosides tested compared with other siglecs. Despite high structural similarity of CD33 related siglecs, they demonstrated different ganglioside selectivity, in particular to the Neu5Acα2-8Neu5Ac motif (Rapoport et al. 2003a). Siglec-7 binds to GD3 ganglioside, LSTb oligosaccharide, sialyl Le^a, and NeuAcα2–8NeuAc, whereas Siglec-9 preferentially binds GD1a ganglioside and LSTc oligosaccharide (Yamaji et al. 2002; Rapoport et al. 2003a; Miyazaki et al. 2004).

Sulfated Gangliosides, High-Affinity Ligands for Neural Siglecs, Inhibit NADase Activity of CD38: Three kinds of novel sulfated gangliosides structurally related to the Chol-1 (α-series) ganglioside GQ1bα were synthesized. These sulfated gangliosides were potent inhibitors of NADase activity of leukocyte cell surface antigen CD38. Among the synthetic gangliosides, GSC-338 (II(3)III(6)-disulfate of iso-GM1b) was surprisingly found to be the most potent structure in both the NADase inhibition and MAG-binding activity. The study indicated that the sulfated gangliosides are useful to study the recognition of the internal tandem sialic acid residues α2-3-linked to Gal(II(3)) as well as the siglec-dependent recognition including a terminal sialic acid residue (Hara-Yokoyama et al. 2003; Ito et al. 2003).

Binding of Soluble Siglecs with Sulfated Oligosaccharides: Soluble siglecs were studied with polyacrylamide glycoconjugates in which: (1) the Neu5Ac residue was substituted by a sulfate group (Su); (2) glycoconjugates contained both Su and Neu5Ac; (3) sialoglycoconjugates contained a tyrosine-O-sulfate residue. Sulfate derivatives of LacNAc did not bind siglecs-1, -4, -5, -6, -7, -8, -9, and -10; binding of 6'-O-Su-LacNAc to siglec-8 was stronger than binding of 3'SiaLacNAc. The relative affinity of 3'-O-Su-TF binding to siglecs-1, -4, and -8 was similar to that of 3'SiaTF. 3'-O-Su-Le(c) displayed two-fold weaker binding to siglec-1 and siglec-4 than 3'SiaLe(c). The interaction of soluble siglecs with sulfated oligosaccharides containing sialic acid showed that siglecs-1, -4, -5, -6, -7, -9, and -10 did not interact with

these compounds; binding of 6-O-Su-3'SiaLacNAc and 6-O-Su-3'SiaTF to siglec-8 was weaker than that of the corresponding sulfate-free sialoside probes. Siglec-8 displayed affinity to 6'-O-Su-LacNAc and 6'-O-Su-SiaLe^x, and defucosylation of the latter compound led to an increase in the binding. Sialoside probes containing tyrosine-O-sulfate residue did not display increased affinity to siglecs-1 and -5 compared with glycoconjugates containing only sialoside. Cell-bound siglecs-1, -5, -7, and -9 did not interact with 6-O-Su-3'SiaLacNAc, whereas the sulfate-free probe 3'SiaLacNAc demonstrated binding. In contrast, the presence of sulfate in 6-O-Su-6'SiaLacNAc did not affect binding of the sialoside probe to siglecs. 6'-O-Su-SiaLe^x displayed affinity to cell-bound siglecs-1 and -5; its isomer 6-O-Su-SiaLe^x bound more strongly to siglecs-1, -5, and -9 than SiaLe^x (Rapoport et al. 2006).

Potent Inhibitors of MAG: Ten of the known human siglecs or their murine orthologs were evaluated for their specificity for over 25 synthetic sialosides representing most of the major sequences terminating carbohydrate groups of glycoproteins and glycolipids. Each siglec was found to have a unique specificity for binding 16 different sialoside-streptavidin-alkaline phosphatase probes. Competitive inhibition studies revealed that Siglec-4 binds with 500–10,000-fold higher affinity to a series of mono- and di-sialylated derivatives of the O-linked T-antigen (Galβ1-3 GalNAc α OThr) as compared with α-methyl-NeuAc (Blixt et al. 2003).

Group B Streptococcal Capsular Sialic Acids Interact with Siglecs: Group B Streptococcus (GBS) is classified into nine serotypes that vary in capsular polysaccharide (CPS) architecture, but share the presence of terminal sialic acid (Sia) residue. The position and linkage of GBS Sia closely resembles that of cell surface glycans found abundantly on human cells. CD33-rSiglecs are expressed on host leukocytes that engage host Sia-capped glycans and send signals that dampen inflammatory gene activation. GBS evolved to display CPS Sia as a form of molecular mimicry limiting the activation of an effective innate immune response. Carlin et al. (2007) demonstrated that GBS of several serotypes interact in a Sia- and serotype-specific manner with certain human CD33-rSiglecs, including hSiglec-9 and hSiglec-5 expressed on neutrophils and monocytes. Modification of GBS CPS Sia by O-acetylation (OAc) has been recognized, and the degree of OAc can markedly impact the interaction between GBS and hSiglecs-5, -7, and -9. Thus, production of Sia-capped bacterial polysaccharide capsules that mimic human cell surface glycans in order to engage CD33-rSiglecs may be an example of bacterial mechanism of leukocyte manipulation (Carlin et al. 2007).

17.11 Functions of CD33-Related Siglecs

17.11.1 Endocytosis

Although it is assumed that CD33-related Siglecs have important roles in modulating leukocyte behavior, including inhibition of proliferation or cellular activation, modulation of cytokine secretion, and induction of apoptosis, their precise signaling functions remain unknown (Crocker et al. 2007; Crocker 2005; Varki and Angata 2006). Nevertheless, studies have demonstrated their interactions with various sialylated pathogens and suggested their potential importance in host defense and pathogenicity. For example, several pathogens, including *Neisseria meningitides*, Group B Streptococci, and *Campylobacter jejuni*, have been shown to bind to CD33-related Siglecs (Jones et al.; 11 2003; Avril et al. 2006; Carlin et al. 2007). Furthermore, there is increasing evidences that indicate endocytic capacities of human CD33-related Siglecs (Lock et al. 2004; Walter et al. 2005; Biedermann et al. 2007). This might be of physiological relevance for clearance of sialylated antigens and modulation of antigen presentation. Endocytosis of CD33 based immunotherapy results in cellular uptake of the drug (GO; Mylotarg^{TM}), which is cleaved intracellularly to release the toxic moiety (Linenberger 2005; Damle and Frost 2003). CD33 is present on tumor cells of 85–90% of adult and pediatric patients with acute myeloid leukemia (AML), and the therapeutic success of GO depends largely on its CD33-dependent uptake. Other CD33-related Siglecs, such as Siglec-8 for targeting eosinophils in allergic inflammation or other disease states, or Siglec-9 for treatment of AML or inflammatory diseases suggest are potential targets in clinical exploitation (Biedermann et al. 2007; Nutku et al. 2003; Von Gunten et al. 2005). The mechanisms underlying the endocytosis of CD33-related Siglecs remain elusive. Studies of CD33, Siglec-5, Siglec-F, and Siglec-9 have shown that they all endocytose slowly when bound to antibody. Also, CD33, Siglec-F, and Siglec-9 use their ITIMs for endocytosis (Walter et al. 2005; Biedermann et al. 2007; Tateno et al. 2007). However, it is not clear whether the ITIMs stimulate endocytosis when they are phosphorylated or when they are not phosphorylated. Walter et al. (2008b) identified proteins that bind to CD33 in an ITIM-dependent manner and assessed their importance for CD33 internalization in human myeloid cells by specific silencing of target genes through expression of siRNA.

It was shown that endocytosis is largely limited and determined by the intracellular domain while the extracellular and transmembrane domains play a minor role. Tyrosine phosphorylation, most likely through Src family kinases, increases uptake of CD33 depending on the integrity of two cytoplasmic ITIMs. Simultaneous depletion of protein tyrosine phosphatases (SHP1 and SHP2), which bound to phosphorylated CD33, increased internalization of CD33 slightly in some

cell lines, whereas depletion of spleen tyrosine kinase (Syk) had no effect, implied that SHP1 and SHP2 can dephosphorylate the ITIMs or mask binding of phosphorylated ITIMs to an endocytic adaptor. Studies indicated that restraint of CD33 internalization through intracellular domain is relieved partly when the ITIMs are phosphorylated and thus showed that Shp1 and Shp2 can modulate this process (Walter et al. 2008c). Walter et al. (2008b) indicated that phosphorylation-dependent ubiquitylation regulates cell surface expression and internalization, and thus possibly function, of CD33/Siglec-3, suggesting an important role of ubiquitin in endocytosis of ITIM-bearing inhibitory immunoreceptors.

17.11.2 Phagocytosis of Apoptotic Bodies

Elimination of apoptotic bodies is one of the important functions of macrophages. A specific apoptotic glycosylation pattern may play an assistant or even a causative role in phagocytosis of apoptotic bodies. Taking into account that siglecs, a mannose receptor and galectins expressed on macrophages could be involved in engulfment of apoptotic bodies, their potential expression on THP-1 cells was assessed by means of polyacrylamide glycoconjugates. A strong binding of cells occurred to siglec ligands (3'SiaLac, 6'SiaLac, [Neu5Acα2-8]2) and galectin ligands (LacNAc, GalNAcβ1 - 4GlcNAc, Galβ1 - 3GalNAcβ and asialoGM1), where as Galβ1 - 3GalNAcβ-terminated chains represented the apoptotic bodies; the other tested galectin ligands did not appear to be the target for THP-1 cells (Rapoport et al. 2003b).

Macrophage lectins were probed with neoglycoconjugates. Glyc-PAA-fluo where carbohydrate is linked to fluorescein-labeled polyacrylamide. The neoglycoconjugates containing a Neu5Acα2-3Gal fragment bound to macrophages isolated from blood of healthy donors. Besides, carbohydrate chains containing the same fragment were revealed on apoptotic bodies. Phagocytosis of apoptotic bodies by macrophages was inhibited with sialooligosaccharide ligands of siglec-5 and mAbs to siglec-5. Thus, siglec-5 expressed on macrophages could participate in phagocytosis of apoptotic bodies. In addition, siglecs of tumor-associated macrophages modify engulfment of apoptotic bodies. The phagocytic potency of macrophages isolated from blood of breast cancer patients was lower than engulfment ability of macrophages obtained from healthy donors. Staining of macrophages from blood of tumor patients was more intense than that of macrophages from healthy donors; phagocytosis of apoptotic bodies by tumor-associated macrophages was inhibited by carbohydrates that are known to be ligands for siglecs (Rapoport et al. 2005).

17.12 Siglecs as Targets for Immunotherapy

The restricted expression of several siglecs to one or a few cell types makes them attractive targets for cell-directed therapies. Anti-CD33 antibodies have been used alone-and more effectively, attached to chemotherapy agents or radioisotopes-to treat those with AML (Nemecek and Matthews 2002). The anti-CD33 (Siglec-3) antibody gemtuzumab (Mylotarg) is approved for treatment of acute myeloid leukemia (AML) and antibodies targeting CD22 (Siglec-2) are currently in clinical trials for treatment of B cell non-Hodgkins lymphomas and autoimmune diseases. O'Reilly and Paulson (2009) reviewed the properties of siglecs that make them attractive for cell-targeted therapies. In normal myelopoiesis, expression of CD33 is restricted to advanced stages of differentiation, whereas primitive stem cells do not express CD33. It was shown that leukaemic stem cells in patients with $CD33^+$ AML express CD33 (Hauswirth et al. 2007). Antibody-targeted chemotherapy is a therapeutic strategy in cancer therapy that involves a monoclonal antibody specific for a tumor-associated antigen, covalently linked via a suitable linker to a potent cytotoxic agent. Tumor-targeted delivery of a cytotoxic agent in the form of an immunoconjugate is expected to improve its antitumor activity and safety.

Antibody-targeted chemotherapy with gemtuzumab ozogamicin (GO) (CMA-676, a CD33-targeted immunoconjugate of N-acetyl-γ-calicheamicin dimethyl hydrazide [CalichDMH], a potent DNA-binding cytotoxic antitumor antibiotic) is a clinically validated therapeutic option for patients with AML. Calicheamicin is a cytotoxic natural product isolated from Micromonospora echinospora that is at least 1000-fold more potent than conventional cytotoxic chemotherapeutics. Gemtuzumab ozogamicin is indicated for the treatment of elderly patients with relapsed AML (Damle 2004).

Immunoconjugates of calicheamicin targeted against tumor-associated antigens exhibit tumour-specific cytotoxic effects and cause regression of established human tumor xenografts in nude mice. CD33-specific binding triggers internalization of GO and subsequent hydrolytic release of calicheamicin. Calicheamicin then translocates to the nucleus, where calicheamicin binds DNA in the minor groove and causes double-strand DNA breaks, leading to cell death. GO is part of clinical practice for AML, but is frequently associated with severe side effects. The histone deacetylase inhibitor valproic acid potently augments gemtuzumab ozogamicin-induced apoptosis in acute myeloid leukemic cells. The synergistic proapoptotic activity of cotreatment of AML cells with VPA and GO indicates the

potential value of this strategy for AML (Ten Cate et al. 2007). Finally, compared with either agent alone, antibody BC8 combined with GO resulted in marked tumor growth inhibition and superior survival rates of mice bearing human AML xenografts. Further study of this antibody combination for clinical use in AML is warranted (Walter et al. 2008a). Lintuzumab (HuM195) is an unconjugated humanized murine mAb directed against cell surface myelomonocytic CD33 (Feldman et al. 2005).

17.13 Molecular Diversity and Evolution of Siglec Family

The Siglecs can be divided into two groups: an evolutionarily conserved subgroup (Siglecs-1, -2, and -4) and a CD33/Siglec-3-related subgroup (Siglecs-3 and -5-13 in primates), which appear to be rapidly evolving (Varki et al. 2006). A human Siglec-like molecule (Siglec-L1) lacks a conserved arginine residue known to be essential for optimal sialic acid recognition by previously known Siglecs. Loss of the arginine from an ancestral molecule was caused by a single nucleotide substitution that occurred after the common ancestor of humans with the great apes but before the origin of modern humans. The chimpanzee Siglec-L1 ortholog remains fully functional and preferentially recognizes N-glycolylneuraminic acid, which is a common sialic acid in great apes and other mammals. Reintroducing the ancestral arginine into the human molecule regenerates the same properties. Thus, the single base pair mutation that replaced the arginine on human Siglec-L1 is likely to be evolutionarily related to the previously reported loss of N-glycolylneuraminic acid expression in the human lineage suggesting additional changes in the biology of sialic acids that may have taken place during human evolution (Angata et al. 2001b). Although a few Siglecs are well-conserved throughout vertebrate evolution and show similar binding preference regardless of the species of origin, most others, particularly the CD33-related subfamily of Siglecs, show marked inter-species differences in repertoire, sequence, and binding preference. The diversification of CD33-related Siglecs may be driven by direct competition against pathogens, and/or by necessity to catch up with the changing landscape of endogenous glycans, which may in turn be changing to escape exploitation by other pathogens (Angata 2006).

Comparison of different mammalian species has revealed differential and complex evolutionary paths for Siglec protein family, even within the primate lineage. The combination of microarray technology with the zebrafish model system can provide useful information on how genes are coordinated in a genetic network to control zebrafish embryogenesis and can help to identify novel genes that are important for organogenesis (Lo et al. 2003). Lehmann et al. (2004) investigated the occurrence of corresponding genes in bony fish. Interestingly, only unambiguous orthologs of mammalian siglec-4, a cell adhesion molecule expressed exclusively in the nervous system, could be identified in the genomes of fugu and zebrafish, whereas no obvious orthologs of the other mammalian siglecs were found. As in mammals, fish siglec-4 expression is restricted to nervous tissues. Expressed as recombinant protein, fish siglec-4 binds to sialic acids with a specificity similar to the mammalian orthologs. Relatively low sequence similarities in the cytoplasmic tail as well as an additional splice variant found in fish siglec-4 suggest alternative signaling pathways compared to mammalian species. Observations suggest that this siglec occurs at least in the nervous system of all vertebrates (Lehmann et al. 2004).

A comparison of the CD33rSiglec gene cluster in different mammalian species showed that it can be divided into subclusters, A and B. The two subclusters, inverted in relation to each other, each encodes a set of CD33rSiglec genes arranged head-to-tail. Two regions of strong correspondence provided evidence for a large-scale inverse duplication, encompassing the framework CEACAM-18 (CE18) and ATPBD3 (ATB3) genes that seeded the mammalian CD33rSiglec cluster. Phylogenetic analysis was consistent with the predicted inversion. Rodents appear to have undergone wholesale loss of CD33rSiglec genes after the inverse duplication. In contrast, CD33rSiglecs expanded in primates and many are now pseudogenes with features consistent with activating receptors. In contrast to mammals, the fish CD33rSiglecs clusters show no evidence of an inverse duplication. They display greater variation in cluster size and structure than mammals. The close arrangement of other Siglecs and CD33rSiglecs in fish is consistent with a common ancestral region for Siglecs. Expansion of mammalian CD33rSiglecs appears to have followed a large inverse duplication of a smaller primordial cluster over 180 million years ago, prior to eutherian/marsupial divergence. Inverse duplications in general could potentially have a stabilizing effect in maintaining the size and structure of large gene clusters, facilitating the rapid evolution of immune gene families (Cao et al. 2009).

A comprehensive comparative study of Siglecs between the human and mouse genomes suggests that the mouse genome contains eight Siglec genes, whereas the human genome contains 11 Siglec genes and a Siglec-like gene. Although a one-to-one orthologous correspondence between human and mouse Siglecs 1, 2, and 4 is confirmed, the Siglec-3-related Siglecs showed marked differences between human and mouse. Angata et al. (2001a) found only four Siglec genes and two pseudogenes in the mouse chromosome 7 region syntenic to the Siglec-3-related gene cluster on human chromosome 19, which, in contrast, contains seven Siglec genes, a Siglec-like gene, and thirteen pseudogenes. Although some duplications of Siglec-3-related genes predated separation of the primate and rodent

lineages (about 80–100 million years ago), this gene cluster underwent extensive duplications in the primate lineage thereafter (Angata et al. 2001a). A temporary lettered nomenclature for additional mouse Siglecs suggests that mSiglec-F is likely a hSiglec-5 ortholog and the previously reported mSiglec-3/CD33 and mSiglec-E/MIS are likely orthologs of hSiglec-3 and hSiglec-9, respectively. The other Siglec-3-like gene in the cluster (mSiglec-G) is probably a hSiglec-10 ortholog. Another mouse gene (mSiglec-H), without an apparent human ortholog, lies outside of the cluster. Thus, although some duplications of Siglec-3-related genes predated separation of the primate and rodent lineages (about 80–100 million years ago), this gene cluster underwent extensive duplications in the primate lineage thereafter (Angata et al. 2001a).

Humans are genetically very similar to "great apes", (chimpanzees, bonobos, gorillas and orangutans), our closest evolutionary relatives. The human T cells give much stronger proliferative responses to specific activation via the T cell receptor (TCR) than those from chimpanzees. Nonspecific activation using phytohemagglutinin was robust in chimpanzee T cells, indicating that the much lower response to TCR simulation is not due to any intrinsic inability to respond to an activating stimulus. CD33-related Siglecs are inhibitory signaling molecules expressed on most immune cells and are thought to down-regulate cellular activation pathways via cytosolic ITIMs. Among human immune cells, T lymphocytes are a striking exception, expressing little to none of these molecules. In stark contrast, T lymphocytes from chimpanzees as well as the other closely related "great apes" (bonobos, gorillas, and orangutans) express several CD33-related Siglecs on their surfaces. Thus, human-specific loss of T cell Siglec expression occurred after our last common ancestor with great apes, potentially resulting in an evolutionary difference with regard to inhibitory signaling. This was confirmed by studying Siglec-5, which is prominently expressed on chimpanzee lymphocytes, including CD4 T cells (Nguyen et al. 2006b).

Altheide et al. (2006) presented further evidence for accelerated evolution in Sia-binding domains of CD33-related Sia-recognizing Ig-like lectins. Other gene classes are more conserved, including those encoding the sialyltransferases that attach Sia residues to glycans. Despite this conservation, tissue sialylation patterns are shown to differ widely among these species, presumably because of rapid evolution of sialyltransferase expression patterns. Sia modifications on these glycopeptides also appear to be undergoing rapid evolution. This rapid evolution of the sialome presumably results from the ongoing need of organisms to evade microbial pathogens that use Sia residues as receptors. The rapid evolution of Sia-binding domains of the inhibitory CD33-related Sia-recognizing Ig-like lectins is likely to be a secondary consequence, as these inhibitory receptors presumably need to keep up with recognition of the rapidly evolving "self"-sialome.

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Part VII

Novel Super-Families of Lectins

Fibrinogen Type Lectins

18

Anita Gupta

18.1 Ficolins

Ficolins are one of the most important groups of proteins capable of recognizing pathogens, and they function in the innate immune defence as pathogen-associated molecular pattern recognition molecules (Bohlson et al. 2007; Thiel et al. 1997; Runza et al. 2008). Ficolins comprises of a collagen like domain at the N-terminus and a FBG (fibrinogen-like domain), which is the ligand-binding site, at the C-terminus, and they form trimer-based multimers that are N-terminally linked by disulfide bonds (Ohashi and Erickson 2004; Hummelshoj et al. 2007). Three human ficolins (L-, M and H -ficolins) have been characterized. The amino acid sequence homologies between L-ficolin and M-ficolin, and between H-ficolin and either L-ficolin or M-ficolin, are 80% and 48% respectively. These ficolins are associated with the mannose-binding lectin-associated serine protease, and the complexes activate the lectin complement pathway. Interestingly, ficolins collaborate with CRP (C-reactive protein), which is highly up-regulated during the acute-phase response, and the interaction stabilizes CRP binding to bacteria, resulting in the activation of the lectin complement pathway (Ng et al. 2007). In addition to humans, ficolins have been identified in different mammalian species including rodents and pigs, which have two related ficolin genes called A and B and α and β, respectively, orthologous to the human L- and M-ficolin genes, respectively (Endo et al. 2004). To date, H-ficolin has only been identified in humans where as the mouse and rat homologues of H-ficolin gene are pseudogenes, which accounts for the absence of the corresponding protein in rodents (Endo et al. 2004).

18.1.1 Ficolins versus Collectins

Collectins and ficolins bind to oligosaccharide structures on the surface of microorganisms, leading to the killing of bound microbes through complement activation and phagocytosis. The human collectins, which are oligomeric proteins composed of CRDs attached to collagenous regions, are structurally similar to ficolins (L-ficolin, M-ficolin, and H-ficolin). However, they make use of different CRD structures: C-type lectin domains for the collectins and fibrinogen-like domains for the ficolins. Collectins and ficolins bear no significant sequence homology except for the presence of collagen-like sequences over the N-terminal halves of the polypeptides that enable the assembly of these molecules into oligomeric structures. Collectins and ficolins both contain lectin activities within the C-terminal halves of their polypeptides, the C-type CRDs and fibrinogen β/γ (homology) (FBG) domain, respectively. These domains form trimeric clusters at the ends of the collagen triple helices emanating from a central hub, where the N-terminal ends of the polypeptides merge. The collectins and ficolins seem to have evolved to recognize the surface sugar codes of microbes and their binding, to the arrays of cell surface carbohydrate molecules, targets the microbe for subsequent clearance by phagocytic cells (Holmskov et al. 2003).

18.1.2 Characterization of Ficolins

Ficolins resemble collectins in structure, but their collagen-like stalks are followed by a domain homologous to the fibrinogen β and γ chains (Matsushita and Fujita 2001). While a collagen-like domain is found at the N-terminus, the fibrinogen-like domain (FD1), which is the sugar-binding site, is present at the C-terminus (Le et al. 1998; Teh et al. 2000). Ficolins are assembled from homotrimeric subunits comprising a collagen-like triple helix and a lectin-like domain—composed of three fibrinogen-like (FBG)-domains. Two cysteines at the N-terminal end of the polypeptide chains form interchain disulfide bonds that mediate assembly into higher oligomerization structures (Ohashi and Erickson 2004; Hummelshoj et al. 2007). Ficolins are structurally and functionally similar to MBL. Ficolins present in serum have

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common binding specificity for N-acetylglucosamine (GlcNAc). MBL is also a collagenous lectin found in serum and specific for GlcNAc and mannose binding. Its domain organization is similar to that of ficolins, except that MBL has a CRD instead of a fibrinogen-like domain. Ficolin from monocytes is a polypeptide of ~ 42 kDa, which is similar in size to that of ficolin predicted from its cDNA-derived sequence and bound to *E. coli*. Protein(s) reactive with GlcNAc from porcine serum has M_r mainly 40 kDa and plays an important role(s) in innate immunity against microbial infection with Gram-positive and -negative bacteria (Nahid and Sugii 2006).

In humans, L-ficolin/P35 (or ficolin 2) and H-ficolin (or ficolin 3) are serum proteins, whereas M-ficolin (or ficolin 1) is a secretory protein produced by lung and blood cells (Matsushita et al. 1996; Sugimoto et al. 1998; Liu et al. 2005) and mainly expressed by the monocytic cell lineage (Matsushita et al. 1996; Sugimoto et al. 1998). In addition to humans, ficolins have been identified in different mammalian species including rodents and pigs (Ichijo et al. 1991; Fujimori et al. 1998), which have two related ficolin genes called A and B and α and β, respectively, orthologous to human L- and M-ficolin genes, respectively (Endo et al. 2004). Three human ficolins, ficolin-1 (M-ficolin), ficolin-2 (L-ficolin) and ficolin-3 (H-ficolin or Hakata antigen) are encoded by the *FCN1, FCN2* and *FCN3* genes, respectively. If MBL plays a role in innate immunity by acting as an opsonin and activating complement in association with MBL-associated serine protease (MASP) via lectin pathway, human serum ficolins, L-ficolin/P35 and H-ficolin (Hakata antigen), are also associated with MASPs and sMAP, a truncated protein of MASP-2, and activate complement. Thus, serum ficolins are structurally and functionally similar to MBL and have the capacity to activate the lectin pathway and have a role in innate Immunity (Matsushita and Fujita 2001; Krarup et al. 2004).

18.1.2.1 L-Ficolin (Ficolin-2)

L-ficolin (synonymous with ficolin-2 or Ficolin/P35) is synthesized in the liver and found in blood circulation (Matsushita et al. 1996). Adult plasma contains, on average, a level of L-ficolin that is threefold higher than found in cord blood, implying a protective role of this lectin. L-ficolin indeed binds to sugar structures via its FBG domains (Le et al. 1998) and, on binding to carbohydrates on bacteria, promotes clearance by phagocytosis (Matsushita et al. 1996). Like MBL, L-ficolin forms a complex with the MBL-associated proteases (MASPs) and binding of this complex to *Salmonella typhimurium* activated the complement system (Matsushita and Fujita 2001). L-ficolin binds to acetyl groups such as GlcNAc. This protein binds to clinically important bacteria, including *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Streptococcus agalactiae* (Krarup et al. 2005; Lynch et al. 2004; Aoyagi et al. 2005). L-ficolin/P35 binds to an Ra chemotype strain of *S. typhimurium* (TV119) which has an exposed GlcNAc at the non-reducing termini of the polysaccharide, where as it did not bind to LT2, a smooth type strain of *S. typhimurium* with additional O-polysaccharides covering GlcNAc (Taira et al. 2000).

18.1.2.2 H-Ficolin (Ficolin-3)

H-ficolin (synonymous with ficolin-3 or Hakata antigen) was initially identified as a serum auto-antigen, recognized by antibodies in patients suffering from systemic lupus erythematosus and other autoimmune diseases (Sugimoto et al. 1998). It is synthesized in the liver by hepatocytes and bile duct epithelial cells and is secreted into both blood circulation and bile (Akaiwa et al. 1999). It is also synthesized by ciliated bronchial and Type II alveolar epithelial cells and is secreted into bronchus and the alveolar space (Akaiwa et al. 1999). H-ficolin is a lectin that binds to carbohydrate structures found on bacteria and may therefore play an important role in both systemic and mucosal immune defence systems (Endo et al. 2007; Dahl et al. 2001, 1997) Both H- and L-ficolins have also been shown to bind to carbohydrate structures found on Gram-negative bacteria. (Akaiwa et al. 1999; Le et al. 1998; Matsushita et al. 1996) Binding of L-ficolin to *S. typhimurium* directly opsonizes the bacteria for enhanced phagocytosis by polymorphonuclear leucocytes (PMNs) (Matsushita et al. 1996) and this is probably mediated by an L-ficolin receptor(s) on PMNs. H-ficolin shows affinity for GlcNAc, GalNAc and D-fucose, and binds to *S. typhimurium*, *Salmonella minnesota* and *Aerococcus viridans* (Sugimoto et al. 1998; Krarup et al. 2005). The finding that L-ficolin formed a functional complex with the MASPs that bound to *S. typhimurium* and subsequently activated the complement system, implied that L-ficolin could mediate killing and clearance of pathogens more effectively through complement activation and complement receptor-mediated phagocytosis (Matsushita et al. 2000). L ficolin and H-ficolin recognize surface structures on apoptotic cells and initiate the activation of lectin complement pathway (Kuraya et al. 2005). H-ficolin has only been identified in humans where as the mouse and rat homologues of H-ficolin gene are pseudogenes, which account for the absence of corresponding protein in rodents (Endo et al. 2004).

18.1.2.3 M-Ficolin (Ficolin-1)

M-ficolin was initially identified from the membrane fraction of pig uterus (Ichijo et al. 1993). Significant M-ficolin has been detected on U937 cells and on monocytes, but not on lymphocytes and granulocytes. Association with the surface of monocytes or U937 cells is apparently an intrinsic property of M-ficolin instead of that of monocytes or U937

cells. M-ficolin was shown to mediate U937 cell adhesion to the immobilized F(ab′)2 fragment of the anti-FBG antibody and therefore M-ficolin was linked to the cell cytoskeleton either directly or indirectly through its cytoskeleton-linked receptor(s). M-ficolin-mediated U937 cell adhesion may therefore be a novel pathway of monocyte emigration into extravascular tissues.

M-ficolin is synthesized in peripheral blood monocytes. Ficolin mRNA is synthesized by a human monocyte cells, and thus blood monocytes also normally synthesize human ficolin. Peripheral blood monocytes from adult human donors showed that ficolin mRNA is highly expressed in monocytes throughout the first 20 h of adhesion. The origin of ficolin from monocytes, together with its structural similarity to C1q and the collectins, raised the possibility that ficolin is another plasma protein capable of binding to surface structures of micro-organisms (Lu et al. 1996b). However, its expression is down-regulated during monocyte differentiation and its mRNA is not detectable in mature macrophages (Lu et al. 1996b). M-ficolin mRNA has been found to be abundant in peripheral blood monocytes, accounting for 0·44% of the total mRNA in the cells (Hashimoto et al. 1999b). However, M-ficolin mRNA is not detectable in monocyte-derived DCs and was detected only at a very low level in monocyte-derived macrophages in vitro (Hashimoto et al. 1999a, 1999b). M-ficolin expression is a marker of circulating monocytes and has been reported to be located in secretory granules in the cytoplasm of neutrophils, monocytes and type II alveolar epithelial cells in the lung (Liu et al. 2005). M-ficolin shows affinity for GlcNAc, GalNAc and sialic acid, and it binds to *Staph. aureus* through GlcNAc (Liu et al. 2005). M-ficolin binds weakly to *S. typhimurium*, but this binding is not inhibited by GlcNAc (Liu et al. 2005). In addition, the peptide Gly-Pro-Arg-Pro, which mimics the N-terminal sequence of the fibrin α-chain and inhibits fibrin polymerization, prevents binding of the M-ficolin FBG domain to GlcNAc. M-ficolin is expressed by blood monocytes and type II alveolar epithelial cells (Lu et al. 1996a; Theil et al. 2000; Liu et al. 2005; Frederiksen et al. 2005). Interestingly, M-ficolin expression is downregulated when monocytes mature into macrophages, but can be re-induced in mature macrophages when they are treated with bacterial products such as lipopolysaccharide (Frankenberger et al. 2008). M-ficolin, unlike L-ficolin and H-ficolin, was not considered to be a serum protein, but it was recently demonstrated that M-ficolin exists in human plasma and serum under normal conditions (Honore et al. 2008).

M-ficolin is highly homologous to L-ficolin at the amino-acid sequence level and also shows a marked preference for acetylated compounds (Frederiksen et al. 2005; Liu et al. 2005). H-ficolin, in contrast, is less closely related to L-ficolin and shows no binding affinity for acetylated derivatives. So far, it has been only reported to bind to Aerococcus viridans (Tsujimura et al. 2001; Krarup et al. 2005). M-ficolin also has lectin activity and, like L-ficolin (Le et al. 1998) it binds to GlcNAc via the FBG domain. As GlcNAc is a common carbohydrate moiety on Gram-negative bacteria, M-ficolin may also recognize these pathogens.

18.1.2.4 Veficolins

Cerberus rynchops (dog-faced water snake) belongs to Homalopsidae of Colubroidea (rear-fanged snakes). In addition to C-type lectins, the venom gland of *C. rynchops* contains two proteins that showed sequence homology to ficolin**.** These proteins were named as ryncolin 1 and ryncolin 2 (rynchops ficolin) and this new family of snake venom proteins as veficolins (venom ficolins). On the basis of its structural similarity to ficolin, it is speculated that ryncolins may induce platelet aggregation and/or initiate complement activation (OmPraba et al. 2010).

18.1.3 Ligands of Ficolins

Both H- and L-ficolins have also been shown to bind to carbohydrate structures found on Gram-negative bacteria (Akaiwa et al. 1999). L-ficolin binds to GlcNAc (Matsushita et al. 1996; Le et al. 1997, 1998) and N-acetyl-d-galactosamine (GalNAc) (Le et al. 1998). The binding ability is inhibited by acetylated compounds, indicating that this protein specifically recognizes acetyl groups (Krarup et al. 2004). L-ficolin activates the lectin pathway after binding to various capsulated bacteria (Matsushita et al. 1996; Lynch et al. 2004; Aoyagi et al. 2005). A binding specificity for GlcNAc was initially characterized by Matsushita et al. (1996, 2000), in agreement with the finding that L-ficolin recognizes lipoteichoic acid, a GlcNAc-containing cell wall component characteristic of Gram-positive bacteria (Lynch et al. 2004). Binding to a fungal 1,3-β-D-glucan preparation was later reported (Ma et al. 2004), whereas further investigations revealed specificity for N-acetylated carbohydrates and other non-carbohydrate acetylated compounds such as acetylcholine (Krarup et al. 2004). Where as M-ficolin was initially identified as a membrane-associated protein in pig uterus, based on its affinity for TGF-β1 (Ichijo et al. 1993), L-ficolin was shown to bind to corticosteroids (Edgar 1995), and both L- and M-ficolin were found to be elastin-binding proteins (Harumiya et al. 1995, 1996).

L-ficolin activates the lectin-complement pathway upon binding to lipoteichoic acid, a cell component found in all Gram-positive bacteria (Lynch et al. 2004). H-ficolin (ficolin-3), the primary structure of which is 48% identical to that of L-ficolin, binds to GlcNAc, GalNAc and d-fucose (Sugimoto et al. 1998). Unlike L-ficolin, the GlcNAc-binding activity of H-ficolin is not inhibited by acetyl compounds (Krarup et al. 2004). M-ficolin, the primary

structure of which is 80% and 48% identical to those of L-ficolin and H-ficolin, respectively (Sugimoto et al. 1998), binds to GlcNAc, GalNAc and sialic acid (Teh et al. 2000; Liu et al. 2005). This protein also recognizes acetyl groups, like L-ficolin (Frederiksen et al. 2005). Unlike the serum ficolins, M-ficolin has been detected on the surfaces of peripheral blood monocytes and promonocytic U937 cells (Lu et al. 1996a; Teh et al. 2000; Frederiksen et al. 2005). Gout et al. (2010) reported that L-ficolin preferentially recognized disulfated N-acetyllactosamine and tri- and tetrasaccharides containing terminal galactose or N-acetylglucosamine. Binding was sensitive to the position and orientation of the bond between N-acetyllactosamine and the adjacent carbohydrate. The crystal structure of the Y271F mutant fibrinogen domain showed that the mutation does not alter the structure of the ligand binding pocket. These analyses reveal ficolin ligands such as sulfated N-acetyllactosamine (L-ficolin) and gangliosides (M-ficolin) and provide precise insights into the sialic acid binding specificity of M-ficolin, emphasizing the essential role of Tyr^{271} in this respect.

18.1.4 X-ray Structures of M, L- and H-Ficolins

18.1.4.1 Homology to Tachylectin 5A

Several observations indicate that ficolins are not classical lectins and raise questions about their mechanism of action. To answer these questions at molecular level, the X-ray structures of their trimeric recognition domains, alone and in complex with various ligands, were solved to resolutions up to 1.95 and 1.7 Å, respectively. Both domains have three-lobed structures with clefts separating the distal parts of the protomers. Ca^{2+} ions are found at sites homologous to those described for *Tachypleus tridentatus* tachylectin-5A (TL5A), an invertebrate lectin (Kairies et al. 2001). Outer binding sites (S1) homologous to the GlcNAc-binding pocket of TL5A are present in ficolins but show different structures and specificities. In L-ficolin, three additional binding sites (S2–S4) surround the cleft (Garlatti et al. 2007a). The structures revealed that L-ficolin has evolved to be a versatile recognition protein able to recognize a variety of acetylated and carbohydrate targets through three different sites, suggesting that ficolins represent novel types of pattern recognition proteins. Results defined an unpredicted continuous recognition surface able to sense various acetylated and neutral carbohydrate markers in the context of extended polysaccharides such as 1,3-β-D-glucan, as found on microbial or apoptotic surfaces.

18.1.4.2 Domain Structures of M-Ficolin

The human M-ficolin FD1, which contains the ligand-binding site, was over-expressed in Pichia pastoris, purified and crystallized using the vapour-diffusion method at 293 K. The crystals belong to the monoclinic space group P21, with unit-cell parameters a = 55.16, b = 117.45, c = 55.19 Å, β = 99.88°, and contain three molecules per asymmetric unit (Tanio et al. 2006). To investigate the discrimination mechanism between self and non-self by ficolins, Tanio et al. (2007) determined the crystal structure of the human M-ficolin FD1 at 1.9A resolution. Although the FD1 monomer shares a common fold with the fibrinogen γ-fragment and tachylectin-5A, the Asp^{282}-Cys^{283} peptide bond, which is the predicted ligand-binding site on the C-terminal P domain, is a normal trans bond, unlike the cases of the other two proteins. The trimeric formation of FD1 results in the separation of the three P domains, and the spatial arrangement of the three predicted ligand-binding sites on the trimer is very similar to that of the trimeric collectin, indicating that such an arrangement is generally required for pathogen-recognition. The ligand binding study of FD1 in solution indicated that the recombinant protein binds to N-acetyl-d-glucosamine and the peptide Gly-Pro-Arg-Pro and suggested that the ligand-binding region exhibits a conformational equilibrium involving cis-trans isomerization of the Asp^{282}-Cys^{283} peptide bond. The crystal structure and the ligand binding study of FD1 provide an insight of the self- and non-self discrimination mechanism by ficolins (Tanio et al. 2007).

Since GlcNAc, the common ligand of ficolins from several species, is universally expressed on both pathogens and hosts, the discrimination mechanism between self (host) and nonself (pathogen) by ficolins is of particular interest. With regard to this, the trimer formation by the FD1 (M-ficolin FBG domain) is required to recognize pathogen surfaces with high ligand density. In addition, from structural and functional studies of M-ficolin FD1, Tanio et al. (2007) proposed that the ligand-binding region of FD1 exists in a conformational equilibrium between active and non-active states depending on three groups with a pK_a of 6.2, which are probably histidine residues, and suggested that the 2-state conformational equilibrium as well as the trimer formation contributes to the discrimination mechanism between self and non-self of FD1. The GlcNAc binding study of a series of single histidine mutants of FD1 demonstrated that His^{251}, His^{284} and His^{297} are required for the activity, and thus three histidines are the origins of pH dependency of FD1. The analyses of GlcNAc association and dissociation of FD1 provided evidence that FD1 always exchanges between the active and non-active states with the pH-dependent populations in solution. The biological roles of the histidine-regulated conformational equilibrium of M-ficolin are important in terms of self and non-self discrimination mechanism (Tanio and Kohno 2009).

The ligand-bound crystal structures of the CRD of M-ficolin, at high resolution provide the structural insights into its binding properties. Interaction with acetylated carbohydrates

differs from the one described for L-ficolin (Fig. 18.1). Garlatti et al. (2007b) also revealed the structural determinants for binding to sialylated compounds, a property restricted to human M-ficolin and its mouse counterpart, ficolin B. Comparison between the ligand-bound structures obtained at neutral pH and nonbinding conformations observed at pH 5.6 showed how the ligand binding site is dislocated at acidic pH. This means that the binding function of M-ficolin is subject to a pH-sensitive conformational switch. Considering that the homologous ficolin B is found in the lysosomes of activated macrophages (Runza et al. 2006), it is proposed that this switch can play a physiological role in such acidic compartments (Garlatti et al. 2007b).

The structure of human M-ficolin FD1 has been compared with the human fibrinogen γ fragment, tachylectin-5A, L-ficolin and H-ficolin. The overall structure of FD1 is similar to that of other proteins, although the peptide bond between Asp^{282} and Cys^{283}, which is in a predicted ligand-binding site, is a normal *trans* bond, unlike the cases of the other proteins. Analysis of the pH-dependent ligand-binding activity of FD1 in solution suggested that a conformational equilibrium between active and non-active forms in the ligand-binding region, involving cis-trans isomerization of the Asp^{282}-Cys^{283} peptide bond, contributes to the discrimination between self and non-self, and that the pK_a values of His^{284} are 6.1 and 6.3 in the active and non-active forms, respectively (Tanio et al. 2008).

18.1.4.3 Ligand-binding site of Ficolins

The crystal structures of human fibrinogen γ fragment (Pratt et al. 1997) and of the FD1 of *Tachypleus tridentatus* tachylectin-5A (TL5A), an invertebrate lectin (Kairies et al. 2001), have shown that the P domain contributes to ligand binding. In angiopoietin-2, the P domain of the FD1 relates to receptor binding (Barton et al. 2005). These findings suggest that the P domain of ficolins includes the sugar-binding site. Interestingly, although the sugars recognized by ficolins exist on the surface of the host cell, these proteins can discriminate between pathogens and the host cell. The crystal structures of ficolins, complexed with ligands, should clarify the molecular mechanism of the discrimination and advance drug design for general pathogens. The FReD (fibrinogen-related domain) of FIBCD1 (Fibrinogen C domain containing 1) forms noncovalent tetramers and that the acetyl-binding site of FReDs of FIBCD1 is homologous to that of tachylectin 5A and M-ficolin but not to the FReD of L-ficolin (Thomsen et al. 2010).

18.1.5 Functions of Ficolins

The lectin pathway of complement activation in humans is triggered through the action of MBL-associated serine protease-2 in response to recognition of neutral carbohydrates and other motifs present on microbial surfaces. This pathway is initiated by MBL, L-ficolin, H-ficolin, and M-ficolin (Matsushita and Fujita 2001; Liu et al. 2005; Frederiksen et al. 2005). MBL, through its C-type lectin domain, recognizes terminal carbohydrates, provided that their hydroxyl groups at positions C3 and C4 are in equatorial orientation (Weis et al. 1992).

Upon recognition of the infectious agent, the ficolins act through two distinct routes: initiate the lectin pathway of complement activation through attached mannose-binding lectin-associated serine proteases (MASPs), and a primitive opsonophagocytosis thus limiting the infection and concurrently orchestrating the subsequent adaptive clonal immune response. Ficolins are associated with the MASPs and the complexes activate the lectin-complement pathway (Fujita et al. 2004; Liu et al. 2005; Frederiksen et al. 2005) in response to recognition of neutral carbohydrates and N-acetyl groups on pathogens and damaged cells. This results from the ability of MBL and ficolins to associate with and trigger activation of MBL-associated serine protease (MASP)-2. Activated MASP-2 cleaves the complement proteins C2 and C4, thereby triggering the complement cascade (Thiel et al. 1997; Matsushita, et al. 2000). Three other MBL/ficolins-associated proteins have been described, the MASP-1 and MASP-3 proteases (Matsushita et al. 2002; Dahl, et al. 2001) and a truncated form of MASP-2 called MAp19 (19-kDa MBL-associated protein) or sMAp (Stover et al. 1999; Takahashi et al. 1999). MASP-3 has no known physiological substrates whereas MASP-1 cleaves with a low efficiency a few protein substrates, among which are fibrinogen and coagulation factor XIII (Krarup et al. 2008). It has been proposed that MASP-1 might contribute to the activation of the lectin pathway, though this issue is still controversial (Takahashi et al. 2008; Rossi et al. 2001).

Complement activation results in opsonization of microbes and apoptotic cells with C3-derived fragments, thus promoting their clearance through interaction with C3 receptors on phagocytes (Aoyagi et al. 2005; Kuraya et al. 2005). In addition, ficolins may themselves function as opsonins, as suggested by the ability of L-ficolin to enhance phagocytosis of *Salmonella typhimurium* by neutrophils (Matsushita et al. 1996) and the ability of L- and H-ficolins to increase adhesion/uptake of late apoptotic cells by macrophages (Jensen 2007; Honoré et al. 2007; Aoyagi et al. 2005; Kuraya et al. 2005).

The opsonizing effects of ficolins are exerted through receptors present on phagocytic cells that are likely common to other defense collagens such as C1q and the collectins. A likely candidate is the receptor for the collagenous domain of C1q (cC1qR) or calreticulin (Crt) (Stuart et al. 1997), which is thought to function in complex with the endocytic receptor CD91 (Ogden et al. 2001; Vandivier et al. 2002). This hypothesis is supported by studies showing that L- and

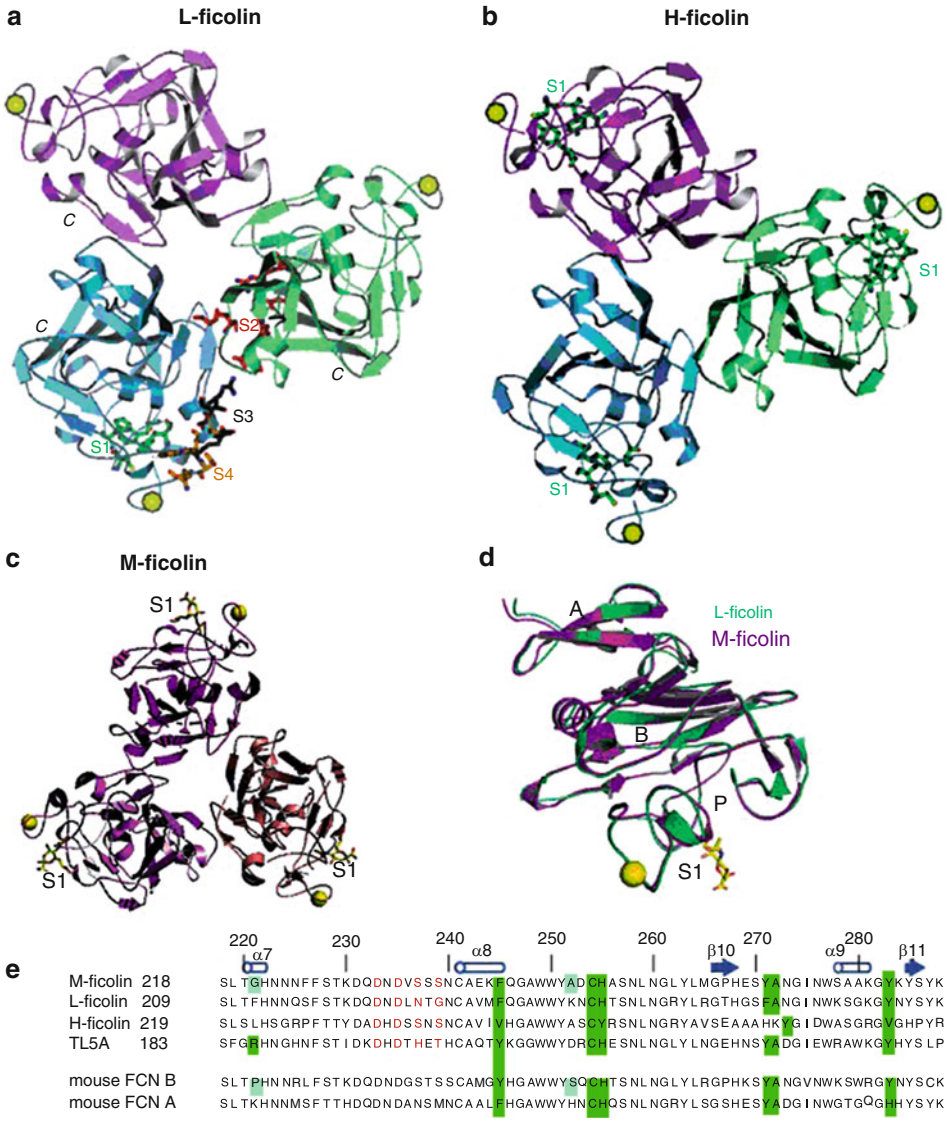

Fig. 18.1 *Homotrimeric structure of the recognition domains of human L-, H- and M-ficolins and location of their binding site(s)*: L-ficolin (**a**) and H-ficolin (**b**) structures seen from the target binding surface (*bottom* view). The side chains of the binding site residues are displayed as ball and sticks and colored *green* (S1), *red* (S2), *black* (S3), and *orange* (S4). To enhance clarity of the side view, only one of each representative binding sites is shown on the L-ficolin trimer. N and C indicate the N- and C-terminal ends of each protomer. Ca^{2+} ions are represented as golden spheres (Reprinted by permission from Macmillan Publishers Ltd: EMBO Journal, Garlatti. et al., © 2007a). (**c**) *Bottom* view of the homotrimeric structure of M-ficolin solved at neutral pH. Ca^{2+} ions are represented as golden spheres. The sialic acid ligand bound to site S1 is shown in a *yellow* ball and stick representation. (**d**) Superposition of the similar fibrinogen-like protomers of M-ficolin (*magenta*) and L-ficolin (*green*). Domains A, B, and P are labeled. (**e**) Sequence alignment of the P domains of human ficolins M, L, and H; mouse ficolins FCN B and FCN A; and TL5A. The residue numbering and the secondary structure elements apply to M-ficolin. Residues involved in the S1 binding site are colored green, and those involved in Ca^{2+} binding are colored red. Small residues allowing accommodation of sialic acid in site S1 are colored blue (Reprinted with permission from Garlatti et al. 2007b © American Society for Biochemistry and Molecular Biology)

H-ficolins bind to cC1qR/CRT (Kuraya et al. 2005) and that H-ficolin binding to Crt can be inhibited by MBL, suggesting that both proteins share a common binding site on CRT (Honoré et al. 2007). In addition, ficolins may themselves function as opsonins, as suggested by the ability of L-ficolin to enhance phagocytosis of *Salmonella typhimurium* by neutrophils and the ability of L- and H-ficolins to increase adhesion/uptake of late apoptotic cells by macrophages (Jensen et al. 2007; Honoré et al. 2007). The other putative function of monocyte surface M-ficolin was implicated by its affinity for GlcNAc, a common sugar structure on Gram-negative bacteria and other pathogens. M-ficolin might act

as a phagocytic receptor or adaptor on circulating monocytes for micro-organism recognition and may potentially mediate monocyte adhesion (Teh et al. 2000).

Studies have shown that MASP-2 binds to a short segment of the collagen-like domain of MBL. Recent studies revealed that Lys^{55} in the collagenous region of MBL is important for interaction with MASPs and CRT. On the other hand residues Lys^{57} of L-ficolin and Lys^{47} of H-ficolin are key components of the interaction with MASPs and CRT, providing strong indication that MBL and the ficolins share homologous binding sites for both types of proteins (Lacroix et al. 2009). Girija et al. (2007) showed that the MASP-2 binding site on rat ficolin-A is also located within the collagen-like domain and encompasses a conserved motif that is present in both MBLs and ficolins. Site-directed mutagenesis revealed that a lysine residue in the X position of the Gly-X-Y collagen repeat, Lys^{56} in ficolin-A, which is present in all ficolins and MBLs known to activate complement, is essential for MASP-2 binding. Similar binding sites and activation kinetics of MASP-2 suggest that complement activation by ficolins and MBLs follows analogous mechanisms.

18.1.5.1 The C3b Deposited by L-Ficolin/MBL forms Alternative Pathway C3 Convertase C3bBb

Serotype III group *B streptococci* (GBS) are the common cause of neonatal sepsis and meningitis. Although deficiency in maternal capsular polysaccharide (CPS)-specific IgG correlates with susceptibility of neonates to the GBS infection, serum deficient in CPS-specific IgG mediates significant opsonophagocytosis. The IgG-independent opsonophagocytosis requires activation of the complement pathway, a process requiring the presence of both Ca^{2+} and Mg^{2+}, and is significantly reduced by chelating Ca^{2+} with EGTA. The role of L-ficolin/mannose-binding lectin-associated serine protease (MASP) complexes in Ca^{2+}-dependent, Ab-independent opsonophagocytosis of serotype III GBS has been defined. The binding of L-ficolin/MASP complexes to the CPS generates C3 convertase C4b2a, which deposits C3b on GBS. The C3b deposited by this lectin pathway forms alternative pathway C3 convertase C3bBb whose activity is enhanced by CPS-specific IgG2, leading to an increased opsonophagocytic killing by further deposition of C3b on the GBS (Aoyagi et al. 2005).

18.1.6 Pathophysiology of Ficolins

Like invertebrate innate immune reactions, the human PGN and 1, 3-β-D-D-glucan recognition proteins function as complement-activating lectins. The counterparts of these proteins from human serum are MBL and L-ficolin. The specific microbial cell component-coupled columns demonstrated that MBL and L-ficolin bind to PGN and 1, 3-β-D-D-glucan, respectively. MBL and L-ficolin were associated with MBL-associated serine proteases-1 and -2 (MASPs) and small MBL-associated protein. The binding of purified MBL/MASP and L-ficolin/MASP complexes to PGN and 1, 3-β-D-glucan, respectively, resulted in the activation of the lectin-complement pathway (Ma et al. 2004; Matsushita and Fujita 2001). A lot of reports showed that dysfunction or abnormal expressions of ficolins may play crucial roles in the pathogenesis of human diseases including: (1) infectious and inflammatory diseases, e.g., recurrent respiratory infections; (2) apoptosis, and autoimmune disease; (3) systemic lupus erythematosus; (4) IgA nephropathy; (5) clinical syndrome of preeclampsia; (6) other diseases associated factor e.g. C-reactive protein. Zhang and Ali (2008) reviewed the structures, functions, and clinical implications of ficolins and summarized the reports on the roles of ficolins in human diseases. Precise identification of ficolins functions will provide novel insight in the pathogenesis of these diseases and may provide novel innate immune therapeutic options to treat disease progression.

In clinical studies, like MBL, no absolute levels of deficiency have yet been defined. In more than 300 children with recurrent respiratory tract infections no correlation has been observed between L-ficolin levels and severity of disease (Atkinson et al. 2004). An association with MBL deficiency in the same patient cohort had already been reported (Cedzynski et al. 2004). In this study, low levels of L-ficolin were more common in patients than in controls and most common in patients with co-existing atopic disorders, suggesting a role for L-ficolin in protection from microorganisms complicating allergic disease. Polymorphisms in the ficolins have been identified, although their clinical significance is as yet unknown (Dommett et al. 2006).

18.2 Tachylectins

18.2.1 Horseshoe Crab Tachylectins

Tachylectin-related proteins belong to fibrinogen type lectins and function in innate immunity of various animals, from ancient sponges to vertebrates. The specific recognition by horseshoe crab tachylectins with a propeller-like fold or a propeller-like oligomeric arrangement is reinforced by the short distance between the individual binding sites that interact with pathogen-associated molecular patterns (PAMPs). There is virtually no conformational change in the main or side chains of tachylectins upon binding with the ligands. This low structural flexibility of the propeller structures must be very important for specific interaction with PAMPs. While MBL and ficolins trigger complement activation through the

lectin pathway in the form of opsonins, tachylectins have no effector collagenous domains and no lectin-associated serine proteases found in the mammalian lectins. Furthermore, no complement-like proteins have been found in horseshoe crabs, except for α_2-macroglobulin. The mystery of the molecular mechanism of the scavenging pathway of pathogens in horseshoe crabs is to be solved (Kawabata and Tsuda 2002).

Kawabata and Iwanaga (1999) purified five types of tachylectins from circulating hemocytes and hemolymph plasma of the Japanese horseshoe crab, *Tachypleus tridentatus*. Tachylectin-1 interacts with Gram-negative bacteria probably through 2-keto-3-deoxyoctonate, one of the constituents of LPS. Tachylectin-1 also binds to polysaccharides such as agarose and dextran with broad specificity. Tachylectin-2 binds to D-GlcNAc or D-GalNAc and recognizes staphylococcal lipoteichoic acids and LPS from several Gram-negative bacteria. In contrast, tachylectins-3 and -4 specifically bind to S-type LPS from several Gram-negative bacteria through a certain sugar moiety on the O-specific polysaccharides (O-antigens). Tachylectin-5 identified in hemolymph plasma has the strongest bacterial agglutinating activity in the five types of tachylectins, and exhibits broad specificity against acetyl group-containing substances. Thus, the innate immune system of horseshoe crab may recognize invading pathogens through a combinatorial method using lectins with different specificities against carbohydrates exposed on pathogens. An encounter of these lectins derived from hemocytes and hemolymph plasma at injured sites, in response to the stimulation of LPS, suggests that they serve synergistically to accomplish an effective host defense against invading microbes and foreign substances (Kawabata and Iwanaga 1999). The structure of tachylectin-2, the first example of a fivefold symmetric β-propeller protein, sheds light on the role played by this lectin in horseshoe crab host defense (Rini and Lobsanov 1999).

18.2.1.1 Tachylectin 5A and 5B

Gokudan et al. (1999) characterized tachylectins 5A and 5B (TLs-5). TLs-5 agglutinated all types of human erythrocytes and Gram-positive and Gram-negative bacteria. TLs-5 specifically recognizes acetyl group-containing substances including noncarbohydrates; the acetyl group is required and is sufficient for recognition. TLs-5 enhanced the antimicrobial activity of a horseshoe crab-derived big defensin. cDNA sequences of TLs-5 indicated that they consist of a short N-terminal Cys-containing segment and a C-terminal fibrinogen-like domain with the highest sequence identity (51%) to that of mammalian ficolins. TLs-5, however, lack the collagenous domain found in a kind of "bouquet arrangement" of ficolins and collectins. Electron microscopy revealed that TLs-5 form two- to four-bladed propeller structures. Kairies et al. (2001) reported the crystal structure of TL5A, a lectin from hemolymph plasma of T. tridentatus. TL5A shares not only a common fold but also related functional sites with γ fragment of mammalian fibrinogen (Fig. 18.2). A fibrinogen-related lectin, named Dorin M, from the hemolymph plasma of soft tick, Ornithodoros moubata, is produced in the tick hemocytes. Dorin M is also expressed in salivary glands and has a fibrinogen-like domain. It exhibits similarity with tachylectins 5A and 5B from a horseshoe crab, Tachypleus tridentatus. In addition, Dorin M is closely related to tachylectins-5 (Rego et al. 2006).

18.2.2 X-ray Structure

TL5A is an ellipsoidal molecule (overall dimensions ~34 × 36 × 53 $Å^3$), subdivided into three distinct but interacting domains (Fig. 18.2 a and b). The N-terminal domain A (residues Asp^{45}–Trp^{89}) comprises two short helices and a small two-stranded antiparallel β-sheet. The N-terminal helix (α1) is diagonally twisted and anchored through a disulfide bond (Cys^{49}–Cys^{80}) to the second strand of β-sheet (β2). The second, short helix β2 connects strand β1 with strand β2. Structural elements N-terminal of Asp^{45} could not be resolved because of poorly defined electron density. Cysteine residues Cys^6 and Cys^{170} were expected to be involved in lectin homodimerization. However, electron density revealed Cys^{170} to be present in the free -SH form. In addition, chemical-labeling experiments also showed that Cys^6 and Cys^{170} are present in the free SH forms in native TL5A. The two disulfide bridges, Cys^{49}–Cys^{80} and Cys^{206} Cys^{219}, were confirmed by sequence analysis of peptides derived from TL5A (Kairies et al. 2001). The central and larger domain B (residues Thr^{90}–Ala^{180} and Pro^{253}–Phe^{264}) is clamped through domains A and P and predominantly made up by a twisted seven-stranded antiparallel β-sheet (strands β3–β7, β9 and β12). Helices β4 and β5 within this domain can be interpreted as a single helix divided by a loop, which participates with a small antiparallel two-stranded β-sheet (β4, β5) to the main seven-stranded β-sheet. The central strand β12 (residues Gln^{254}–Pro^{261}) extends the C terminus of domain P back to domain B, bringing both polypeptide termini in close proximity. The C-terminal domain P (residues Gly^{181}–Leu^{252}) possesses only a few short elements of regular secondary structure, and comprises two major functional sites within TL5A: the Ca^{2+}-binding site and the nearby acetyl group-binding pocket (Fig. 18.2b). The Ca^{2+} ion is bound in a loop region located ~11 Å away from the adjacent ligand binding site, coordinated by seven oxygen ligands in a pentagonal bipyramidal manner. Both carboxylate oxygen atoms of Asp^{198}, the main chain carbonyl oxygens of His^{202} and Thr^{204}, and

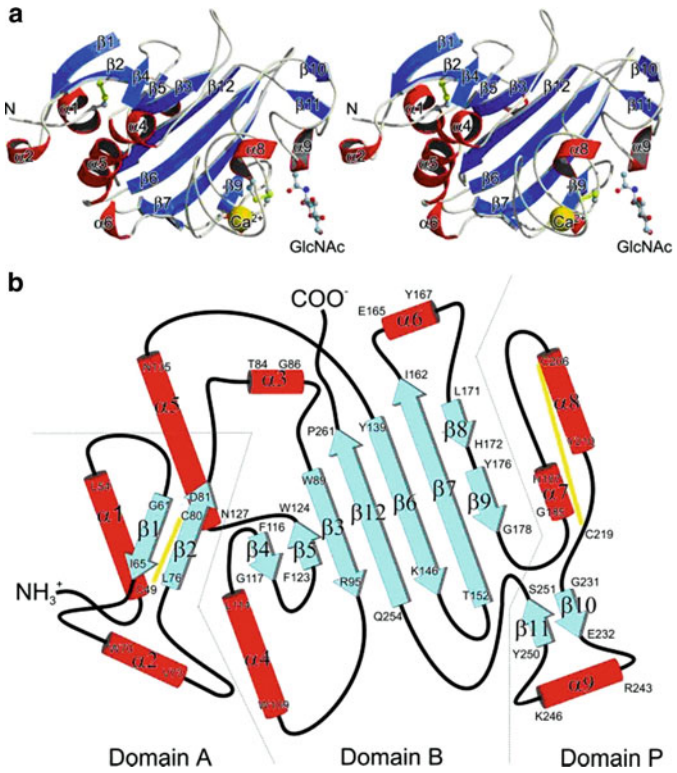

Fig. 18.2 *Crystal structure of TL5A from the Japanese horseshoe crab T. tridentatus*. (**a**) Stereo view of the TL5A ribbon plot. GlcNAc is represented by a ball-and-stick model and the Ca^{2+} ion is represented by a golden sphere. Disulfide bridges are colored yellow. (**b**) Topology diagram showing the arrangement of secondary-structure elements in TL5A. Disulfide bridges are indicated by yellow lines. Domains named in analogy to γ-fibrinogen fragment (Yee et al. 1997) (Reprinted with permission from Kairies et al. 2001© National Academy of Sciences USA)

one water molecule (WAT 40) form the pentagonal base, whereas WAT61 and the side chain oxygen atom OD1 (Asp^{200}) occupy the vertices of the bipyramide (Kairies et al. 2001).

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Chi-Lectins: Forms, Functions and Clinical Applications

19

Rajesh K. Gupta and G.S. Gupta

19.1 Glycoside Hydrolase Family 18 Proteins in Mammals

19.1.1 Chitinases

Chitinases (EC.3.2.1.14) hydrolyze the β-1,4-linkages in chitin, an abundant N-acetylglucosamine (GlcNAc) polysaccharide that is a structural component of protective biological matrices of invertebrate such as insect exoskeletons and fungal cell walls. Chitinases cleave chitin and contain the conserved sequence motif DXXDXDXE, in which the glutamate is the catalytic residue. Chitinases are found in species including archaea, bacteria, fungi, plants, and animals. On the basis of sequence homologies, chitinases fall into two groups: families 18 and 19 of glycosyl hydrolases. Members of family 18 employ a substrate-assisted reaction mechanism (van Aalten et al. 2001), whereas those of family 19 adopt a fold-and-reaction mechanism similar to that of lysozyme (Monzingo et al. 1996), suggesting that these families evolved independently to deal with chitin. The glycoside hydrolase 18 (GH18) family of chitinases is an ancient gene family widely expressed in archea, prokaryotes and eukaryotes. Since chitin is an important structural component of pathogens like fungi as well as a constituent of the mammalian diet, a dual function for mammalian chitinases in innate immunity and food digestion has been envisioned (Suzuki et al. 2002; Boot et al. 2005a). Indeed, for human chitotriosidase, an enzyme predominantly expressed by phagocytes, a fungistatic effect has been demonstrated (van Eijk et al. 2005; Brunner et al. 2008). Several studies have tried to link a common chitotriosidase deficiency to susceptibility for infection by chitin-containing parasites (Bussink et al. 2006). The physiological function of the second mammalian chitinase, acidic mammalian chitinase (AMCase), has attracted considerable attention due to a report linking the protein to the pathophysiology of asthma (Zhu et al. 2004a).

19.2 Chitinase-Like Lectins: Chi-Lectins

In addition to active chitinases, highly homologous mammalian proteins lacking enzymatic activity due to substitution of active-site catalytic residues have been identified. Despite their lack of enzymatic activity, these proteins have retained active-site carbohydrate binding and hence have been named chi-lectins (Renkema et al. 1998; Houston et al. 2003; Bussink et al. 2006). Like the active chitinases, chi-lectins belong to family 18 of glycosyl hydrolases, consisting of a 39-kDa catalytic domain having a TIM-barrel structure, one of the most versatile folds in nature (Sun et al. 2001; Fusetti et al. 2003; Houston et al. 2003). In contrast to both chitinases, chi-lectins lack the conserved additional chitin-binding domain (Boot et al. 2001). Despite the detailed knowledge regarding structure, insight into the exact physiological function of the various chi-lectins is limited (Bussink et al. 2006). Similar to chitotriosidase and AMCase, chi-lectins are secreted locally or into the circulation and hence Chi-lectins play a significant role in inflammatory conditions. For example, human cartilage GP39 (HC-gp39/YKL-40/CHI3L1), a protein expressed by chondrocytes and phagocytes, has been implicated in arthritis, tissue remodeling, fibrosis, and cancer (Johansen 2006). Similarly, the human chi-lectin YKL-39 (CHI3L2) and the murine Ym1 (CHI3L3/ECF-L) have been associated with the pathogenesis of arthritis (Tsuruha et al. 2002) and allergic airway inflammation, respectively (Ward et al. 2001; Homer et al. 2006). Investigation of family 18 glycosyl hydrolases has revealed that active chitinases and chi-lectins are widespread and conserved in the mammalian kingdom. An ancient gene duplication first allowed the specialization of two active chitinases, chitotriosidase and AMCase, and subsequent gene duplications followed by loss-of-enzymatic-function mutations, have led to the evolution of a broad spectrum of chi-lectins in mammals.

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Chitin is not found in vertebrates, but vertebrates do possess a small number of closely-related active chitinases. In mammals these are represented primarily by AMCase and chitotriosidase, both of which appear to have roles in the immune system. Eukaryotes also possess di-N-acetylchitobiase, a lysosomal enzyme involved in the degradation of N-linked glycoproteins, which is distantly related to AMCase and chitotriosidase. In mammals, but not other vertebrates, there are non-enzymatic chitinase-like proteins very similar to AMCase and chitotriosidase, such as YKL-40. Preservation of the hydrophobic substrate binding cleft, and consequently of high affinity binding to chito-oligosaccharides, has been demonstrated in some of these proteins, which have been termed chitinase-like lectins or Chi-lectins. Stabilin-1 interacting chitinase-like protein (SI-CLP, CHID1) is a non-enzymatic protein of unknown function which is distantly related to other mammalian glycoside hydrolase family 18 proteins and is conserved in eukaryotes.

19.3 YKL-40 [Chitinase 3-Like Protein 1 (CHI3L1)]

19.3.1 The Protein

Johansen and associates identified a protein secreted in vitro by the human osteosarcoma cell line MG63 and named it 'YKL-40' based on its three NH2-terminal amino acids tyrosine (Y), lysine (K), and leucine (L) and its molecular weight of 40 kDa (Johansen et al. 1992). Chitinase 3-like protein 1 or YKL-40 contains a single polypeptide chain of 383 amino acids. The complete amino acid and cDNA sequence of human YKL-40 was deduced by Hakala et al. (1993). The sequence of YKL-40 from several other mammals is known. Amino acid sequence of YKL-40 suggested that YKL-40 belongs to the glycosyl hydrolase family 18. YKL-40 shares significant amino acid sequence homology to bacterial chitinases and to six "mammalian chitinase–like proteins": oviduct-specific glycoprotein, chitotriosidase, YKL-39, TSA 1902, inducible silicotic bronchoalveolar lavage protein-p58 also named acidic mammalian chitinase, and mouse Ym1 also named eosinophil chemotactic cytokine (Boot et al. 2005b; Johansen et al. 2006). Interestingly, *Drosophila melanogaster* secretes several proteins, DS47 and imaginal disc growth factors, with sequence homology to YKL-40 (Kawamura et al. 1999).

The gene for human CHI3L1 is located on chromosome 1q31–q32, and consists of 10 exons and spans about 8 kb of genomic DNA (Rehli et al. 1997). The genes of all other human mammalian chitinase–like proteins identified are also located on chromosome 1. The transcriptional regulation of YKL-40 during human macrophage differentiation has been described. There are probably two independent transcription start sites and the promoter sequence that contains binding sites for several known factors and specific binding of nuclear PU.1, Sp1, Sp3, USF, AML-1, and C/EBP proteins (Rehli et al. 2003). The Sp1-family transcription factors seem to have a predominating role in controlling YKL-40 promoter activity.

19.3.2 Cell Distribution and Regulation

CHI3L1 is expressed by a variety of cell types including synovial cells, chondrocytes and smooth muscle cells, neutrophils, macrophages, fibroblast-like synovial cells, chondrocytes, and hepatic stellate cells. The expression of CHI3L1 is totally absent in monocytes (Rehli et al. 1997) and marginally expressed in monocyte-derived dendritic cells (Krause et al. 1996), but is strongly induced during the late stages of human macrophage differentiation. Rehli et al. (1997) demonstrated that promoter elements (in particular, the proximal -377 base pairs of the CHI3L1 promoter region) control the expression of CHI3L1 in the macrophage. CHI3L1 is also expressed in neutrophils (Volck et al. 1998), chondrocytes (Hakala et al. 1993), fibroblast-like synoviocytes (De Ceuninck et al. 2001), vascular smooth muscle cells, vascular endothelial cells (Malinda et al. 1999), ductal epithelial cells (Qin et al. 2007), hepatic stellate cells (Johansen 2006), and colonic epithelial cells (Mizoguchi 2006). In physiological concentrations CHI3L1 tends to promote proliferation of these cell types.

In U87, hypoxia and ionizing radiation induced a significant increase in YKL-40 after 24–48 h. The hypoxic induction of YKL-40 was independent of HIF1. Etoposide, ceramide, serum depletion and confluence led to elevated YKL-40. Inhibition of p53 augmented the YKL-40 expression indicating that YKL-40 is attenuated by p53. In contrast, both basic fibroblast growth factor and tumor necrosing factor-α repressed YKL-40. Diverse types of stress resulted in YKL-40 elevation, which strongly supports an involvement of YKL-40 in the malignant phenotype as a cellular survival factor in an adverse microenvironment (Junker et al. 2005a, b).

CHI3L1 is related to several other mammalian chitinase-like proteins (CLPs): chitotriosidase, YKL-39, Ym1, AMCase, oviduct-specific glycoprotein,, human cartilage glycoprotein 39 (HC-gp39) and stabilin-1-interacting (SI)-CLP. These mammalian chitinase-like proteins possess a conserved sequence motif (DXXDXDXE) on strand 4, and catalytic activity in these chitinases is mediated by the glutamic acid (E), which protonates the glycosidic bond with chitin (van Aalten et al. 2001). AMCase and chitotriosidase have catalytic activity but CHI3L1, YKL-39, Ym1, oviductin, SI-CLP do not have this activity (Kzhyshkowska et al. 2006). Due to substitution of an

essential glutamic acid residue to leucine, CHI3L1 loses chitinase activity, but still can bind to chitin and chito-oligosaccharides with high affinity through a preserved hydrophobic substrate binding cleft (Kawada et al. 2007). SI-CLP is upregulated by IL-4 as well as by glucocorticoids. This feature of SI-CLP makes it an attractive candidate for the examination of individual sensitivity of patients to glucocorticoid treatment and prediction of side effects of glucocorticoid therapy

Exposure to antigens containing chitin- or chitin-like structures sometimes induces strong T helper type-I. responses in mammals which may be associated with the induction of mammalian chitinases

19.3.3 Ligands of YKL-40

YKL-40 binds chitin of different lengths in a similar fashion as with other family 18 chitinases, but has no chitinase activity (Hakala et al. 1993; Renkema et al. 1998). The amino acids essential for the catalytic activity in chitinases are three acidic residues Asp, Glu, and Asp. The corresponding residues in human YKL-40 are Asp^{115}, Leu^{119}, and Asp^{186}. The recombinant chitin-binding domain binds to chitin, but not to glucan, xylan, or mannan and the binding of the recombinant chitin-binding domain to chitin was inhibited by N-acetylglucosamine, di-N-acetylchitobiose, and hyaluronan, but not by N-acetylgalactosamine or chondroitin. Furthermore, the recombinant domain interacts specifically with hyaluronan and hybrid-type N-linked oligosaccharide chains on glycoproteins, and that the oligosaccharide-binding characteristics are similar to those of wheat germ agglutinin, a lectin that binds to chitin (Ujita et al. 2003). YKL-40 binds specifically to collagen triple helices and regulates cleavage and fibril formation. Furthermore, Bigg et al., have reported an ability of YKL-40 to bind to collagen type 1, 2 and 3 (Bigg et al. 2006). YKL-40 binds chitin and chito oligosaccharides using 9 GlcNAc-binding subsites. The short chito-oligosaccharides which prime hyaluronic acid synthesis and are retained at reducing ends of hyaluronic acid molecules in mammals are suggested carbohydrate ligands for YKL-40; hyaluronic acid is associated with similar processes to YKL-40. Heparan sulphate, found on proteoglycans, is also a potential ligand and cell surface receptor for YKL-40 (Fusetti et al. 2003; Houston et al. 2003). The putative heparin-binding site is GRRDKQH (residues 143–149) (Fusetti et al. 2003). YKL-40 has therefore been defined as a CLP or chitinase-like lectin (Chi-lectin). YKL-39 is a protein of unknown function closely related to YKL-40 and present in humans and other primates.

YKL-40 is a glycoprotein in which most of the glucosamine is incorporated into N-linked complex oligosaccharides (De Ceuninck et al. 1998). Two possible sites of glycosylation are found in YKL-40, but only the NH2-terminal is glycosylated with two units of N-acetylglucosamine through β(1–4) linkage. Binding of either short or long oligosaccharides to human YKL-40 is possible, and the presence of two distinct binding sites with selective affinity for long and short oligosaccharides suggests that YKL-40 could function by cross-linking two targets. Glycosylation is a unique feature of the YKL-40 structure as the residue corresponding to Arg^{84} does not exist in chitinases and is mutated to Pro in other mammalian chitinase–like proteins. YKL-40 also binds heparin and amino acid sequence analysis reveals that YKL-40 contains one heparin binding motif (GRRDKQH at position 143–149) and two potential hyaluronan binding sites on the external face of the folded protein (Fusetti et al. 2003; Malinda et al. 1999). The putative heparin-binding site is located in a surface loop. It has been suggested that heparan sulfate is a more likely ligand of YKL-40, and unsulfated fragments of heparan sulfate can be accommodated in the binding groove of YKL-40 (Fusetti et al. 2003).

19.3.4 The Crystal Structure of YKL-40

Family 18 chitinases have catalytic domains of triosephosphate isomerase (TIM barrel) fold with a conserved DxDxE motif (Vaaje-Kolstad et al. 2004) and catalyze the hydrolytic reaction by substrate-assisted mechanism (Fig. 19.1a) (Terwisscha van Scheltinga et al. 1996; van Aalten et al. 2001), whereas family 19 chitinases have high percentage of α-helices and adopt the single displacement catalytic mechanism (Hoell et al. 2006). The crystallographic structures of human YKL-40 (Fusetti et al. 2003; Houston et al. 2003) and goat YKL-40 (Mohanty et al. 2003) display the typical fold of family 18 glycosyl hydrolases (Henrissat and Davies 1997). The structure is divided into two globular domains: a big core domain which consists of a $(\beta/\alpha)_8$ domain structure with a TIM barrel fold, and a small α/β domain, composed of five antiparallel β-strands and one α-helix, inserted in the loop between strand β7 and helix α7. This gives the active site of YKL-40 a groove-like character (Fig. 19.1b and Fig. 19.2). The TIM barrel domain consisting an $(\alpha/\beta)_8$-barrel fold has been found in many different proteins, most of which are enzymes. The TIM barrel domains share low sequence identity and have a diverse range of functions. The specific enzyme activity is determined by the eight loops at the carboxyl end of β-strands. In some TIM barrels, an additional loop

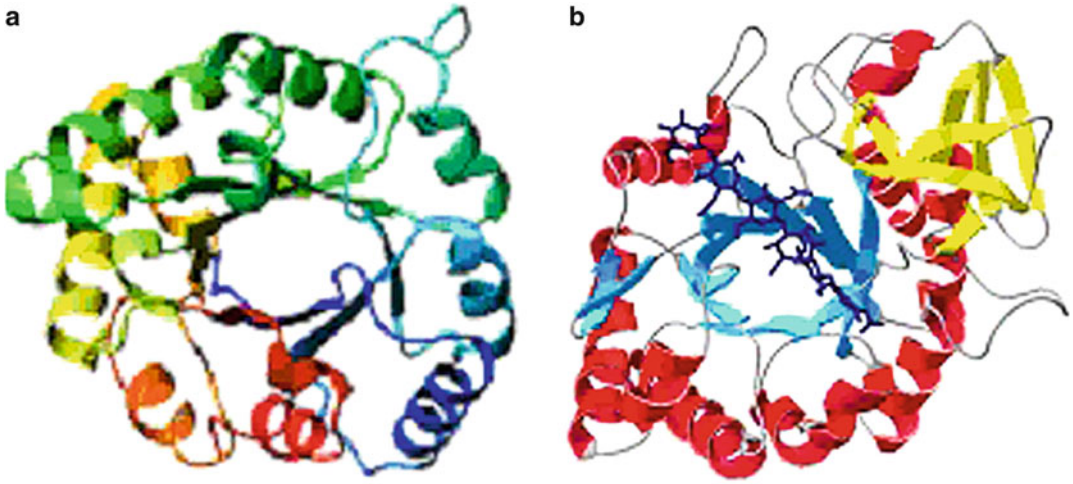

Fig. 19.1 (**a**) Top view of a triosephosphateisomerase (TIM) barrel (PDB code: 8TIM), colored from *blue* (N-terminus) to *red* (C-terminus). (**b**) Structure of human YKL-40 with bound GlcNAc oligomer. β-strands are colored as described in Fig. 19.1a. The GlcNAc oligomer is shown in *dark blue* (PDB code: 1HJW)

from a second domain approaches the active site of the TIM domain and participates in binding and catalysis (Li and Greene 2010).

Mammary Gland Protein-40 (MGP40): X-ray structure of chitolectin, mammary gland protein (MGP-40), is consistent with the $(\beta/\alpha)_8$ barrel topology of the Family 18 glycosidase proteins (Houston et al. 2003; Sun et al. 2001; van Aalten et al. 2001; Varela et al. 2002). MGP40 is an Asn-linked glycoprotein itself. The single disaccharide is covalently linked to the protein (at Asn^{39}) and forms hydrogen bonds with Arg^{84} and Ile^{40}, influencing the backbone conformation of loop Val^{75}-Phe^{85}, which in turn alters the disposition of Trp^{78}. Since Trp^{78} is an essential member of the binding site, the altered positioning of the site leads to constriction of the binding site, thereby leading to the inability of the MGP40 to bind sugar. Therefore, the oligosaccharides cannot bind to the putative binding site. Finally, Arg^{84}, which is hydrogen-bonded to the covalently bond sugar, is conserved in this particular class of chitolectins. The active chitinases in the Family 18 group which bind and cleave sugars possess a Pro in place of Arg^{84}, which in turn leads to inability of hydrogen bonding to the covalent linked sugar. Subsequently, there is a "relaxed" backbone conformation of the Val^{75}-Phe^{85} loop in the Family 18 chitinases and thereby no constriction of the binding site (Mohanty et al. 2003).

Human Cartilage Glycoprotein-39 (HUMGP39): The X-ray crystal structures of another chitolectin HumGP39 has been reported independently by two different groups (Houston et al. 2003; Fusetti et al. 2003). These structures are also consistent with $(\beta/\alpha)8$ barrel topology. The HumGP39 protein structures show a disaccharide covalently linked to it at $Asn^{19.}$ However, these structures depict in addition an oligosaccharide bound in the active site. Crystal structures of this protein show complexes with di, tri, tetra, penta and hexasaccharide bound at the active site. Based on these studies, it appears that the chitolectin group of proteins from Family 18 can bind chitin oligosaccharides. In comparison to MGP40 and HumGP39, the active site of glycosidases shows a difference of a glutamic acid, an essential acid/base residue for chitin cleavage and replaced with leucine or glutamine in chito-lectins.

The primary sequences of MGP40 and HumGP39 are highly identical (83 % identity). It was observed that since Arg^{84} is conserved between the two proteins, it is not the cause for the lack of oligosaccharide binding in MGP40 (Mohanty et al. 2003). Haq et al. (2007) compared the aromatic residues in both the proteins, focusing on Trp and Tyr residues present in the floor of the binding groove that are likely to interact strongly with the sugar molecules. Haq et al. (2007) suggested that MGP40 is capable of binding oligo-saccharides contrary to the conclusion of Mohanty

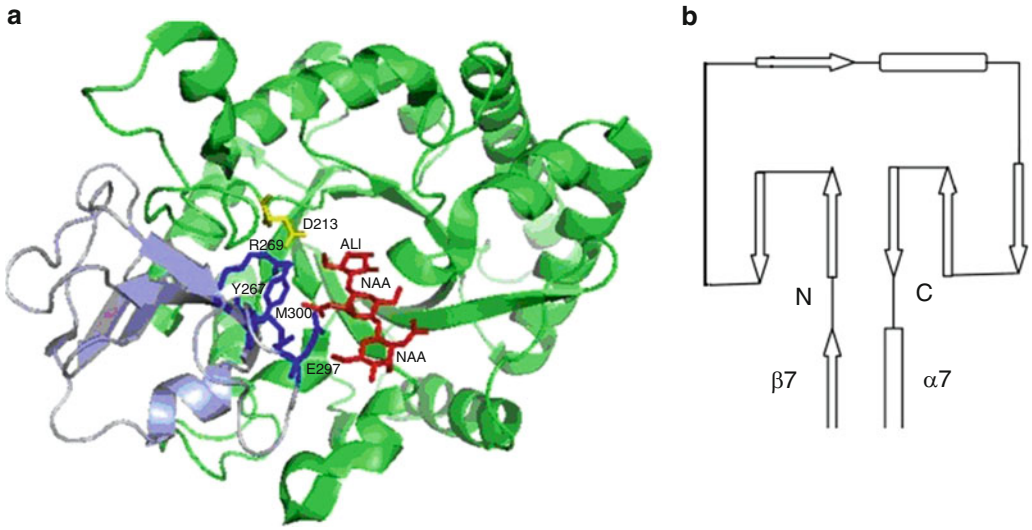

Fig. 19.2 *Structural analysis of Chitinase insertion domain (CID)*: (**a**) Ribbon model of human chitotriosidase (PDB: 1HKM) in complex with the substrate (NAA-NAA-ALI), showing the TIM barrel and CID. The helices and strands on the TIM barrel are coloured in *green* and those on the CID are coloured in *light blue*. Some residues (Tyr^{267}, Arg^{269}, Glu^{297}, and Met^{300}) in *blue* on the CID and Asp^{213} in *yellow* on the TIM barrel interact with the substrate in *red*. (**b**) Schematic representation of the CID between β7 and α7 on the TIM barrel, which is composed of two anti-parallel β-strands followed by one β-strand, one short α-helix, and lastly three anti-parallel β-strands. The arrows indicate β-strands and the rectangles are α-helices. The lines stand for the loops connecting α-helices or β-strands (Reprinted from Li and Greene 2010 © PLoS One)

et al. (2003) and that the conformation of residues 209–213 is not altered upon ligand binding as proposed by Mohanty et al. (2003). Based on the two HumGP39 structures, it was inferred that there are two distinct binding sites; a distal site for trisaccharides and the main site for tetra- and larger saccharides. It was proposed that the Trp in the +1 position functions as a "gate" to the main binding site that is in the "pinched" conformation when the oligosaccharide is not bound and is in the "stacked" conformation while interacting with the oligosaccharide.

Chitinase Insertion Domain (CID): While catalytic domain in chitinase activity has been studied extensively, the function of the chitinase insertion domain is least understood. For example, human chitotriosidase (PDB code: 1HKM), as a family 18 chitinase in the subfamily A, has a TIM domain and a chitinase insertion domain (CID), which is a module inserted into the TIM barrel (Fig. 19.2). Therefore the presence or absence of the insertion domain appears to be subfamily specific (Suzuki et al. 1999; Li and Greene 2010). Chitinase insertion domain sequences include four experimentally determined structures and span five kingdoms. The role of conserved residues was explored by conducting a structural analysis of a number of holo-enzymes. Hydrogen bonding and van der Waals calculations revealed a distinct subset of four conserved residues constituting two sequence motifs that interact with oligosaccharides. The other conserved residues may be key to the structure, folding, and stability of this domain. Sequence and structural studies of the chitinase insertion domains conducted within the framework of evolution identified four conserved residues which clearly interact with the substrates. Evolutionary studies propose a link between the appearance of chitinase insertion domain and the function of family 18 chitinases in the subfamily A (Li and Greene 2010).

19.4 Human Cartilage 39-KDA Glycoprotein (or YKL-39)/(CHI3L2)

The homologous human cartilage 39-kDa glycoprotein (HC gp-39) from articular cartilage chondrocytes primary culture has the N-terminal sequence YKL, which was termed YKL-19. The cDNA of HC gp-39 contained an open reading frame coding for a 383-amino acid long peptide which contains regions displaying significant homology with a group of bacterial and fungal chitinases and a similar enzyme found in the nematode, Brugia malayi. In addition significant homologies were observed with three mammalian secretory proteins, suggesting that a related protein family exists in mammals. The human protein does not possess any glycosidic activity against chitinase substrates. The mRNA of HC gp-39 is present in human articular chondrocytes as well is in liver, but undetectable in muscle tissues, lung, pancreas, mononuclear cells, or fibroblasts. Neither the protein nor mRNA for HC gp-39 was detectable in normal newborn or adult human articular cartilage obtained at surgery, while mRNA for HC gp-39 was detectable both in synovial specimens and in cartilage obtained from patients with rheumatoid arthritis (Hakala

et al. 1993; Renkema et al. 1998). One major site of synthesis of HC gp-39is the involuting mammary gland upon cessation of lactation (Morrison and Leder 1994). The 1434-nt sequence of YKL-39 cDNA predicts a 385-residue initial translation product and a 364-residue mature YKL-19. The amino acid sequence of YKL-39 is most closely related to YKL-40, followed by macrophage chitotriosidase, oviductal glycoprotein, and macrophage YM-1. These proteins share significant sequence identity with bacterial chitinases and have the probable structure of an $(\alpha\beta)_8$ barrel. The YKL-39 lacks the active site glutamate, which is essential for the activity of chitinases, and as expected has no chitinase activity. The highest level of YKL-39 mRNA expression is seen in chondrocytes, followed by synoviocytes, lung, and heart. YKL-39 accounts for 4 % of the protein in chondrocyte-conditioned medium, prostromelysin accounts for 17 %, and YKL-40 accounts for 33 %. In contrast to YKL-40, YKL-39 is not a glycoprotein and does not bind to heparin.

HC gp-39 and chitotriosidase are expressed in lipid-laden macrophages accumulated in various organs during Gaucher disease. In addition, these proteins can be induced with distinct kinetics in cultured macrophages. Studies depict remarkable phenotypic variation among macrophages present in the atherosclerotic lesion. Furthermore, chitotriosidase enzyme activity was shown to be elevated up to 55-fold in extracts of atherosclerotic tissue. Although a function for chitotriosidase and HC gp-39 has not been identified, it was hypothesized that these proteins play a role in cell migration and tissue remodeling during atherogenesis (Boot et al. 1999). However, little is known about the distribution of HC-gp39 and its role in fetal development (Ling and Recklies 2004).

19.5 Ym1 and Ym2: Murine Proteins

19.5.1 The Protein

Ym1 (or CH13L3) and Ym2 (or CH13L4) are murine chi-lectins, which are produced by macrophages, dendritic cells and mast cells. Ym1 is a murine protein secreted in large quantities by peritoneal macrophages upon nematode infection and is a marker for alternatively activated macrophages, which exert anti-inflammatory effects and promote wound healing, as well as combating parasitic infections. It is a single chain polypeptide (45 kDa) with a strong tendency to crystallize at its isoelectric point (pI 5.7). Upon nematode infection, murine peritoneal macrophages synthesize and secrete Ym1 protein, which is a functional marker for alternatively activated macrophages in T_{H2}-mediated inflammatory responses. Ym1 shares significant structural similarity to the family 18 chitinases. The function of Ym1 is not known, and there is conflicting evidence regarding both chitinase activity (weak or absent) and sugar-binding activity. SPR studies suggested that Ym1 exhibits calcium-independent binding at low pH to glucosamine (GlcN) and galactosamine (GalN), and especially to oligomers of these sugars, including heparin, but not to the unsubstituted or N-acetylated sugar. Treatment of macrophages and mast cells in vitro with IL-4 induced expression of Ym1 and Ym2 mRNA (HogenEsch et al. 2006). While Ym2 mRNA expression increased 976-fold, the mRNA of Ym1 increased 24-fold in the skin of cpdm/cpdm mice. Macrophages, dendritic cells and mast cells are cellular sources of Ym1 and Ym2 proteins. Eosinophils and neutrophils did not contain detectable concentrations of these proteins (Boot et al. 2005b; HogenEsch et al. 2006). Ym2 is another murine protein of unknown function closely related to Ym1. The Ym1 gene is mapped to a central region of mouse chromosome 3 (in the region syntenic to human chromosome 1p13) and that of another murine chitinase-like gene, Brp39, to a central region of mouse chromosome 1 (in the region syntenic to human chromosome 1q31).

Ym1 and Ym2 (Ym1/2) are induced by IL-4 in mouse bone marrow-derived mast cells and highly induced in bronchoalveolar lavage fluid (BALF) and the lung. Ym1/2 expression was completely inhibited by dexamethasone (Dex) in BALF and weakly inhibited in the lung. Although Dex pretreatment inhibited the Ym1/2 expression level in an animal model, it did not reduce IL-4 induction of Ym1/2 expression in primary cultured macrophages. It had no inhibitory effect on the phosphorylation level of STAT6 in macrophages. The inhibitory effect of Dex on Ym1/2 protein expression in the murine model of asthma does not involve the STAT6 signaling pathway (Lee et al. 2008).

Oral infections of mice with *Trichinella spiralis* induce activation of peritoneal exudate cells to transiently express and secrete Ym1. Co-expression of Ym1 with Mac-1 and scavenger receptor pinpoints macrophages as its main producer. A single open reading frame of 398 amino acids with a leader peptide (21 residues) typical of secretory protein has been deduced. Ym1 exhibits binding specificity to saccharides with a free amine group, such as GlcN, GalN, or GlcN polymers, but it does not bind to other saccharides. The interaction is pH-dependent but Ca^{2+} and Mg^{2+} ion-independent. The binding avidity of Ym1 to GlcN oligosaccharides was enhanced due to the clustering effect. Specific binding of Ym1 to heparin suggests that heparin/heparan sulfate may be its physiological ligand in vivo during inflammation and/or tissue remodeling. Although it shares 30 % homology with microbial chitinases, no chitinase activity was found associated with Ym1 (Chang et al. 2001).

Crystals discovered within the aged lung at the sites of chronic inflammation in a mouse model of chronic

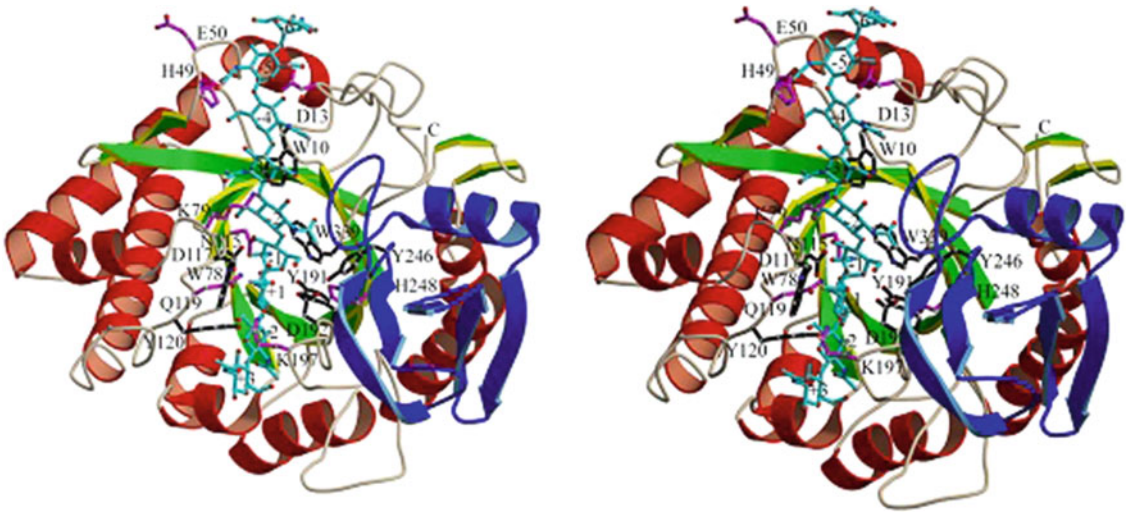

Fig. 19.3 Stereo view of the refined Ym1 structure with a model of N-acetylglucosamine$_9$ (NAG9). The NAG9 model was generated through superposition of the hCGP39–NAG8 complex, the SmChiA–NAG8 complex, and the SmChiB–NAG5 complex. The NAG9 model is labeled with the − 6 to +3 sugar-binding subsites and is shown as a stick drawing with cyan carbons. Helices in the TIM-barrel domain are colored in *red*, strands in *green*, and the additional α + β domain in *blue*. The conserved carbohydrate-interacting residues are shown in *magenta*, where non-conserved residues are shown in *black* (Reprinted with permission from Tsai et al. 2004 © Elsevier)

granulomatous disease, were identified as the chitinase-like protein Ym1. Ym1, found as a neutrophil granule protein, had weak β-N-acetylglucosaminidase activity, indicating that it might contribute to the digestion of glycosaminoglycans. Crystal formation is likely to be a function of excess neutrophil turnover at sites of inflammation in the chronic granulomatous disease mouse. Failure to remove subcutaneous Ym1 crystals injected into knockout mice indicates that a failure of digestion may also contribute to crystallization (Harbord et al. 2002).

19.5.2 Crystal Structure of Ym1

Co-crystallization and soaking experiments with various glucosamine or N-acetylglucosamine oligomers yield only the uncomplexed Ym1. The refined Ym1 structure at 1.31A resolution clearly displays a water cluster forming an extensive hydrogen bond network with the "active-site" residues (Fig. 19.3). This water cluster contributes notable electron density to lower resolution maps and this might have misled and given rise to a previous proposal for a monoglucosamine-binding site for Ym1. But crystals of Ym1 complexed with GlcN or GlcNAc polymers could not be obtained. A structural comparison of family 18 glycosidase like proteins reveals a lack of several conserved residues in Ym1, and illustrates the versatility of the divergent active sites. Therefore, Ym1 may lack N-acetylglucosamine-binding affinity, and this suggests that a new direction should be taken to unravel the function of Ym1 (Tsai et al. 2004).

Mouse Breast Regression Protein 39: Mouse breast regression protein 39 (BRP-39; CHI3l1) and its human homologue YKL-40 are chitinase-like proteins that lack chitinase activity. The biological properties of BRP-39/YKL-40 have only been rudimentarily defined. BRP-39$^{-/-}$ mice have markedly diminished antigen-induced T_{H2} responses and that epithelial YKL-40 rescued the T_{H2} responses in these animals. Mechanistic investigations demonstrated that BRP-39 and YKL-40 play an essential role in antigen sensitization and immunoglobulin E induction, stimulate dendritic cell accumulation and activation, and induce alternative macrophage activation. These proteins also inhibit inflammatory cell apoptosis/cell death while inhibiting Fas expression, activating protein kinase B/AKT, and inducing Faim 3. These proteins may prove therapeutic targets in T_{H2}- and macrophage-mediated disorders.

19.5.3 Oviductin

The high-molecular-weight oviductins, consisting of the amino-terminal 39-kDa catalytic domain followed by a heavily glycosylated serine/threonine-rich domain, are secreted by nonciliated oviductal epithelial cells and have been shown to play a role in fertilization and early embryo development (Buhi 2002). Among glycoside hydrolase family 18 proteins, it has a unique O-glycosylated mucin-like region following the chitinase-like domain. Carbohydrate binding has yet to be investigated in oviductin. The deduced amino acid sequence of an estrogen-dependent sheep oviductal glycoprotein (Mr 90,000–116,000) revealed the

presence of several potential sites for glycan substitution on a protein backbone of Mr 66,500, and identity with chitinases. The oviductal glycoprotein contained N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose, and sialic acid both in α2,3 and α2,6 linkages, typical of sialomucins. The oviductal glycoprotein was resistant to digestion with O-glycanase alone and chondroitinase ABC, with the latter indicating that it was not a proteoglycan. Results suggest that the secreted glycoprotein contains saccharide residues typical of sialomucins, and despite primary amino acid sequence identity, the oviductal glycoprotein does not share an enzymatic relationship with chitinases (DeSouza and Murray 1995).

19.6 Functions of CHI3L1 (YKL-40)

Although CHI3L1 was first identified in 1993 (Hakala et al. 1993), its biological function has been largely undetermined. CHI3L1 possesses a functional carbohydrate-binding motif which allows binding with a polymer or oligomer of GlcNAc, but CHI3L1 lacks enzymatic activity entirely. It is not yet known to have a specific receptor. Expression of YKL-40 appears to be induced by a change in the extracellular matrix environment, and is thought to have a role in development, tissue remodelling and inflammation. However, its pattern of expression is associated with pathogenic processes related to inflammation, extracellular tissue remodeling, fibrosis and solid carcinomas. It is assumed that YKL-40 plays a role in cancer cell proliferation, survival, invasiveness and in the regulation of cell-matrix interactions. YKL-40 is suggested as a marker associated with a poorer clinical outcome in genetically defined subgroups of different tumors (Kazakova and Sarafian 2009; Lee et al. 2011).

19.6.1 Role in Remodeling of Extracellular Matrix and Defense Mechanisms

Mammalian chitinase-like proteins have a postulated role in remodeling of extracellular matrix and defense mechanisms against chitin-containing pathogens. The expression of these proteins is increased in parasitic infections and allergic airway disease. YKL-40 has been indicated as a biomarker of sepsis (Hattori et al. 2009). YKL-40 is a growth factor for fibroblasts and has an anti-catabolic effect preserving extracellular matrix during tissue remodeling (Ling and Recklies 2004). YKL-40 stimulates migration and adhesion of endothelial cells and vascular smooth muscle cells (VSMCs), suggesting a role in angiogenesis (Malinda et al. 1999; Nishikawa and Millis 2003), and may play a role in regulating response of cancer cells to hypoxia (Saidi et al. 2008).

19.6.2 Growth Stimulating Effect

Since CHI3L regulates the growth of imaginal disc cells in *D.melanogaster,* it has been predicted that CHI3L1 can have a growth stimulating effect (Kawamura et al. 1999). Growth stimulating effects of CHI3L1 have been demonstrated on connective tissue cells (Recklies et al. 2002) and on endothelial cells (Malinda et al. 1999). CHI3L1 also stimulates angiogenesis and reorganization of vascular endothelial cells (Malinda et al. 1999). Insulin-like growth factor-1 works synergistically with CHI3L1 to enhance the response of human synovial cells isolated from patients with osteoarthritis (Recklies et al. 2002). In addition, CHI3L1 also promotes the activation of 2 major signaling pathways associated with mitogenesis and cell survival: MAPK (mitogen-activated protein kinase) pathways and PI-3 K (phosphoinositide 3-kinase)-mediated pathways in fibroblast cells. The purified human CHI3L1 efficiently phosphorylates MAPK p42/p44 in human synovial cells, fibroblasts, articular chondrocytes (Recklies et al. 2002) and human colonic epithelial cells (Eurich et al. 2009) in a dose-dependent manner. It has been suggested that G-protein-regulated MAPK networks are involved in the action of most non-nuclear oncogenes and subsequent carcinogenesis and tumor progression (Pearson et al. 2001). The networks are involved in the activation of MAPK p42/p44 which may enhance the carcinogenic change of epithelial cells during upregulated CHI3L1 expression under inflammatory conditions (Eurich et al. 2009).

19.7 Chi-Lectins As Markers of Pathogenesis

19.7.1 A Marker of Inflammation

The CHI3L1 plays a role in the pathological conditions leading to arthritis and tissue fibrosis (Recklies et al. 2002). Increased circulating levels of CHI3L1 have been reported in the serum of patients with several inflammatory conditions including IBD (Crohn's disease (CD) and ulcerative colitis (UC) (Vind et al. 2003), asthma (Chupp et al. 2007; Ober et al. 2008) and liver cirrhosis (Johansen et al. 1997). Serum CHI3L1 is rarely detectable in healthy individuals (Vind et al. 2003), and therefore CHI3L1 has been proposed as a useful marker for indicating inflammatory activity and poor clinical prognosis for IBD (Vind et al. 2003). CHI3L1 is strongly expressed by macrophages in the synovial membrane of patients with rheumatoid arthritis (RA) and a polarized IFNγ-mediated proinflammatory Th1-type immune response has been observed in half the patients with RA. CHI3L1 seems to be the cross-tolerance inducing protein in chronic arthritis which effectively downregulates the pathogenic immune responses. It is possible that nasal administration of

CHI3L1 represents an attractive approach for suppressing the clinical manifestation of chronic types of inflammation as well as autoimmune diseases.

Human cartilage GP39 (HC-gp39) has been implicated in arthritis, inflammatory bowel disease, tissue remodeling, fibrosis, and cancer (Hakala et al. 1993; Recklies et al. 2002; Johansen 2006). Prominent sites of HC-gp39 production are degenerate articular cartilage and inflamed or hyperplastic synovium, fibrotic liver tissue and gliomas, where a correlation of HC-gp39 production with malignancy has been reported. The presence of HC-gp39 at sites of inflammation has been described in several studies (Hormigo et al. 2006; Johansen et al. 1999a, 1999b), and the use of serum levels of this protein as a disease marker for the progression of joint erosion has been proposed. Cintin et al. (2002) have reported that serum levels of HC-gp39 correlate negatively with survival in patients with colorectal carcinomas; a similar correlation has been suggested for patients with breast carcinomas (Johansen et al. 1995). Similarly, the human chi-lectin YKL-39 (CHI3L2) and the murine Ym1 (CHI3L3/ECF-L) have been associated with the pathogenesis of arthritis (Tsuruha et al. 2002) and allergic airway inflammation, respectively (Chang et al. 2001; Ward et al. 2001; Homer et al. 2006).

Acidic mammalian chitinase (AMCase) and murine lectin Ym1 are upregulated in Th2-environment, and enzymatic activity of AMCase contributes to asthma pathogenesis. Chitotriosidase and YKL-40 reflect the macrophage activation in atherosclerotic plaques. Serum level of YKL-40 is a diagnostic and prognostic marker for numerous types of solid tumors. YKL-39 is a marker for the activation of chondrocytes and the progression of the osteoarthritis in human. The genetic contribution of YKL-40 gene to atopic susceptibility strongly suggests that the g.-247C/T polymorphism in the CHI3L1 promoter region is associated with the risk of atopy (Sohn and Wu 2009).

Expression in Osteoarthritis: Among numerous substances increasingly proposed for diagnostic purposes, very few may be considered as true disease markers in osteoarthritis (OA); COMP, antigenic keratin sulphate, hyaluronic acid, YKL-40, type III collagen N-propeptide and urinary glucosyl-galactosyl pyridinoline seem to be the most promising (Punzi et al. 2005). The presence of YKL-40 in cartilage and synovium in OA patients correlates with histopathological changes and may reflect local disease activity. In addition, the levels of YKL-40 in serum and synovial fluid also seem to correlate with disease severity. The functional role of YKL-40 is not yet clear, but its production as part of the inflammatory response in articular chondrocytes may modulate the cellular response to proinflammatory cytokines, acting to limit connective tissue degradation. Further elucidation of its roles and relationships may enable YKL-40 to act as a useful biomarker in the development of therapies for OA (Huang and Wu 2009).

Biomarkers for Asthma: Ober and Chupp (2009) provided an overview of genetic basis of asthma and immune-mediated diseases with polymorphisms in the genes encoding these proteins, CHIT1, CHI3L1, and CHIA, respectively. Polymorphisms in the CHIT1, CHIA, and CHI3L1 genes influence chitotriosidase enzyme activity, acid mammalian chitinase activity, and YKL-40 levels, respectively. Regulatory SNPs in CHI3L1 have been also associated with asthma, atopy, and immune-mediated diseases, and nonsynonymous SNPs in CHIA were associated with asthma. No CHIT1 polymorphisms, including a common nonfunctional 24-bp duplication allele, have been associated with asthma. These genes represent novel asthma susceptibility genes. Additional studies of this gene in populations of diverse ancestries are warranted (Ober et al. 2009). There is ample evidence to support an association of acidic mammalian chitinase (AMC) and YKL-40 with allergic bronchial asthma in patients. Recent studies in a mouse asthma model revealed that anti-inflammatory drugs like corticosteroid and cysteinyl leukotriene receptor antagonist were able to suppress elevated pulmonary levels of mammalian chitinases. Taken together, mammalian chitinases and chitinase-like proteins may be useful as biomarkers for asthma (Shuhui et al. 2009). Assessments of YKL-40 may allow physicians to more accurately diagnose and predict the course of asthma and thereby allow therapy to be appropriately tailored for a given patient (Hartl et al. 2009)

An overview of the chitinase and chitinase-like proteins, has been related to the genetic studies of asthma and immune-mediated diseases with polymorphisms in the genes encoding these proteins, CHIT1, CHI3L1, and CHIA, respectively. Polymorphisms in the genes of these proteins influence chitotriosidase enzyme activity, acid mammalian chitinase activity, and YKL-40 levels, respectively. Regulatory SNPs in CHI3L1 were also associated with asthma, atopy, and immune-mediated diseases, and nonsynonymous SNPs in CHIA were associated with asthma. No CHIT1 polymorphisms, including a common nonfunctional 24-bp duplication allele, have been associated with asthma. These genes represent novel asthma susceptibility genes (Ober and Chupp 2009).

Cardiovascular Diseases: Several inflammatory cytokines are involved in vascular inflammation resulting in endothelial dysfunction which is the earliest event in the atherosclerotic process leading to manifest cardiovascular disease. Human cartilage glycoprotein-39 (YKL-40) is secreted into

circulation by macrophages, neutrophils, chondrocytes, vascular smooth muscle cells and cancer cells. The YKL-40 is a serum biomarker in diseases with fibrosis, inflammation and tissue remodelling. In contrast to C-reactive protein (CRP) produced in the liver in response to inflammation, YKL-40 is produced by lipid-laden macrophages inside the vessel wall. YKL-40 is an inflammatory glycoprotein involved in endothelial dysfunction by promoting chemotaxis, cell attachment and migration, reorganization and tissue remodeling as a response to endothelial damage. YKL-40 protein expression is seen in macrophages and smooth muscle cells in atherosclerotic plaques with the highest expression seen in macrophages in the early lesion of atherosclerosis. Several studies demonstrate that elevated serum YKL-levels are independently associated with the presence and extent of coronary artery disease and even higher YKL-40 levels are documented in patients with myocardial infarction. Moreover, elevated serum YKL-40 levels have also been found to be associated with all-cause as well as cardiovascular mortality. YKL-40 levels are elevated both in patients with type-1 and type-2 diabetes, known to be at high risk for the development of cardiovascular diseases, when compared to non-diabetic persons.

Atherosclerotic plaque macrophages express YKL-40, particularly macrophages that have infiltrated deeper into the lesion, and the highest YKL-40 mRNA expression is found in macrophages in early atherosclerotic lesion (Boot et al. 1999). Studies have suggested that high serum YKL-40 levels could be a prognostic biomarker of short survival. This is demonstrated in 80-year-old people and in patients with *Streptococcus pneumoniae* bacteraemia, and local or metastatic cancer (Johansen et al. 2009; Kastrup et al. 2009). Serum YKL-40 levels are elevated in patients with acute myocardial infarction (MI) ((Nøjgaard et al. 2008; Wang et al. 2008) and stable coronary artery disease (CAD),and associated with the number of diseased vessels assessed by coronary angiography. Elevated serum YKL-40 is a risk factor for acute coronary syndrome and death in patients with stable CAD (Kastrup et al. 2009).

The serum YKL-40 was increased in chronic heart failure (CHF), and YKL-40 detected high risk patients for adverse outcomes in CHF (Bilim et al. 2010; Johansen et al. 2010). The YKL-40 is elevated and associated with mortality in patients with stable coronary artery disease (CAD). The influence of statin treatment and lipid status has been studied on serum YKL-40 and Hs-CRP in patients with stable CAD. HsCRP, but not YKL-40, is associated with the cholesterol levels in statin treated patients. In general population, elevated plasma YKL-40 levels are associated with increased risk of ischemic stroke and ischemic cerebrovascular disease, independent of plasma CRP levels (Kjaergaard et al. 2010), indicating that YKL-40 could be a superior prognostic biomarker in patients with stable CAD, since it is independent of changes in cholesterol levels in both statin and non-statin treated patients (Mygind et al. 2011).

A positive association between elevated circulating YKL-40 levels and increasing levels of albumin-uria has been described in patients with type 1 diabetes indicating a role of YKL-40 in the progressing vascular damage resulting in microvascular disease. YKL-40 has been related to endothelial dysfunction, atherosclerosis, cardiovascular disease and diabetes and offers future perspectives in research (Catalán et al. 2011; Rathcke et al. 2009; Rathcke and Vestergaard 2009; Mathiasen et al. 2010). However, Plasma YKL-40 was identified as an obesity-independent marker of type 2 diabetes related to fasting plasma glucose and plasma IL-6 levels (Nielsen et al. 2008).

Neurological Diseases: Elevated levels of CHI3L1 (YKL-40) have been observed in the cerebrospinal fluid (CSF) of human and non-human primates with lentiviral encephalitis. Immunohistochemistry showed that CHI3L1 was associated with astrocytes. It was demonstrated that CHI3L1 is induced in astrocytes in a variety of neurological diseases but that it is most abundantly associated with astrocytes in regions of inflammatory cells (Bonneh-Barkay et al. 2010; Gaidamashvili et al. 2004; Nigro et al. 2005). Disease-modifying therapies for Alzheimer's disease (AD) would be most effective during the preclinical stage before significant neuronal loss occurs. Data demonstrate that YKL-40 is elevated in AD and, together with Aβ42, has potential prognostic utility as a biomarker for preclinical AD (Craig-Schapiro et al. 2010).

Respiratory Disorders: The exaggerated expression of YKL-40, the human homolog of BRP-39, has been reported in chronic obstructive pulmonary disease (COPD). We hypothesized and confirmed that BRP-39/YKL-40 plays an important role in the pathogenesis of cigarette smoke (CS)-induced emphysema. Studies demonstrate a regulatory role of BRP-39/YKL-40 in CS-induced inflammation and emphysematous destruction. Studies also highlight that maintaining the physiologic levels of YKL-40 in the lung will be therapeutically important to prevent excessive inflammatory responses or emphysematous alveolar destruction (Matsuura et al. 2011). Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease that is hallmarked by fibrosis, inflammation and tissue remodelling. The -329 A/G polymorphism was associated with serum and BALF YKL-40 levels in IPF patients. High serum and BALF YKL-40 levels are associated with poor survival in IPF patients and could be useful prognostic markers for survival in IPF (Korthagen et al. 2011).

19.7.2 CHI3L1 as Biomarker in Solid Tumors

CHI3L1 or YKL-40 is expressed and secreted by several types of solid tumors including glioblastoma, colon cancer, breast cancer and malignant melanoma (Table 19.1). Although the exact function of CHI3L1 in inflammation and cancer is largely unknown, CHI3L1 plays a pivotal role in exacerbating the inflammatory processes and in promoting angiogenesis and remodeling of the extracellular matrix. CHI3L1 may be highly involved in the chronic engagement of inflammation which potentiates development of epithelial tumorigenesis presumably by activating the mitogen-activated protein kinase and the protein kinase B signaling pathways. Anti-CHI3L1 antibodies or pan-chitinase inhibitors may have the potential to suppress CHI3L1-mediated chronic inflammation and the subsequent carcinogenic change in epithelial cells (Eurich et al. 2009).

Clinical studies with different types of advanced tumors suggest that serum levels of CHI3L1 may be a new biomarker in cancer. In many cases, serum CHI3L1 provides independent information of survival. The highest serum CHI3L1 is detected in patients with advanced cancer and with the poorest prognosis. However, serum CHI3L1 cannot be used as a single screening test for cancer (Johansen et al. 2006). When evaluated with other prognostic factors of survival after recurrence of breast cancer, serum CHI3L1and serum LDH were the most significant independent factors. It was suggested that serum CHI3L1 may be of value in the follow-up of patients with breast cancer and in evaluating potential metastatic spread. CHI3L1 expression is strongly elevated in serum and biopsy material from glioblastomas patients. Though, the elevated plasma CHI3L1 is a biomarker of poor prognosis in cancer patients, but in general population, elevated plasma CHI3L1 predicts increased risk of gastrointestinal cancer and decreased survival after any cancer diagnosis (Johansen et al. 2009).

It has been strongly hypothesized that CHI3L1 plays a pivotal role as a growth stimulating factor for solid tumors or has a suppressive/protective effect in the apoptotic processes of cancer cells (Johansen et al. 2006) and inflammatory cells (Lee et al. 2009). In addition to CHI3L1 expression in a wide variety of human solid tumors, elevated levels of CHI3L1 in serum and/or plasma have been detected in patients with different types of solid tumors. Therefore, it is reasonable to predict that the serum level of CHI3L1 can be a reliable marker of progression of certain kinds of tumors and of a "bad prognosis" in patients with certain types of malignant tumors (Johansen et al. 2006).

The CHI3L1 can be used as a tumor marker for ovarian cancer (Høgdall et al. 2009), small cell lung cancer (Johansen et al. 2004), metastatic breast cancer (Johansen et al. 2009, 2010), and metastatic prostate cancer (Brasso et al. 2006). In addition, CHI3L1 is one of the most significant prognosis markers for cervical adenocarcinoma (Mitsuhashi et al. 2009), recurrent breast cancer (Johansen et al. 1995) and metastatic breast cancer (Jensen et al. 2003), as well as advanced stages of breast cancer (Coskun et al. 2007). Interestingly, the CHI3L1 serum level could be a useful and sensitive biomarker for recurrence in locally advanced breast carcinoma (Coskun et al. 2007), ovarian carcinoma (Gronlund et al. 2006), endometrial cancer (Diefenbach et al. 2007), squamous cell carcinoma of the head and neck (Roslind et al. 2007, 2008), metastatic prostate cancer or melanoma (Johansen et al. 2007; Schmidt et al. 2006), Hodgkin's lymphoma (Biggar et al. 2008) and colon cancer. Johansen and colleagues suggest that CHI3L1 may be used as a sensitive predictor of any cancer (Johansen

Table 19.1 *Expression of CHI3L1 (or YKL40) in solid tumors* (Eurich et al. 2009; Johansen et al. 2006)

Solid tumor	Location	Reference(s)
Glioma, Oligodendroglioma, glioblastoma	Brain	Tanwar et al. 2002; Nutt et al. 2005; Saidi et al. 2008; Bhat et al. 2008; Zhang et al. 2010
Squamous cell carcinoma of the head and neck	Head and neck	Johansen et al. 2006; Roslind et al. 2008.
Lung cancer (small cell carcinoma)	Lung	Junker et al. 2005
Breast cancer	Breast	Roslind et al. 2008; Qin et al. 2007; Johansen et al. 2006, 2008; Bhat et al. 2008, Junker et al. 2005; Cintin et al. 2002; Svane et al. 2007; Yamac et al. 2008
Hepatocellular carcinoma	Liver	Johansen et al. 2009
Colorectal cancer	Colon	Cintin et al. 2002; Bhat et al. 2008
Kidney tumor	Kidney	Bhat et al. 2008; Berntsen et al. 2008
Ovarian tumor, endometrial cancer	Ovary	Høgdall et al. 2009; Mitsuhashi et al. 2009; Coskun et al. 2007; Diefenbach et al. 2007; Peng et al. 2010; Zou et al. 2010
Primary prostate cancer, Metastatic prostate cancer	Prostate	Brasso et al. 2006; Johansen et al. 2007; Kucur et al. 2008
Papillary thyroid carcinoma, thyroid tumor	Thyroid	Huang et al. 2001
Extracellular myxoidchondrosarcoma	Bone	Sjögren et al. 2003
Multiple myeloma	Bone marrow	Mylin et al. 2006, 2009
Hodgkin's lymphoma	Lymph node	Biggar et al. 2008
Malignant melanoma	Melanocyte	Johansen et al. 2006; Schmidt et al. 2006
Myxoid liposarcoma	Fat cells	Sjögren et al. 2003

et al. 2009; Qin et al. 2007). They categorized patients into 5 distinct levels according to the amount of plasma CHI3L1 detected by ELISA, and found that participants with the highest level of plasma CHI3L1 had a median survival rate of only 1 year after the cancer diagnosis (Johansen et al. 2009). In contrast, the patients with the lowest level of plasma CHI3L1 had a survival rate of more than 4 years. Although the variation of CHI3L1 serum levels in healthy subjects in this study was relatively small, subsequent measurements would be required to determine cancer risk since the serum level of CHI3L1 could also be elevated in patients with other inflammatory diseases or autoimmune disorders (Johansen et al. 2008; van Aalten et al. 2001). From the results, it has been highly predicted that serum CHI3L1 levels seem to be a potential and promising biomarker for malignant tumors.

It is believed that IBD is a risk factor of cancer development based on the severity of the disease course. As previously reported, serum CHI3L1 concentration is elevated in patients with IBD (Vind et al. 2003) and primary colorectal cancer (Cintin et al. 2002). People with CD have a 5.6-fold increased risk of developing colon cancer; therefore screening for colon cancer by colonoscopy is strongly recommended for patients who have had CD for several years (Collins et al. 2006). Inflammation was recently recognized as an important factor in the pathogenesis of malignant tumors (Bromberg and Wang 2009).

Eurich et al. (2009) reviewed some examples of inflammation-mediated carcinogenesis and diseases which express CHI3L1 during the course of inflammation and the subsequent tumorigenesis. CHI3L1 protects cancer cells from undergoing apoptosis and also has an effect on extracellular tissue remodeling by binding specifically with collagen types I, II, and III (Bigg et al. 2006). Studies indicate that CHI3L1 is strongly associated with cell survival and cell migration during the drastic tissue remodeling processes by interacting with extracellular matrix components (Ling and Recklies 2004). The canonical (Wnt/β-catenin) pathway is known to play a crucial role in UC-related tumor progression (van Dekken et al. 2007). Reports strongly suggest that CHI3L1 may have a direct but not a secondary role for inflammation-associated tumorigenesis by continuously activating the Wnt/β-catenin canonical signaling pathway in CECs. As demonstrated, CHI3L1 expression is enhanced by proinflammatory cytokine interleukin-6 (De Ceuninck et al. 1998; Mizoguchi 2006), which also has a critical tumor-promoting effect during early colitis-associated cancer tumorigenesis (Grivennikov et al. 2009). The chitin-binding motif (CBM) of CHI3L1 is specifically associated with the CHI3L1-mediated activation of the Akt-signaling in CEC by transfecting the CBM-mutant CHI3L1 vectors in SW480 CECs. Downstream, CHI3L1 enhanced the secretion of IL-8 and TNFα in a dose-dependent manner. The 325 through 339 amino-acids in CBM are crucial for the biological function of CHI3L1 and is a critical region for the activation of Akt, IL-8 production, and for a specific cellular localization of CHI3L1. The activation may be associated with the development of chronic colitis (Chen et al. 2011).

19.7.3 Chitinase 3-Like-1 Protein (CHI3L1) or YKL-40 in Clinical Practice

Anti-Inflammatory Role of CHI3L1 (YKL-40): Chitinase 3-like-1 gene is significantly upregulated in inflamed colon of the dextran sulfate sodium-induced colitis model. CHI3L1 is mainly expressed in colonic epithelial cells and macrophages in the inflamed colon of dextran sulfate sodium-induced colitis. The CHI3L1 possesses an ability to enhance the adhesion and internalization of intracellular bacteria into colonic epithelial cells. In vivo neutralization of CHI3L1 significantly suppressed the development of dextran sulfate sodium-induced colitis by decreasing the bacterial adhesion and invasion into colonic epithelial cells. The CHI3L1 inhibited cellular responses to the inflammatory cytokines IL-1 and TNF-α. Stimulation of human skin fibroblasts or articular chondrocytes with IL-1 or TNF-α in presence of CHI3L1 resulted in a marked reduction of both p38 mitogen-activated protein kinase and stress-activated protein kinase/Jun N-terminal kinase phosphorylation, whereas nuclear translocation of NF-kB proceeded unimpeded. HC-gp39 suppressed the cytokine-induced secretion of MMP1, MMP3 and MMP13, as well as secretion of the chemokine IL-8 (Ling and Recklies 2004). Kawada et al. (2007) provided insight into the physiological role of mammalian chitinases in host/microbial interactions and suggested that inhibition of chitinase activity can be a novel therapeutic strategy of allergic and inflammatory disorder (Kawada et al. 2007). Unexpected roles have been identified for CHI3L1 in intestinal inflammation. While galectin-1 contributes to the suppression of intestinal inflammation by the induction of effector T cell apoptosis, in contrast, galectin-4 is involved in the exacerbation of this inflammation by specifically stimulating intestinal $CD4^+$ T cells to produce IL-6. CHI3L1 enhances the host/microbial interaction that leads to the exacerbation of intestinal inflammation (Mizoguchi and Mizoguchi 2007).

CHI3L1 plays a unique role during the development of intestinal inflammation, the protein is induced in both colonic lamina propria macrophages and CECs during the course of intestinal inflammation in experimental colitis models as well as in patients with IBD (Mizoguchi 2006). It has been suggested that a genetic defect against intracellular bacterial infection is strongly associated with the development of CD. CHI3L1 molecule particularly enhances the adhesion of chitin-binding protein-expressing bacteria to CECs through

the conserved amino-acid residues (Kawada et al. 2008). Therefore, overexpression of CHI3L1 may be strongly associated with the intracellular bacterial adhesion and invasion on/into CECs in CD patients (Xavier and Podolsky 2007). In contrast, in an aseptic condition such as bronchial asthma, epithelium-expressing CHI3L1 seems to play a regulatory role by rescuing Th2-type immune responses (Lee et al. 2009). Further studies are required to understand the exact role of epithelium-expressing CHI3L1 in inflammatory conditions.

CHI3L1 was recently introduced into clinical practice, yet its application is still restricted (Kazakova and Sarafian 2009). CHI3L1 may participate in the innate immune response as it is regarded as an acute phase protein, since its plasma concentration is increased in inflammatory diseases (Chupp et al. 2007; Kronborg et al. 2002; Johansen 2006; Rathcke et al. 2006). The CHI3L1/YKL-40 is induced specifically during the course of inflammation in such disorders as inflammatory bowel disease, hepatitis and asthma and has been found to be either the cause or a biomarker for asthma. When the asthma subjects were stratified, serum CHI3L1 levels in the exacerbation group were higher than those in the stable and control groups (Chupp et al. 2007; Fontana et al. 2010). The CHI3L1 gene is abnormally expressed in the hippocampus of subjects with schizophrenia and may be involved in the cellular response to various environmental events that are reported to increase the risk of schizophrenia. The functional variants at the CHI3L1 locus influence the genetic risk of schizophrenia. CHI3L1 is a potential schizophrenia-susceptibility gene and suggest that the genes involved in the biological response to adverse environmental conditions are likely to play roles in the predisposition to schizophrenia (Zhao et al. 2007).

19.8 Evolution of Mammalian Chitinases (-Like) of GH18 Family

Homologous lectins to chitinases of the glycosyl hydrolase family 18 have been isolated from plants. Insects utilize multiple family 18 chitinolytic enzymes and also non-enzymatic chitinase-like proteins for degrading/remodeling/binding to chitin in different insect anatomical extracellular structures, such as the cuticle, peritrophic membrane, trachea and mouth parts during insect development, and possibly for other roles including chitin synthesis. Drosophila protein database revealed the presence of 18 chitinase-like proteins. Among these, seven are novel chitinase-like proteins that contain four signature amino acid sequences of chitinases, including both acidic and hydrophobic amino acid residues critical for enzyme activity (Zhu et al. 2004a). *D. melanogaster* imaginal disc growth factor proteins stimulate cell proliferation in common with mammalian chitinase-like protein YKL-40, but are only distantly related to mammalian glycoside hydrolase family 18 proteins, displaying around 25 % sequence identity. Chitinases may also affect gut physiology through their involvement in peritrophic membrane turnover. The chitinase from larvae of tomato moth (Lacanobia oleracea) shows 75–80 % identity with other Lepidopteran chitinases. L. oleracea chitinase caused chronic effects when fed, causing reductions in larval growth and food consumption by 60 % (Fitches et al. 2004). A chitinase-like protein from oyster, Clp1, which binds chitin and exhibits similar functional properties to YKL-40, has been described.

Gene duplication and loss according to a birth-and-death model of evolution is a feature of the evolutionary history of the GH18 family. Mammals are not known to synthesize chitin or metabolize it as a nutrient, yet the human genome encodes eight GH18 family members. Some GH18 proteins lack an essential catalytic glutamic acid and are likely to act as lectins rather than as enzymes. Both types of proteins widely occur in mammals although these organisms lack endogenous chitin. Molecular phylogenetic analyses suggest that both active chitinases (chitotriosidase and AMCase) result from an early gene duplication event. Further duplication events, followed by mutations leading to loss of chitinase activity, allowed evolution of chi-lectins. The homologous genes encoding chitinase (-like) proteins are clustered in two distinct loci that display a high degree of synteny among mammals. Despite the shared chromosomal location and high homology, individual genes have evolved independently. Orthologs are more closely related than paralogues, and calculated substitution rate ratios indicate that protein-coding sequences underwent purifying selection. Substantial gene specialization has occurred in time, allowing for tissue-specific expression of pH optimized chitinases and chi-lectins (Bussink et al. 2007). Bussink et al. (2007) reported that several family 18 chitinase-like proteins are present only in certain lineages of mammals, exemplifying evolutionary events in the chitinase protein family (Bussink et al. 2007).

The current human GH18 family likely originated from ancient genes present at the time of the bilaterian expansion (~550 M years). The family expanded in the chitinous protostomes *C. elegans* and *D. melanogaster*, declined in early deuterostomes as chitin synthesis disappeared, and expanded again in late deuterostomes with a significant increase in gene number after the avian/mammalian split. This comprehensive genomic study of animal GH18 proteins reveals three major phylogenetic groups in the family: chitobiases, chitinases/chitolectins, and stabilin-1 interacting chitolectins. Only the chitinase/chitolectin group is associated with expansion in late deuterostomes. Finding that the human GH18 gene family is closely linked to the human MHC paralogon on chromosome 1, together with the recent association of GH18 chitinase activity with Th2 cell inflammation, suggests that its late expansion could be related to an

emerging interface of innate and adaptive immunity during early vertebrate history (Funkhouser and Aronson 2007).

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Novel Groups of Fuco-Lectins and Intlectins

20

Rajesh K. Gupta and G.S. Gupta

20.1 F-type Lectins (Fuco-Lectins)

A novel lectin family "F-type" constituted by a large number of proteins exhibit greater multiples of F-type motif, either tandemly arrayed or in mosaic combinations with other domains, including a putative transmembrane receptor that suggests an extensive functional diversification of this lectin family. F-type domains are found in proteins from a range of organisms from bacteria to vertebrates, but exhibit patchy distribution across different phylogenetic taxa suggesting that F-type lectin genes have been selectively lost even between closely related lineages, and making it difficult to trace the ancestry of the F-type domain. F-type lectins were first characterized in eels, which are teleost fish. Teleost fish species commonly have a number of F-type lectins (containing multiple tandem F-type domains) resulting from gene duplication events that have occurred independently in different teleost fish lineages. The F-type domain has clearly gained functional value in fish, whereas the fate of F-type domains in higher vertebrates is not clear, rather it has become defunct. While the functions of many F-type lectins have not yet been characterized, a pathogen-recognition role has been established for F-type lectin-like proteins in both invertebrates (e.g. the horseshoe crab *Tachypleus tridentatus*) and vertebrates (e.g. the Japanese eel *Anguilla japonica*). The F-type lectin fold from *Anguilla anguilla* agglutinin (AAA) (Bianchet et al. 2002) is widely distributed in proteins from horseshoe crabs to amphibians, with some representatives proposed to play a role in immunity (Honda et al. 2000; Saito et al. 1997). Although AAA and the rat MBL specifically recognize terminal L-fucose, they do so through differing binding interactions (Ng et al. 1996), which illustrate convergent functional properties among unrelated lectin families. Interestingly, the F-type fold is also shared with several proteins of distinct functional properties, including C-terminal domain of blood coagulation factors V and VIII, C-terminal domain of bacterial sialidases, N-terminal domain of fungal galactose oxidase, a human ubiquitin ligase subunit APC10/DOC1, a domain of single-strand DNA repair protein XRCC1, the b1 domain of neuropilin, and yeast allantoicase (Odom and Vasta 2006) pointing towards an ancient origin for F-type fold. The structural analogy of these seemingly unrelated proteins is indicative of the archaic origin of this lectin fold. Proteins from fish and amphibians with a range of domain organizations contain F-type domains, most of which are likely to bind fucose based on the conservation of key residues. The single-domain F-type lectins found in eels, which represent an early branch of teleost fish, are generally not found in modern teleosts, having been replaced by tandem two-domain proteins in species including striped bass (*Morone saxatilis*), zebrafish (*Danio rerio*), pufferfish (*Tetraodon nigroviridans* and *Fugu rubripes*) and stickleback (*Gasterosteus aculeatus*). In two-domain F-type lectins, both domains feature the fucose and Ca^{2+}-binding residues, but the N-terminal domain lacks one inter-strand disulfide bond, and the C-terminal domain lacks the adjacent cysteines in loop 4, which may affect sugar binding. In four-domain tandem F-type lectins, which are exclusive to trout species (e.g. *Oncorhynchus mykiss*), the first and second domains lack the adjacent cysteines, and the second domain has strikingly lost all three residues from His/Arg/Arg triad, suggesting that fucose binding has been lost in this domain.

20.1.1 F-type Lectins in Mammalian Vertebrates

20.1.1.1 Characterization

The fate of F-type domains in higher vertebrates is not clear. Two genes encoding three-domain F-type proteins are predicted in the genome of the opossum (*Monodelphis domestica*), an early-branching mammal. The genes are present in tandem within a genomic region that is absent in placental mammals and in birds, but which in *Xenopus tropicalis* contains a gene encoding a three-domain F-type lectin ~40 % identical to the opossum sequences. In humans

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this region would lie within chromosome 12, between the conserved genes *Wbp11* and *Foxj2*. Some remnants of F-type lectin genes are evident in this region of human genome. Human macrophages express a membrane lectin, or sugar-specific receptor, which specifically mediates the binding and endocytosis of mannose- and fucose-terminated glycoproteins and is involved in the phagocytosis of pathogens (Leu et al. 1985). A similar lectin activity was sought on cultured human DC (Avraméas et al. 1996).

A lectin with a high affinity for fucose residues has been purified from rat liver. The rat hepatic fucose binding protein (FBP) contains two polypeptide subunits with M_r of 88 kDa and 77 kDa. Peptide maps of the subunits, however, showed that they are structurally very similar but distinct from other rat hepatic lectins (Lehrman et al. 1986). The hepatic FBP has a high affinity for Fuc-BSA and galactosyl-BSA but a low affinity for N-acetylglucosaminyl-BSA. In contrast, the mannose/N-acetylglucosamine lectin binds N-acetylglucosaminyl-BSA and Fuc-BSA but not galactosyl-BSA (Lehrman and Hill 1986). The interaction between a rat alveolar macrophage lectin (M_r of 180 kDa) and its ligands is dependent on Ca^{2+} over a optimal pH range. The apparent K_D for fucosyl bovine serum albumin is 20.4×10^{-10} M. D-Mannose, L-fucose, and N-acetyl-D-glucosamine were the most effective inhibitors, and D-galactose was the least. The lectin isolated from alveolar macrophages is widely distributed in other rat tissues. Hepatocytes are devoid of this lectin, but hepatic Kupffer cells and endothelial cells contain significant amounts (Haltiwanger and Hill 1986). Of all rat tissues examined, only liver contained the fucose-binding lectin, whereas both liver and blood serum contained the mannose/N-acetylglucosamine lectin. The fucose-binding lectin was neither responsible for the uptake nor more than one lectin was acting. With the identification of another lectin (Mr = 180 kDa) only two lectins appeared to be involved. The hepatic mannose/N-acetylglucosamine lectin had a higher affinity for L-fucosyl-bovine serum albumin than the majority of the lectin in hepatocytes. This lectin, called the high affinity form, showed K_D of 2.3×10^9 for L-fucosyl-bovine serum albumin compared to 3.5×10^8 for the normal form. The two forms, however, had identical molecular weights (32 kDa) under reducing and nonreducing conditions and produced identical peptide maps after protease digestion (Haltiwanger et al. 1986).

20.1.1.2 Fucose Binding Proteins/Lectins in Fertilization

Bolwell et al. (1979) suggested that fertilization in *Fucus serratus* is based on an association between fucosyl- and mannosyl-containing ligands on the egg surface and specific carbohydrate-binding receptors on the sperm surface like *Fucus serratus,* the fucose-binding proteins (FBP) have been identified in mammamlian spermatozoa. In boar spermatozoa, during ionophore induced early stage acrosome reaction (AR), the FBP is first localized at the border between equatorial segment and anterior acrosome. With the propagation of AR the FBP was dramatically expressed and visible over the entire surface of acrosome and equatorial segment. Results suggested that FBP is responsible for the specific binding of the ghost-sperm unit to the zona pellucida (Friess et al. 1987). Oviductal sperm in cattle (Bos Taurus) revealed a prominent FBP of 16.5 kDa, which was labeled over live uncapacitated sperm acrosome, while capacitated sperm did not. The presence of a Le^a-binding protein with an apparent mass of 16.5 kDA appeared to originate from seminal plasma (Ignotz et al. 2001).

20.2 F-type Lectins in Fish and Amphibians

20.2.1 Anguilla Anguilla Agglutinin (AAA)

The fucose-binding lectins are secretory proteins and exhibit only weak similarities to frog pentraxin, horseshoe crab tachylectin-4, and fly fw protein. There are at least seven closely related members whose messages are abundantly expressed in liver and in significant levels in gill and intestine. The serum fucolectins are derived from liver. The message levels were increased by lipopolysaccharide, suggesting a role for fucolectins in host defense. Eel fucolectins have a SDS-resistant tetrameric structure consisting of two disulfide-linked dimers (Honda et al. 2000). Eel serum lectins have been useful as anti-H hemagglutinins and also in lectin histochemistry as fucose-binding lectins (fucolectins). This domain of unknown function has been identified in Japanese eel (Anguilla japonica) fucolectins and at least one frog pentraxin. The AAA acts as a defensive agent, recognizes blood group fucosylated oligosaccharides including A, B, H and Lewis B-type antigens, but does not recognize Lewis A antigen. AAA has low affinity for monovalent haptens.

20.2.1.1 AAA: A Homotrimer with Three Fucose Binding Sites

AAA is a non-covalent homotrimer in which all three fucose binding sites are oriented in the same direction. This arrangement resembles that of the C-type CRDs in mannose binding protein and may confer high affinity for pathogen surfaces displaying repetitive arrays of oligosaccharide ligands. The AAA F-type CRD has a β-barrel structure, with one three-stranded β-sheet and one five-stranded sheet, connected by two disulfide bonds. One end of the barrel features five loop regions (here termed loops 1–5) that form a ring enclosing the site of fucose binding, which is a positively-charged hollow (Fig. 20.1). A Ca^{2+} ion is bound within a sub-domain that largely lacks regular

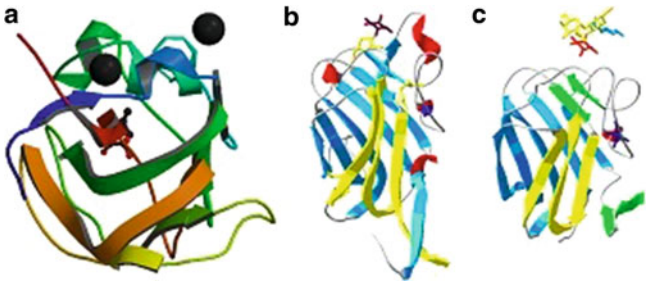

Fig. 20.1 (**a**) Structure of Anguilla anguilla agglutinin asymmetric unit (PDB ID 1 K12); (**b**) Fucose Binding lectin with bound fucose: β-strands in three-stranded sheet are colored *yellow* and those in the five-stranded sheet are colored *blue*. 310 helices are colored *red*. The fucose ligand is shown in *purple* and the Ca^{2+} ion in *blue* (PDB ID: 1 K12); (**c**) Structure of F-type domain 1 from *S. pnemoniae* SP2159 with bound blood group A tetrasaccharide. B-strands in the three-stranded sheet are colored *yellow*, those in the five-stranded sheet are colored *blue*, and those in other sub-structures are colored *green*. The 310 helix is colored red. Ca^{2+} is shown in *blue*. The ligand is colored by monosaccharide: *yellow*, Gal/GalNac; *blue*, Glc; *red*, Fuc (PDB ID: 2J1U)

secondary structure, and stabilization of tertiary or quaternary structure by Ca^{2+} may enhance sugar binding activity in AAA and other F-type lectins. Within the positively-charged hollow, side chains from a triad of residues characteristic of fucose binding make hydrogen bonds to the 3-OH, 4-OH and ring oxygen of fucose. In AAA these residues—a histidine in loop 3 and two arginine residues in loop 4—are present in the fucose binding sequence motif **H**. . .**R**GDCCGE**R**, which is found in other F-type domains in more general form **H**. . .**R**XDXXXX(**R**/**K**), where the gap between **H** and **R** is around 26 residues. Carbon atoms C1 and C2 of the fucose ring make van der Waals contacts with the adjacent disulfide-bonded cysteine residues in loop 4 (**H**. . .**R**GDCCGE**R**), and the methyl group (C6) is accommodated in a hydrophobic pocket formed by side chains from loops 1 and 2. Other monosaccharide ligands (e.g. 3-O-methyl-D-galactose, 3-O-methyl-D-fucose) are bound through the same set of interactions. Oligosaccharide ligands, which include the blood group antigens H and Le^a, interact with AAA primarily through the terminal fucose, but non-terminal saccharide residues also interact with a subset of the residues involved in fucose binding as well as additional residues from loop regions 1, 2 and 4. In the seven fucolectins from the Japanese eel (*Anguilla japonica*), various combinations of residue substitutions in loops 1 and 2 open up the methyl binding pocket and/or allow for polar interactions, which may adjust the oligosaccharide binding specificities of these lectins and tune them to recognize different pathogens. Non-eel F-type domains have a shorter loop 1, which may remove some interaction with oligosaccharide ligands and broaden oligosaccharide specificity.

20.2.1.2 Glycans Interacting AAA

AAA is suggested to be associated with innate immunity by recognizing disease-associated cell surface glycans such as bacterial LPSs, and has been widely used as a reagent in hematology and glycobiology. Among the glycans tested, AAA reacted well with nearly all human blood group A_h (GalNAcα1 → 3[L Fucα1 → 2]Gal), B_h (Galα1 → 3[L Fucα1 → 2]Gal), (H LFucα1 → 2Gal) and Le^b (Fucα1 → 2 Galβ1 → 3[Fucα1 → 4]GlcNAc) active glycoproteins (gps), but not with blood group Le^a (Galβ1 → 3[Fucα1 → 4] GlcNAc) substances, suggesting that residues and optimal density of α1-2 linked LFuc to Gal at the non-reducing end of glycoprotein ligands are essential for lectin-carbohydrate interactions. Blood group precursors, Galβ1-3GalNAc (T), GalNAcα1-Ser/Thr (Tn) containing glycoproteins and *N*-linked plasma gps, gave only negligible affinity. Among the mammalian glycotopes tested, A_h, B_h and H determinants were the best, being about 5 to 6.7 times more active than LFuc, but were weaker than *p*-nitrophenylαFuc indicating that hydrophobic environment surrounding the LFuc moiety enhance the reactivity. The hierarchy of potency of oligo- and mono-saccharides can be ranked as follows: *p*-nitrophenyl-αFuc > A_h, B_h and H > LFuc > LFucα1 → 2 Galβ1 → 4Glc (2′-FL) and Galβ1 → 4[L Fucα1 → 3]Glc (3′-FL), while LNDFH I (Le^b hexa-), Le^a, Le^x (Galβ1 → 4 [Fucα1 → 3]GlcNAc), and LDFT (gluco-analogue of Le^y) were inactive. From the present observations, it can be concluded that the combining site of AAA should be a small cavity-type capable of recognizing mainly H/crypto H and of binding to specific polyvalent ABH and Le^b glycotopes (Wu et al. 2004).

20.2.1.3 Comparison of AAA with MBL and UEA-1

MBL from eel serum has a M_r of ~246 kDa and is composed of identical subunits of ~24 kDa, two of each were always covalently linked. AAA, a FBL from eel serum, had a M_r of about 121 kDa and consisted four subunits of 30 kDa, which was made of two identical subunits of 15 kDa. Upon isoelectric focusing MBL displays four bands ranging from pH 4.8 to 5.2. FBL shows 17–20 bands between pH 5.5 and 6.2. Hemagglutination activity of MBL was inhibited only by mannan, whereas FBL activity was inhibited by several glycosubstances. MBL and FBL activity was constant between pH 4–5 and 10. Temperatures above 55º C totally destroyed MBL activity whereas FBL activity remained constant up to 75 º C (Gercken and Renwrantz 1994).

The binding site of AAA has been compared with related fucose-specific lectin from *Ulex europaeus* (UEA-I). Both AAA and lectin from *Ulex europaeus* (UEA-I) recognize Fuc α1-2Gal β-HSA. In addition, AAA cross-reacts

with neoglycolipids bearing lacto-N-fucopentaose (LNFP) I [H type 1] and II [Le^a], and lactodifucotetraose (LDFT) as glycans. UEA-I, on the other hand, binds to a LDFT-derived neoglycolipid but not to the other neoglycolipids tested. According to these results, AAA reacts with fucosylated type 1 chain antigens, whereas UEA-I binds only to the α1-2-fucosylated LDFT-derived neoglycolipid. AAA showed a broad reaction in the superficial pyloric mucosa from secretors and non-secretors, but AAA reactivity was more pronounced in Le(a^+b^-) individuals. On the other hand, UEA-I stained the superficial pyloric mucosa only from secretor individuals. Both reacted with most human carcinomas of different origin (Baldus et al. 1996).

20.2.2 FBP from European Seabass

A Binary Tandem Domain F-Lectin from Striped Bass (Morone Saxatilis): The FBP32 is a recently-characterized two-domain F-type lectin from striped bass, which is expressed abundantly in liver, and to a minor extent in other tissues. FBP32 is present in serum and up-regulated by inflammatory challenge. Gene duplication has produced at least one FBP32 paralogue (the more widely expressed FBP32II) in striped bass, while multiple two-domain F-type lectins are present in other fish species as a result of independent gene duplications. FBP32 binds specifically, in a Ca^{2+}-independent manner, to terminal fucose, but does not share the specificity of AAA for the H and Le^a antigens. FBP32 is monomeric and the presence of two F-type domains within one polypeptide may serve to increase ligand binding affinity, as an alternative mechanism to the oligomerization seen in AAA. Odom and Vasta (2006) described the molecular characterization of a unique L-Fuc-binding lectin from serum of striped bass (*Morone saxatilis*) that not only shares the CRD motif of AAA but possesses two similar yet distinct CRDs in the polypeptide subunit. In contrast with the N-terminal CRD, which presents the highest similarity to the AAA CRD, the C-terminus-CRD presents unique features. Among F-type tandem lectins, MS-FBP32 and other tandem binary homologues appear unique in that although their N-terminal domain shows close similarity to the fucose recognition domain of the eel agglutinin, their C-terminal domain exhibits changes that potentially could confer a distinct specificity for fucosylated ligands. In contrast with the amniotes, in which F-type lectins appear conspicuously absent, the widespread gene duplication in teleost fish suggests these F-type lectins acquired increasing evolutionary value within this taxon.

The crystal structure of the complex of MS-FBP32 with L-fucose shows a cylindrical 81-A-long and 60-A-wide trimer divided into two globular halves: one containing N-terminal CRDs (N-CRDs) and the other containing C-terminal CRDs (C-CRDs). The resulting binding surfaces at the opposite ends of the cylindrical trimer have the potential to cross-link cell surface or humoral carbohydrate ligands. The N-CRDs and C-CRDs of MS-FBP32 exhibit significant structural differences, suggesting that they recognize different glycans. Analysis of the carbohydrate binding sites provides the structural basis for the observed specificity of MS-FBP32 for simple carbohydrates and suggests that the N-CRD recognizes more complex fucosylated oligosaccharides and with a relatively higher avidity than the C-CRD. Modeling of MS-FBP32 complexed with fucosylated glycans that are widely distributed in nature suggests that the binary tandem CRD F-type lectins functions as opsonins by cross-linking "non-self" carbohydrate ligands and "self" carbohydrate ligands, such as sugar structures displayed by microbial pathogens and glycans on the surface of phagocytic cells from the host (Bianchet et al. 2010) (Fig. 20.2).

A lectin specific for fucose and galactose from serum of *Dicentrarchus labrax* showed hemagglutinating activity (HA) against rabbit erythrocytes. The HA was calcium-independent and comprised of two components, but only 34 kDa component (DLL2) showed activity against rabbit erythrocytes. The HA is a dimeric structure stabilized by disulfide bonds. The Ca^{2+}-independent fucose-binding specificity, a significant amino acid sequence homology of the N-terminal end, and cross-reaction of eel fucolectin with antibodies to DLL2 suggested that this lectin belongs to fucolectin family (Cammarata et al. 2001).

20.2.3 Other F-type Lectins in Fish

A F-type lectin (DlFBL) has been isolated from sea bass (Dicentrarchus labrax) serum. DlFBL exhibits two tandemly arranged CRDs that display F-type sequence motif. DlFBL is specifically expressed and localized in hepatocytes and intestinal cells. Exposure of formalin-killed *E. coli* to DlFBL enhanced their phagocytosis by D. labrax peritoneal macrophages relative to the unexposed controls, suggesting that DlFBL may function as an opsonin in plasma and intestinal mucus (Salerno et al. 2009).

A fucose-binding lectin, designated SauFBP32, was purified from the serum of the *gilt head bream Sparus* with subunit M_r of 35 and 30 kDa under reducing and non-reducing conditions, respectively. The native lectin is a monomer under the selected experimental conditions and agglutinated rabbit erythrocytes (Cammarata et al. 2007). A 23 kDa L-fucose-binding lectin from serum of Nile tilapia (*Oreochromis niloticusL.*), designated as TFBP, was isolated by Argayosa and Lee (2009). The fucose-binding proteins were detected in the soluble protein extracts from the gills, gut, head kidneys, liver, serum and spleen using a fucose-binding protein. The fucose-binding lectin from gill

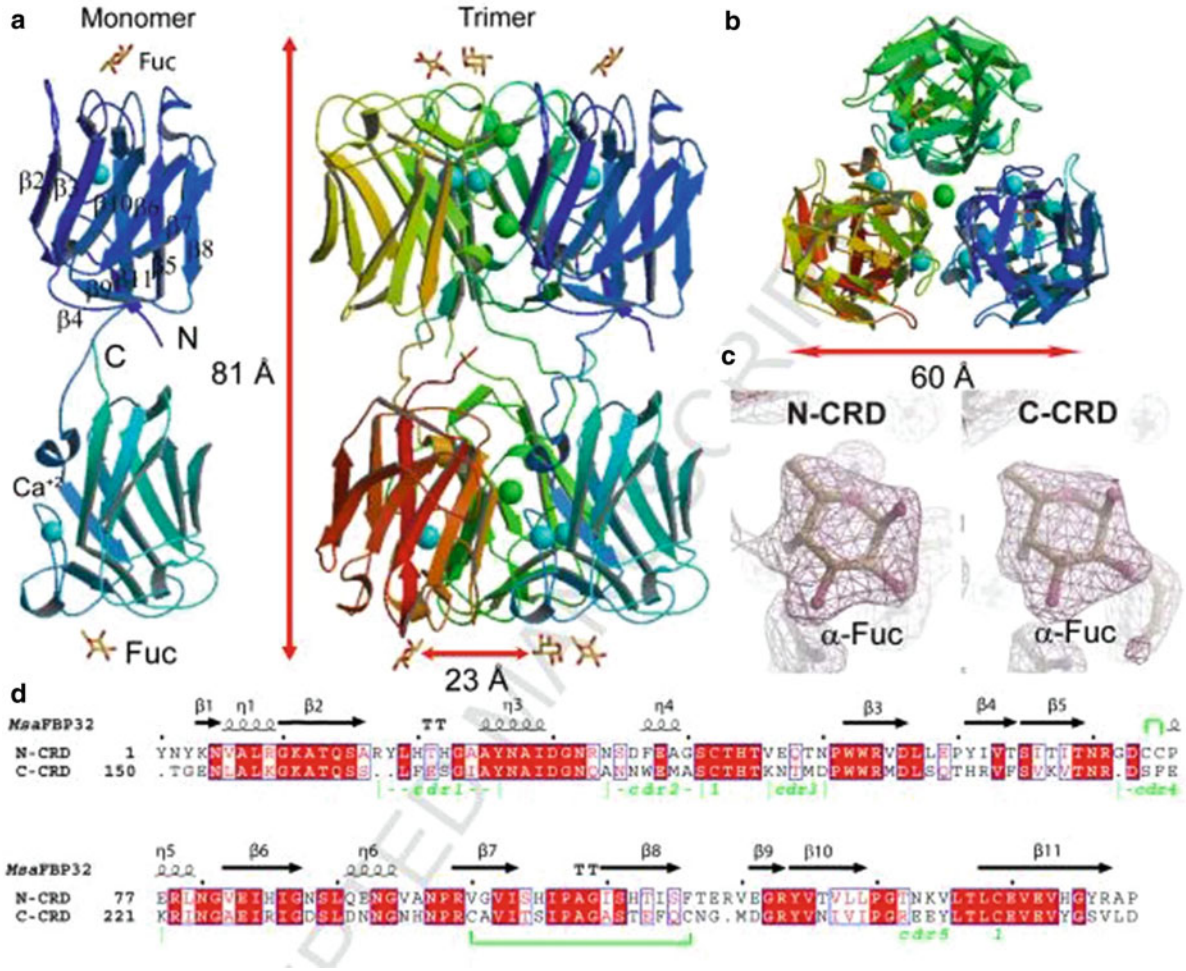

Fig. 20.2 *Structure of* MS*FPB32:* (**a**) View of MSFPB32 asymmetric unit of the crystal (trimer) and of an isolated monomer. (**b**) top view of the ASU trimer (N-CRD side). Monomers are colored with different hues. L-Fucose atoms are colored *yellow* for carbon atoms, and *red* for oxygen atoms. Ca^{+2} ions are colored *cyan* and Cl^{-} ions are colored *green*. (**c**) 2mFo-DFc sigmaA-weigthed electronic density at the CRDs binding sites. The *magenta* map is contoured at 0.35 e/Å^3. (**d**) Sequence alignment of the two MSFPB32 CRDs resulting in a 48.6 % and a 51 % of identity for the N- and the C-CRD respectively. The first line indicates the observed secondary structure elements of each CRD. Coils indicate 3–10 helices, T indicates turns and arrows indicate β-sheets strands. *Red* solid boxes over the sequences indicate identity and boxed *red* characters indicate amino acids with similar polarity. The last line shows the positions of the different CRDs. Disulfide bridges are indicated either by number "1" under the connected cysteines or a green line between them when they are within one CRD (Printed with permission from Bianchet et al. 2010 © Elsevier)

of bighead carp (*Aristichthys nobilis*) designated GANL had a M_r of 37 kDa under reducing conditions. GANL is a homomultimeric glycoprotein with a native molecular mass of 220 kDa and a carbohydrate content of ~13.4 %. The purified lectin only agglutinated rabbit native erythrocytes, and did not require Ca^{2+}. Its activity was inhibited by only fucose. GANL contains a high proportion of Asp, Glu, Leu, Val, and Lys. The 10 residues of N-terminal region were AGEQGGQCSA. GANL agglutinates and inhibits the growth of *Vibrio harveyi* but has no antifungal activity (Pan et al. 2010).

20.2.4 F-Type Lectins in Amphibians

Xenopus species possess proteins containing F-type domains in copies of 1 (*X laevis* X-epilectin), 2 (*X tropicalis* II-FBPL), 3 (*X tropicalis* III-FBPL) or 4 (*X laevis* II-FBPL), as well as 5 F-type domains in combination with a pentraxin domain (*X laevis* PXN-FBPL). *Xenopus* F-type domains sometimes lack fucose-binding His residue and/or a small number of Ca^{2+}-binding residues. A posterior epidermal marker, X-epilectin in *X. laevis* gene encodes for a fucolectin, which specifically binds fucose residues. The expression of this gene is switched on during gastrulation

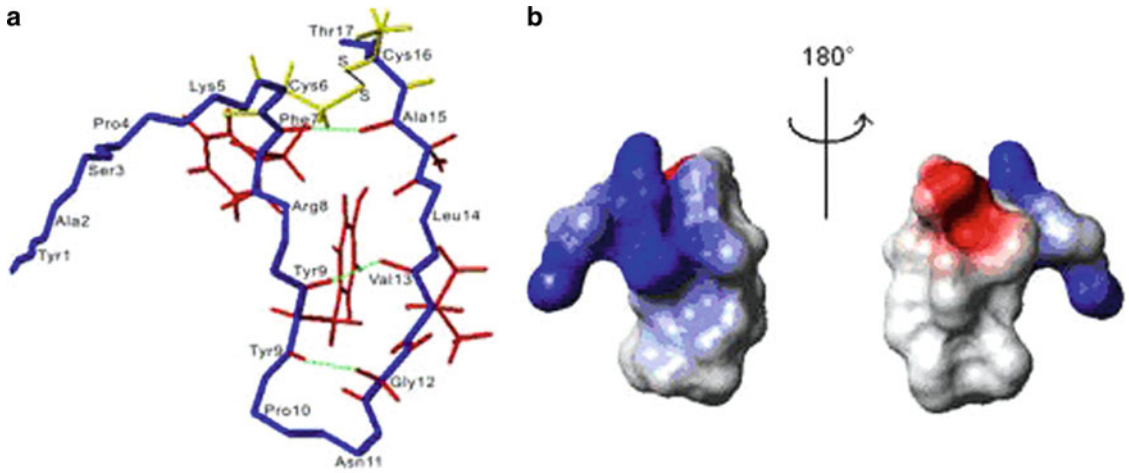

Fig. 20.3 The solution structure of odorranalectin. Backbone, side-chains of residues 4–16 and side-chains of residues 1–3, 4 were shown in *blue*, *red* and *green*, respectively. (**a**) The mean structure calculated from the 20 lowest-energy structures which highlighted three hydrogen bonds (*green broken lines*) and one disulfide bonds (*black solid line*). (**b**) Electrostatic surface of odorranalectin. Positively charged region and negatively charged region were shown in *blue* and *red*, respectively (Adapted from Li et al. 2008)

and up-regulated during neurula stages and found expressed ubiquitously throughout the epidermis. In adult, X-epilectin is mainly expressed in intestinal components, kidney, spinal cord and skin. Change of fate of animal caps into cement gland or dorsal mesoderm induces a down-regulation of X-epilectin expression in explants treated respectively with ammonium chloride and activin A. X-epilectin expression is down-regulated by Noggin and tBR, an effect which is inhibited by BMP4 over-expression, suggesting X-epilectin expression is mediated by BMP signaling pathway (Massé et al. 2004).

A lectin-like peptide named odorranalectin was identified from skin secretions of *Odorrana grahami*. The lectin was composed of 17 aa with a sequence of YASPKCFRY-PNGVLACT. L-fucose could specifically inhibit the haemagglutination induced by this lectin. In mice, odorranalectin mainly conjugated to liver, spleen and lung. The cyclic peptide of odorranalectin in solution NMR adopts a β-turn conformation stabilized by one intramolecular disulfide bond between Cys6-Cys16 and three hydrogen bonds between Phe7-Ala15, Tyr9-Val13, Tyr9-Gly12 (Fig. 20.3). NMR titration and mutant analysis showed that residues K5, C6, F7, C16 and T17 on odorranalectin consist of binding site of L-fucose. The structure of odorranalectin in bound form is more stable than in free form. Smaller peptides which can mimic the function of lectins are promising candidates for drug targeting (Li et al. 2008).

20.3 F-type Lectins in Invertebrates

20.3.1 Tachylectin-4

The F-type domain is found in a range of invertebrate species, often within lineage-specific protein contexts. Sugar binding has been demonstrated in an F-type lectin from the Japanese horseshoe crab *Tachypleus tridentatus*. Like many invertebrates, the horseshoe crabs hemolymph fluid, containing solutes and hemocytes, bathes the internal organs. The horseshoe crab hemocytes are a single type of granular cell, which mounts an innate immune response upon recognition of bacterial LPS. Tachylectin-4 from horseshoe crab hemocyte has Ca^{2+} dependendent hemagglutinating activity against human A-type erythrocytes and was more potent than hemocyte lectin with an affinity to *N*-acetylglucosamine, tachylectin-2. The tachylectin-4 is an oligomeric glycoprotein of 470 kDa, composed of subunits of 30 and 320.5 kDa. The activity was inhibited by L-Fucose, *N*-acetylneuraminic acid and bacterial S-type LPS but not by R-type LPS lacking O-antigen. Colitose (3-deoxy-L-fucose), a unique sugar present in the O-antigen of *E. coli* O111:B4 with structural similarity to L-fucose, is the most probable candidate for a specific ligand of tachylectin-4. The ORF of 1344-bp cDNA coding for tachylectin-4 coded for the mature protein with 232 amino acids. Tachylectin-4 is homologous to the

NH2-terminal domain with unknown functions of *Xenopus laevis* pentraxin 20.

20.3.2 F-type Lectins from *Drosophila melanogaster*

Furrowed and CG9095 from *Drosophila melanogaster* contain single F-type domains within an architecture that also includes a C-type lectin-like domain and a number of complement control repeats. These proteins, which have homologs in other insects (e.g. bee and mosquito) are the only F-type proteins which have transmembrane domains. Signaling by these receptors is influenced by O-fucosylation, but the F-type domains in the receptors do not contain complete His/Arg/Arg triads (the C-type lectin-like domain in these receptors is also unlikely to bind sugar). It has been stated that F-type domains are not present in the nematode worm *Caenorhabditis elegans*, but a highly divergent F-type domain, which is very unlikely to bind fucose, is present in the CG9095 orthologue, C54G4.4. The divergence of F-type domains between CG9095 proteins in different species is suggestive of a non-sugar-binding function of the F-type domain in these receptors.

20.3.3 F -Type Lectins in Sea Urchin

In the sea urchin *Strongylocentrotus purpuratus*, a single F-type domain is present in complex protein architectures, as well as in a simple single-domain fucolectin. The complex proteins include CRL, which is involved in the complement system, and a protein containing a CCP and an EGF domain, both of which have very similar F-type domains, and a protein containing scavenger receptor Cys-rich, Kringle and other domains. F-type domains in the sea urchin are distinctive due to a number of insertions and the absence of fucose-binding His residue and adjacent cysteines. The sea urchin lectin from *Toxopneustes pileolus* is galactose and fucose specific. Incubation of rat peritoneal mast cells with this lectin in presence of CaCl2 inhibited the histamine release induced by GlcNAc-specific *Datura stramonium* agglutinin (DSA). It is suggested that the lectin binds to D^{+}-Gal residues of DSA to interfere with mast cell activation induced by DSA, a glycoprotein with arabinose and Gal residues (Suzuki-Nishimura et al. 2001). Discoidin I and discoidin II are N-acetylgalactosamine (GalNAc)-binding proteins from *Dictyostelium discoideum*. They consist of two domains: an N-terminal discoidin domain and a C-terminal H-type lectin domain. The N-terminal discoidin domain presents a structural similarity to F-type lectins such as eel agglutinin, where an amphiphilic binding pocket suggests possible carbohydrate-binding activity (Mathieu et al. 2010).

20.3.4 Bindin in Invertebrate Sperm

In free-spawning, invertebrate's sperm–egg incompatibility is a barrier to mating between species, and divergence of gamete recognition proteins (GRPs) can result in reproductive isolation. Bindin is a major protein for species-specific recognition between sperm and congenetic egg during fertilization in many free-spawning marine invertebrates. Bindins have identical 24-residue signal peptides and conserved 97-residue N-terminal sequences, and they differ in mass because of the presence of between 1 and 5 tandemly repeated 134-residue fucose-binding lectin (F-lectin) domains. Oyster bindin is a single copy gene, but F-lectin repeat number and sequence are variable within and between individuals. Eight residues in F-lectin fucose-binding groove are subject to positive diversifying selection, indicating a history of adaptive evolution at lectin's active site. There is one intron in middle of each F-lectin repeat, and recombination in this intron creates many combinations of repeat halves. Alternative splicing creates many additional size and sequence variants of the repeat array. Males contain full-length bindin cDNAs of 5 possible sizes, but only one or two protein mass forms exist in each individual. Sequence analysis indicates that positive selection, alternative splicing, and recombination can create thousands of bindin variants within *C. gigas*. The extreme sequence variation in F-lectin sequence of oyster bindin within-species is a novel finding (Springer et al. 2008). The full-length bindin cDNA from oyster *Crassostrea angulata* comprises 1,049 bp with a 771-bp ORF encoding 257 amino acids. The deduced amino acid sequence contained a putative signal peptide of 24 amino acids. The length of the bindin genomic DNA was 8,508 bp containing four exons and three introns. Three haplotypes of F-lectin repeat were detected from seven sequences of F-lectin repeat of six male oysters. Intron-4 may play an important role in recombination. The amount of intraspecific polymorphism in male GRPs may be a consequence of the relative efficiency of local (molecular recognition) and global (electrical, cortical, and physical) polyspermy blocks that operate during fertilization (Moy et al. 2008; Wu et al. 2010).

20.4 F-type Lectins in Plants

The fucose binding proteins extracted from Lotus tetragonolobus seeds consists of two distinct classes of components which correspond to tetrameric glycoproteins of 118–120 kDa with potent temperature dependent

hemagglutinating activity and a highly aggregated dimeric component of 58 kDa with macrophage activating properties (Leu et al. 1980). Immobilized fucose-binding lectin Lotus tetragonolobus agglutinin (LTA1), also known as lotus lectin LTA is effective in isolating glycans containing the Le^x antigen and is useful in analyzing specific fucosylation of glycoconjugates (Yan et al. 1997). A well ordered, two-dimensional lattice is formed from fucose-specific isolectin A from Lotus tetragonolobus cross-linked with difucosyllacto-N-neohexaose, an oligosaccharide possessing the Le^x determinant, which is an oncofetal antigen. Using the symmetry and dimensions of the lattice and its appearance in filtered electron micrographs, molecular models were used to determine the orientation of the lectin in the lattice, and to define the range of lectin-oligosaccharide interactions consistent with the structural data (Cheng et al. 1998). Detailed discussion on plant lectins is beyond scope of this book.

20.5 F-type Lectins in Bacteria

The F-type domain is found in a diverse type of bacterial species, in a variety of architectural contexts. Bacterial F-type domains frequently conserve the fucose binding motif, but they do not include disulfide bonds. Mutagenesis experiments have suggested that genes encoding putative glycoside hydrolases are necessary for full virulence of gram positive *S. pneumonia* in a murine lung model of pneumonia. One such gene (SP2159) of *S. pneumoniae* TIGR4 is "fucolectin-related protein" but here called SpGH98. This gene is part of a fucose utilization operon that is conserved among three sequenced strains of *S. pneumoniae*. This protein is a 1038-amino acid protein of which the ~600 amino acid N-terminal region shows amino acid sequence identity with family glycoside hydrolase 98 (GH98). Following GH98 catalytic module is a triplet of modules (Fig. 20.4) which have ~ 50–60 % identity with one another and ~25 % amino acid sequence identity to AAA (Bianchet et al. 2006). This protein contains a C-terminal triplet of fucose binding modules that have significant amino acid sequence identity with the Anguilla anguilla fucolectin. Boraston et al. (2006) dissected the modular structure of SpGH98 by heterologous production of two single modules (SpX-1 and SpX-3), a tandem construct (SpX-20.2) and the triplet (SpX-20.2.3). Functional studies of these fucose binding modules reveal binding to fucosylated oligosaccharides and suggest the importance of multivalent binding. The crystal structures of ligand bound forms of one fucose binding module uncovered the molecular basis of fucose, ABH blood group antigen, and LeY antigen binding. These studies, extended by fluorescence microscopy, showed specific binding to mouse lung tissue

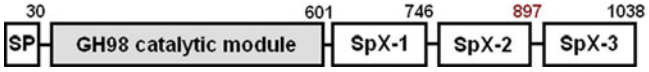

Fig. 20.4 Modular arrangement of SpGH98. The number above the box shows the number of amino acid residue that defines the module boundaries. SP denotes the signal peptide (Boraston et al. 2006)

and defined a new family of carbohydrate binding modules (CBM) now classified as family 47 (Boraston et al. 2006).

Among other Gram-positive bacteria, an F-type domain is found in a protein from *Solibacter usitatus* Ellin6076, combined with a number of bacterial immunoglobulin-like domains and FG-GAP repeats. A lectin is associated with the bacterial cell surface of Rhizobium lupini strain LL13 with a Mr ~19,000. This protein specifically aggregated L-Fuc-BSA-coated microspheres (Wisniewski et al. 1994). Among Gram-negative purple bacteria, an F-type domain follows the structurally similar coagulation factor V/VIII domain in a protein from Saccharophagus degradans 2–40, an organism which degrades a range of polysaccharide substrates, whereas in *Acidiphilium cryptum* JF-5 an F-type domain is present at the C-terminus of the fucolectin tachylectin-4 pentraxin-1 protein, and in Gluconobacter oxydans 621 H F-type domains are present in the proteins GOX0967 and GOX0982. Among bacteria of the planctomycetes phylum, an F-type domain is present at the centre of a large protein that is a periplamic component of a sugar transport system in *Blastopirellula marina* DSM 3645, and a more distantly related domain is present in a probable cytochrome c precursor (NPβ868124) in *Rhodopirellula baltica* SH 20.

PA-IIL is a fucose-binding lectin from *Pseudomonas aeruginosa* that is closely related to the virulence factors of the bacterium and has high affinity for the monosaccharide ligand. Structural studies revealed a new carbohydrate-binding mode with direct involvement of two calcium ions (Mitchell et al. 2002). The crystal structure of the tetrameric PA-IIL in complex with fucose and calcium allowed a proposal which suggested hydrogen-bond network in the binding site. Computational methods indicated that extensive delocalization of charges between the calcium ions, the side chains of the protein-binding site and the carbohydrate ligand is responsible for the high enthalpy of binding and therefore for the unusually high affinity observed for this unique mode of carbohydrate recognition (Mitchell et al. 2005). Mutagenesis of amino acids forming the specificity binding loop allowed identification of one amino acid that is crucial for definition of lectin sugar preference. Altering specificity loop amino acids causes changes in saccharide-binding preferences of lectins derived from PA-IIL, via creation or blocking possible binding interactions (Adam et al. 2007).

The fucose-specific lectin LecB is implicated in tissue binding and biofilm formation by the opportunistic pathogen

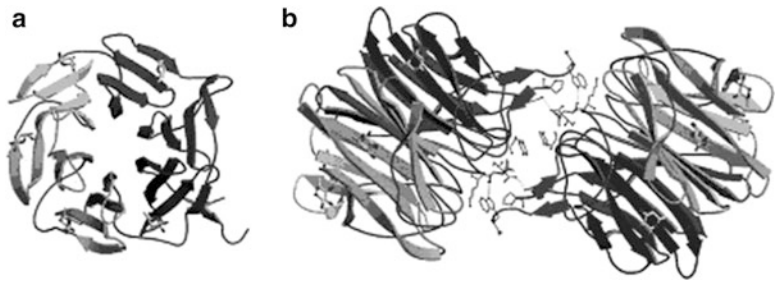

Fig. 20.5 (**a**) Ribbon diagram of monomer A of AAL complexed with fucose shown as sticks. (**b**) Dimer of AAL with stick representation of the amino acids involved in the interaction of monomers (Adapted with permission from Wimmerova et al. 2003 © The American Society for Biochemistry and Molecular Biology)

Pseudomonas aeruginosa, which causes severe respiratory tract infections mainly in immuno-compromised patients or cancer patients undergoing chemotherapy (Kolomiets et al. 2009). The plant pathogen *Ralstonia solanacearum*, which causes lethal wilt in many agricultural crops, produces a potent L-fucose-binding lectin (RSL: subunit Mr 9.9 kDa) exhibiting sugar specificity similar to that of PA-IIL of the human aggressive opportunistic pathogen *Pseudomonas aeruginosa* (Sudakevitz et al. 2002). The properties of the first lectin are related to fungal lectins. The second one, RS-IIL (subunit Mr 120.6 kDa) is a tetrameric lectin with high sequence similarity to fucose-binding lectin PA-IIL of *Pseudomonas aeruginosa*. RS-IIL recognizes fucose but displays much higher affinity to mannose and fructose, which is opposite to the preference spectrum of PA-IIL (Sudakevitz et al. 2004).

20.6 Fuco-Lectins in Fungi

20.6.1 Fuco-Lectin from Aleuria Aurantia (AAL)

A fucose-binding lectin was purified from fruiting bodies of *Aleuria aurantia*, **a** widespread ascomycete fungus. The lectin combines the terminal fucose in the carbohydrate chain. AAL shows sugar-binding specificity for L-fucose. Recombinant form of AAL is a fucose-binding lectin composed of two identical 312 aa subunits (Kochibe and Furukawa 1980). Each subunit contains five binding sites for fucose. One of the binding sites in rAAL had unusually high affinities towards fucose and fucose-containing oligosaccharides with K_D values in the nM range. (Olausson et al. 2008). Isolectin A from *Lotus tetragonolobus* (LTL-A), and AAL were found to be specific for fucose residues. While α-L-Fuc-(1,3)-β-D-GlcNAc and Lewis^x (Le^X) bound to both lectins, sialyl-Lewis^x (sLe^X) bound to AAA only. AAA bound to the ligands more tightly. Binding strength for both lectins decreased from α-L-Fuc-(1,3)-β-D-GlcNAc to Le^X and was lowest for sLe^X. STD NMR experiments suggest that only the L-fucose residues are in intimate contact with the protein (Haselhorst et al. 2001).

AAL: Overall Fold and Organization: The crystal structure of the lectin complexed with fucose revealed that each monomer consists of a six-bladed β-propeller fold and of a small antiparallel two-stranded β-sheet that plays a role in dimerization. Five fucose residues were located in binding pockets between the adjacent propeller blades (Fig. 20.5a) (Paoli 2001). The global shape is a short cylinder, or tore, with an approximate diameter of 45 Å and a height of 35 Å. In the β-propeller fold, each consecutive β-sheet has its first strand (number 1) lining the central cavity of the protein and the last one (number 4) most exposed to solvent at the cylinder surface. Loops connecting the strands within each module are rather short, with the exception of blade III (amino acids 108–162) that displays longer loops. The consecutive blades are connected by long segments that run from the outside of the protein to the central tunnel. AAL has been described as a dimer in solution and also confirmed as a dimer in crystal. The two monomers are very similar, and superimposition of their backbones gives an r.m.s. value of 0.26 Å. A pseudo-2-fold axis of symmetry generates this dimer in the crystal (Fig. 20.5b). The small domain created by the antiparallel association of the N-terminal and C-terminal peptides plays a key role in the dimerization, additional contact being mediated by four loops (those interconnecting blades I and II and blades II and III and the loops between strands β_2-I and β_3-I and between strands β_2-II and β_3-II). Hydrophobic contacts involve the C terminus Trp^{312} from each monomer with Lys^{83} from the other. In addition, tyrosine residue, Tyr^6, interacts via aromatic ring stacking with its counterpart on the other monomer through 2-fold axis. Four main hydrogen bonds are also established between the side chains of Asp^{263} and Ser^{283} and between the Trp^{312} nitrogen side chain and backbone carbonyl backbone of Leu^{59}. Several amino acids at the N and C termini of

the peptide chains protrude from the base of the β-propeller cylinder, associating in a small anti-parallel two-stranded β-sheet that forms a separated domain. The inner cavity of AAL has a tunnel shape with a diameter of about 8 Å in its middle part and almost closed off on the N terminus side of the first inner β-strands. This cavity has a strong hydrophobic character, being formed mostly by the conserved alanine residues of the first strands of each propeller blade. The core is filled with a set of about 50 water molecules forming a well ordered hydrogen bond network.

Due to repeats in the amino acid sequence, there are strong similarities between the sites. Oxygen atoms O-3, O-4, and O-5 of fucose are involved in hydrogen bonds with side chains of amino acids conserved in all repeats, whereas O-1 and O-2 interact with a large number of water molecules. The nonpolar face of each fucose residue is stacked against the aromatic ring of a Trp or Tyr amino acid, and the methyl group is located in a highly hydrophobic pocket. Depending on the precise binding site geometry, the alpha- or beta-anomer of the fucose ligand is observed bound in the crystal. Experiments conducted on a series of oligosaccharides confirmed the broad specificity of the lectin, with a slight preference for alphaFuc1-2Gal disaccharide. This multivalent carbohydrate recognition fold is a new prototype of lectins that is proposed to be involved in the host recognition strategy of several pathogenic organisms including not only the *Aspergillus* but also the phytopathogenic bacterium *Ralstonia solanacearum* (Wimmerova et al. 2003).

20.7 Intelectins

20.7.1 Intelectin-1 (Endothelial Lectin HL-1/ Lactoferrin Receptor or *Xenopus* Oocyte Lectin)

The murine gene encoding Intelectin (ITLN) was identified by Komiya et al. (1998) and was renamed Intelectin-1 (ITLN-1), following the isolation of Intelectin-2 by Lee et al. (2001). Murine intelectin-1 was called as HL-1 (Endothelial lectin HL-1) and suggested it to be a human homolog of *Xenopus* oocyte lectin XL-35. The protein has been identified independently as Omentin of adipose tissue. The protein is structurally identical to the intestinal receptor for lactoferrin (Wrackmeyer et al. 2006). The cDNA was cloned by Suzuki et al. (2001) and called LFR [Lactoferrin (LF) receptor (R)].

The mature human intelectin-1 (hITLN-1) is a secretory glycoprotein consisting of 295 amino acids and *N*-linked oligosaccharides, and its basic structural unit is a 120-kDa homotrimer in which 40-kDa polypeptides are bridged by disulfide bonds. The *hITNL* gene was split into 8 exons on chromosome 1q21.3. The hITNL showed high levels of homology with mouse intelectin, *Xenopus laevis* cortical granule lectin/oocyte lectin, lamprey serum lectin, and ascidian galactose-specific lectin. These homologues commonly contained no CRD, which is a characteristic of C-type lectins, although some of them have been reported as Ca^{2+}-dependent lectins. Recombinant hITNL revealed affinities to D-pentoses and a D-galactofuranosyl residue in presence of Ca^{2+}, and recognized bacterial arabinogalactan of *Nocardia* containing D-galactofuranosyl residues. These results suggested that hITNL is a galactofuranose binding lectin that plays a role in the recognition of bacterial- specific components in the host, and is not a member of C-type lectin family (Tsuji et al. 2001).

Galactofuranosyl residues, which are not found on mammalian tissues, are recognized as dominant immunogens (Daffe et al. 1993; Leitao et al. 2003). The hITLN-1 is expected to recognize not only *N. rubra* (Daffe et al. 1993), but also *Aspergillus fumigatus* (Leitao et al. 2003), *Mycobacterium tuberculosis* (Pedersen and Turco 2003), *Streptococcus oralis* (Abeygunawardana et al. 1991), *Leishmania major*, and *Trypanosoma cruzi* (Suzuki et al. 1997), all of which contain galactofuranosyl residues in cell walls. Some observations indicate that hITLN-1 and mouse intelectin-1 (mITLN-1) may play an immunological role against selected microorganisms or foreign antigens (Pemberton et al. 2004a, 2004b; Datta et al. 2005; Kuperman et al. 2005; Wali et al. 2005). Since intelectin homologues are generally oligomeric (Abe et al. 1999; Suzuki et al. 2001; Tsuji et al. 2001; Nagata 2005), this structure may influence their function.

Most mammalian ITLNs have 10 conserved Cys residues but two of these are not found in the N-terminal regions of mouse and rat ITLNs. The recombinant hITLN-1 is a trimer, disulfide-linked through Cys-31 and Cys-48, and N-glycosylated at Asn-163. Despite 84.9 % amino acid identity to hITLN-1, recombinant and intestinal mITLN-1 are unglycosylated 30-kDa monomers. Elution profiles of recombinant hITLN-1, as well as recombinant and intestinal mITLN-1 indicated that the two intelectins have different saccharide-binding specificities. Despite 84.9 % amino acid identity between these two proteins, the oligomeric structure and carbohydrate-recognition specificity of mITLN-1 differ from those of hITLN-1 (Tsuji et al. 2007). In mammals, the galactose-binding C-type lectins, a macrophage lectin or asialoglycoprotein receptors, bind to asialoglycoproteins. However, the binding affinity of hITNL-1 to asialoglycoproteins was weak (Tsuji et al. 2001). This was thought to be because the galactosyl residues of asialoglycoproteins are galactopyranosides although hITNL has affinity to the galactofuranosyl residue.

The hITLN-1 mRNA level increases in airway epithelial cells from individuals with asthma (Kuperman et al. 2005). A high percentage of malignant pleural mesothelioma show

up-regulated expression of hITLN-1 (Wali et al. 2005). A marked induction in Intelectin gene expression was observed among human primary mesothelial cells as a consequence of crocidolite asbestos exposure and simian virus 40 infection. However, expression of hITNL-1 is decreased in human airway epithelium of smokers compared to nonsmokers (Carolan et al. 2008). Others have shown that infection with *T. spiralis* (Pemberton et al. 2004a, 2004b) or *Trichuris muris* (Datta et al. 2005) induced mITLN-1 and mITLN-2 mRNA expression. These observations and the galactofuranose binding by hITLN-1 and mITLN-1 suggest that ITLN homologues may have an immunoregulatory role in host defense. For instance, the serum ITLN homologue of ascidian (galactose-specific lectin) functions as an opsonin (Abe et al. 1999). However, Voehringer et al. (2007) indicated no significant modification of immune responses or clearance of microorganisms in mITLN transgenic mice. Although hITLN-1 is a soluble intestinal protein (Tsuji et al. 2001), hITLN-1 is also a glycosylphosphatidylinositol-anchored intestinal lactoferrin receptor (Suzuki et al. 2001), HL-1 of vascular endothelial cells (Lee et al. 2001), or omentin of adipose tissue (Schäffler et al. 2005) and human visceral fat (Tsuji et al. 2007). However, the physiological function of mITLN-1 may differ from that of hITLN-1. It was reported that swine ITLN, which is expected to be oligomeric, is associated with lipid rafts on the enterocyte brush border (Wrackmeyer et al. 2006). It is possible that hITLN-1 is similarly located. Additional investigation will be required to further establish the physiological function of ITLN-1 in both humans and mice.

Human hITLN-1 mRNA is expressed in heart, small intestine, colon, and thymus and exclusively localized in endothelial cells (Lee et al. 2001). Primary cultures of human aortic endothelial cells are positive for HL-1 expression, but several other human cell types are not. The brush border membrane is organized in stable glycolipid-based lipid raft microdomains, and like divalent lectin galectin-4, intelectin was enriched in microvillar "superrafts", i.e., membranes that resist solubilization with Triton X-100 at 37º C. Wrackmeyer et al. (2006) reported that trimeric Intelectin serves as an organizer and stabilizer of the brush border membrane, preventing loss of digestive enzymes to the gut lumen and protecting the glycolipid microdomains from pathogens.

Intelectin is expressed in Paneth and goblet cells of small intestine and serves a protective role in the innate immune response to parasite infection (Komiya et al. 1998; Peebles 2010; Wrackmeyer et al. 2006). Secretory granules of lysozyme-positive Paneth cells in the bottom of crypts as well as goblet cells along crypt-villus axis were intensively labeled with intelectin antibodies, but quantitatively, the major site of intelectin deposition was the enterocyte brush border. Tsuji et al. (2009) found that human intelectin-1 is a serum protein and binds to *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Human ITLN-1-binding to BCG was inhibited by Ca^{2+}-depletion, galactofuranosyl disaccharide, ribose, or xylose, and was dependent on the trimeric structure of human ITLN-1. Human ITLN-1-transfected cells express ITLN-1 on the cell surface and secrete it in the culture supernatant. Study indicates that intelectin is a host defense lectin that assists phagocytic clearance of microorganisms.

As the *X. laevis* oocyte lectin, a homologue of hITNL, has been shown to participate in the formation of fertilization envelope that blocks sperm entry, hITNL may also participate in fertilization. However, hITNL was also expressed on various tissues other than oocytes, and it has been demonstrated that the other homologues are also present in various tissues (Komiya et al. 1998; Abe et al. 1999). Thus, hITNL and its homologues may not only participate in the formation of fertilization envelope but also have other physiological functions.

The hITLN is plentifully expressed in heart. Since *viridans streptococci* invading blood attack heart and cause subacute infectious endocarditis, and since the surface polysaccharide of *Streptococcus oralis* contains galactofuranosyl residues (Abeygunawardana et al. 1991), hITNL may function in heart as a defense protein against these pathogens (Komiya et al. 1998).

20.7.2 Intelectin-2 (HL-2) and Intelectin-3

Intelectin-2 shows 91 % sequence homology with Intelectin, later named Intlectin-1 (Pemberton et al. 2004). Intelectin-2 also called HL-2 (Endothelial lectin HL-2) was identified as the human homologue of *Xenopus* oocyte lectin XL-35 (Lee et al. 2001). The Intelectin-2 gene is absent from C57BL/10 genome but present in genome of BALB/c mice. The protein is expressed by intestinal goblet cells, and its expression is up-regulated after infection with the nematode parasite *Trichinella spiralis*. BALB/c mice, which express Intelectin-2 are resistant to this nematode, whereas C57BL/10 mice, which do not express Intelectin-2 are susceptible to *Trichinella spiralis* infection. The deduced amino acid sequence of each homologue of HL-1 and HL-2 is about 60 % identical and 80 % similar to that of XL35, and none of these sequences contains the C-type lectin motif, although it is known that XL35 requires calcium for ligand binding.

Sheep Intlectins: Intelectin 2 (sITLN2) from sheep abomasal mucosa shared 76–83 % homology with other mammalian intelectins. Expression of sheep abomasal ITLN2 mRNA was up-regulated on post-challenge of worm-free sheep with *Teladorsagia circumcincta*. Infection with *T. circumcincta* was also associated with increased levels of abomasal transcripts encoding sheep mast cell

protease-1, ovine galectin-14 and IL4, which indicated a Th2 type response. The amino acid sequences of sheep sITLN1 and sITLN3 share 86 % and 91 % homology with sheep sITLN2 respectively. While sITLN1 and sITLN3 transcripts are expressed ubiquitously in most of the normal sheep tissues, sITLN2 transcript was restricted to the abomasal mucosa in normal sheep tissues. The three sITLN were absent in unchallenged naïve lambs but induced in abomasal mucosa following challenge of both *Teladorsagia circumcincta* and *Dictyocaulus filaria* natural infection suggesting that intelectins may play an important role in mucosal response to nematode infections in ruminants (French et al. 2009).

Intelectins are capable of binding bacteria via galactofuranose residues and function as intestinal receptors for the antimicrobial glycoprotein lactoferrin (Lf). Lf binds strongly to enterohemorrhagic *E. coli* (EHEC) and the Lf receptor expressed in terminal rectum, the site of predilection of EHEC in cattle. Bovine intelectins (bITLN1 and bITLN2) were expressed in abomasum and rectum, but expression appeared minimal in the jejunum. There was significantly higher expression of bITLN2 in terminal rather than proximal rectum. Thus two bovine ITLNs are expressed along with Lf in the gastrointestinal tract, where they may interact with microbial pathogens (Blease et al. 2009).

20.7.3 Intelectins in Fish

Lin et al. (2009) characterized seven zebrafish intelectins (zINTLs) and made comparative analysis of intelectins from various species. zINTL1-3 are highly expressed in one or several adult tissues. zINTL4-7, however, were expressed at quite low levels both in adults and various development stages. Of the seven zINTLs, zINTL3 was expressed predominantly in the liver and highly up-regulated upon infection, suggesting its important roles in immunity. Based on the characterization of intelectin members in various species it was indicated that intelectin family may be a deuterostome specific gene family; and their expression patterns, quaternary structures and glycosylations vary considerably among various species, though their sequences are highly conserved. Moreover, these varied features have evolved multiple times independently in different species, resulting in species-specific protein structures and expression patterns (Lin et al. 2009). Rainbow trout plasma Intelectin exhibited calcium-dependent binding to N-acetylglucosamine (GlcNAc) and mannose conjugated Toyopearl Amino 650 M matrices. The lectin appeared at ~37 kDa and ~ 72 kDa bands. Similar analysis of plasma revealed a single 72 kDa band under reducing conditions. MALDI-TOF MS demonstrated five, ~37 kDa isoforms (pI 5.3–6.1). A 975 bp cDNA sequence encoded a 325 amino acid secretory protein with homology to human and murine intelectins, which bind bacterial components and were induced during parasitic infections. Rainbow trout IntL was detected ubiquitously in many tissues. Rainbow trout IntL plays a role in innate immune defense against bacterial and chitinous microbial organisms (Russell et al. 2008).

Two types of IntL genes have been identified from catfish. The genomic structure and organization of IntL2 were similar to those of the mammalian species and of zebrafish and grass carp, but orthologies could not be established with mammalian IntL genes. The IntL genes are highly conserved through evolution. Sequence analysis also indicated the presence of the fibrinogen-related domain in the catfish IntL genes. Phylogenetic analysis suggested the presence of at least two prototypes of IntL genes in teleosts, but only one in mammals. The catfish IntL1 gene is widely expressed in various tissues, whereas the channel catfish IntL2 gene was mainly expressed in the liver. While the catfish IntL1 is constitutively expressed, the catfish IntL2 was drastically induced by i.p. injection of *Edwardsiella ictaluri* and/or iron dextran. While IntL1 was expressed in all leukocyte cell lines, no expression of IntL2 was detected in any of the leukocyte cell lines, suggesting that the up-regulated channel catfish IntL2 expression after bacterial infection may be a consequence of the initial immune response, and/or a downstream immune response rather than a part of the primary immune responses (Takano et al. 2008).

20.7.4 Eglectin (XL35) or Frog Oocyte Cortical Granule Lectins

Xenopus Laevis Egg Cortical Granule: *Xenopus laevis* egg cortical granule, calcium-dependent, galactosyl-specific lectin participates in forming the fertilization layer of the egg envelope and functions in establishing a block to polyspermy. This oligomeric lectin is released extracellularly at fertilization and binds to its polyvalent glycoprotein ligand that is cross-linked in the jelly coat layer surrounding the oocyte. The lectin is expressed again at gastrulation and may function as well in cell–cell or cell–matrix adhesion events in the embryo (Outenreath et al. 1988; Lee et al. 1997). A cDNA encoding XL35 was isolated from a *Xenopus* oocyte cDNA library (Lee et al. 1997). The amino acid sequence it encodes did not display the C-type lectin motif, although it does require calcium for binding. The cDNAs encoding two human homologues of the *Xenopus* oocyte lectin, XL35, were isolated from a small intestine cDNA library and termed HL-1 and HL-2. The deduced amino acid sequence of each homologue is about 60 % identical and 80 % similar to that of XL35, and none of these sequences contains the C-type lectin motif, although XL35 requires calcium for ligand binding. HL-1 transcripts

are present at relatively high levels in heart, small intestine, colon, thymus, ovary, and testis. HL-2 transcripts, by contrast, are expressed only in small intestine (Lee et al. 2001).

The translated cDNA for the cortical granule lectin had a signal peptide, a structural sequence of 298 amino acids, a molecular weight of 32.7 kDa, contained consensus sequence sites for N-glycosylation and a fibrinogen domain. The lectin cDNA expressed during early stages of oogenesis and 2/3 of the lectin was associated with the extracellular perivitelline space and the egg/embryo fertilization envelope. Lectin mRNA levels were from 100- to 1000-fold greater in ovary than in other adult tissues. The lectin did not have sequence homology to any known lectin families but had 41–88 % amino acid identity with nine translated cDNA sequences from an ascidian, lamprey, frog, mouse, and human. Based on the conserved carbohydrate binding and structural properties, it was named as eglectin (Chang et al. 2004).

Several nucleic acid sequences that predict proteins homologous to XL35 have been reported in frog, human, mouse, lamprey, trout, ascidian worm. These proteins also showed high degrees of amino acid sequence homology to a common fibrinogen-like motif that may involve carbohydrate binding. Several independent studies on these lectins strongly suggest that the lectins are expressed and stored in specialized vesicles that may be released upon infection by pathogens. In addition, some family members have been shown to bind to oligosaccharides from bacterial pathogens. Therefore, this family of lectins likely participates in pathogen surveillance as part of the innate immune system. These were named as homologues of XL35 (Lee et al. 2004).

Xenopus Embryonic Epidermal Lectin (XEEL): The *Xenopus laevis* embryonic epidermal lectin (XEEL) belong to the group of mammalian intelectins, frog oocyte cortical granule lectins, and plasma lectins in lower vertebrates and ascidians. A cDNA from a *Xenopus laevis* embryo library encodes a predicted translation product of 342 amino acids containing a signal sequence for secretion. The predicted protein has 62–70 % amino acid identity with the *Xenopus* oocyte cortical granule lectin (XCGL), the mouse intelectin, the human HL-1/intelectin and HL-2. Onset of gene expression occurs by gastrulation and the transcripts localize in non-ciliated epidermal cells all over the tail bud embryos. The designated XEEL is secreted from the embryonic epidermis (Nagata et al. 2003).

XCL-1: Ishino et al. (2007) isolated a 35-kDa Ca^{2+}-dependent lectin (XCL-1) from adult *Xenopus* serum. Although XCL-1 gene was not induced in the regenerating tails, Ishino et al. (2007) isolated a cDNA for an XCL-1-related protein (XCL-2). In contrast to the XCL-1 gene, XCL-2 gene expression was significantly increased in the regenerating tails, suggesting its role in tail regeneration. Although both XCL-1 and XCL-2 belong to *Xenopus* lectin family (X-lectins), XCL-1 and XCL-2 exhibit distinct developmental gene expression from two other known X-lectin members, both of which are expressed in the embryonic stage, whereas the XCL-1 and XCL-2 genes are predominantly expressed in the adult and middle/late tadpole stages, respectively, suggesting multiple functions of X-lectins. A gene for a humoral C-type lectin family is transiently expressed in the regenerating legs of the American cockroach (Arai et al. 1998). It suggests that the induction of a gene in regenerating organs is conserved among insects and vertebrates.

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Fuco-Lectins

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Annexins (Lipocortins)

21

G.S. Gupta

21.1 Annexins

21.1.1 Characteristics of Annexins

The annexins or lipocortins are a multigene family of proteins that bind to acidic phospholipids and biological membranes in a Ca^{2+}-dependent manner (Gerke and Moss 2002; Gerke et al. 2005; Raynal and Pollard 1994; Swairjo and Seaton 1994). Annexins are ubiquitous and characterized by an ability to bind to anionic phospholipids at membrane surfaces in response to elevated Ca^{2+}. Annexins are amphipathic and distinct from soluble and integral membrane proteins, but share features of both (Kojima et al. 1994; Brisson et al. 1991). Annexins have molecular weights ranging between 30 and 40 kDa (only annexin 6 is 66 kDa) and possess striking structural features. The characteristic annexin structural motif is a 70-amino-acid repeat, called the annexin repeat. Four annexin repeats packed into an α-helical disk are contained within the C-terminal polypeptide core (Gerke and Moss 2002). While all annexins share this core region, aminoterminal domains of annexins are diverse in sequence and length (ranging from 11 to 196) on each annexin member. It is this diversity of N-terminal amino-acid sequence that gives the individual annexins their functional differences and biological activities and appears to differentiate the cellular function and location (Gerke and Moss 2002; Gerke et al. 2005; Raynal and Pollard 1994). Cysteine 198 is relatively conserved in annexins, and three of four cysteines (198, 242, and 315) in annexin A4 are conserved in annexin A3. Phospholipids are suggested to bind via hydrophilic head groups to annexins, and the phospholipid-binding region is proposed to be localized on the convex surface side where calcium-binding sites are located in the crystal structure of annexin 5 (Huber et al. 1990). The calcium- and phospholipid-binding sites are located in the carboxy terminal domains. Some of the annexins bind to glycosaminoglycans (GAGs) in a Ca^{2+}- dependent manner. While annexin 2 has specific and high-affinity heparin-binding activity (Kassam et al. 1997), annexin A4 binds to heparin, heparan sulfate and chondroitin sulfate (CS) columns in a Ca^{2+}- dependent manner, annexin 5 to heparin and heparan sulfate columns in a Ca^{2+}-dependent manner and annexin 6 to heparin and heparan sulfate columns in a Ca^{2+}-independent manner and to CS columns in a Ca^{2+}-dependent manner (Ishitsuka et al. 1998) (see Table 21.1). Reports suggest that some annexin species may function as recognition elements for L-α-dipalmitoylphosphatidylethanolamine (PE)-derivatized GAGs under some conditions. The crystal structure of several of the annexins has been reported (Favier-Perron et al. 1996; Luecke et al. 1995; Swairjo et al. 1995). It has been established that the annexins are composed of two distinct sides. The convex side faces the biological membrane and contains the Ca^{2+}- and phospholipid-binding sites. The concave side faces the cytosol and contains the N and C termini. Although the annexins have been studied mostly as calcium-dependent phospholipid-binding proteins mediating membrane-membrane and membrane-cytoskeleton interactions, annexins A4, A5 and A6 bind also to carbohydrate structures suggesting that these annexin possess lectin-like domains.

Annexins bind to phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol, which are present in the inner leaflet or cytosolic surface of plasma membrane and hardly appear on cell surface, in contrast to phosphatidylcholin and sphingomyelin, which are major components of the outer leaflet of plasma membrane. However, latter are not recognized by annexins. Annexins also bind to organelle membranes such as the Golgi apparatus. This binding can be reversed by the removal of calcium, freeing the annexin from the phospholipid membrane. However, the functional significance of their reversible membrane-binding ability remains unknown in many annexins, although in some it is thought to be important for vesicle aggregation and membrane organization (Liemann and Huber 1997; Rand 2000; Rescher and Gerke 2004; Lim and Pervaiz 2007). Although all annexins share this binding property, there is variation in calcium sensitivity and phospholipid specificity between

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Table 21.1 Proteins that interact with vertebrate annexins (Adapted from Moss and Morgan 2004, © Genome Biol. 5: 219; 2004)

Annexin	Interacting proteins
ANXA1	Epithelial growth factor receptor, formyl peptide receptor, selectin, actin, integrin A4
ANXA2	Tissue plasminogen activator, angiostatin, insulin receptors, tenascin C, caveolin 1
ANXA3	None known
ANXA4	Lectins, glycoprotein 2
ANXA5	Collagen type 2, vascular endothelial growth factor receptor2, integrin B5, protein kinase C, cellular modulator of immune recognition (MIR), G-actin, helicase, DNA (cytosine-5-) methyltransferase 1 (DNMT1)
ANXA6	Calcium-responsive heat stable protein-28 (CRHSP-28), ras GTPase activating protein, chondroitin, actin
ANXA7	Sorcin, galectin
ANXA8	None known
ANXA9	None known
ANXA10	None known
ANXA11	Programmed cell death 6 (PDCD6), sorcin
ANXA13	Neural precursor cell expressed, developmentally down-regulated 4 (NEDD4)

individual annexins. For example, within one cell there can be differences in the distribution of annexins, with annexin A1 having an endosomal localisation, A2 to be found in cytosol and A4 being associated with the plasma membrane (Liemann and Huber 1997). Furthermore annexins are exported from cytosol to the outside of cells across the plasma membrane by unknown mechanisms. Although annexins lack hydrophobic signal peptides, secretion and expression on cell surface experienced by some annexins are e6dent in some cell types.

Like galectins, certain members of annexin family can be found both inside and outside cells. In particular, annexins A1, A2, A4, A5, and A11 can be found in the nucleus. This localization is consistent with the findings that annexin A1 possesses unwinding and annealing activities of a helicase and that annexin A2 is associated with a primer recognition complex that enhances the activity of DNA polymerase α. Despite these efforts and accomplishments, however, there is little e6dence or information on an endogenous carbohydrate ligand for these lectins that show nuclear and/or cytoplasmic localization (Wang et al. 2004).

21.1.2 Classification and Nomenclature

Annexins are expressed in a wide range of organisms such as higher plants, slime molds, metazoans, insects, birds, and mammals. Studies of the amino acid sequence of the annexins have established the homology of these proteins. Over 20 types have been found in all eukaryotic kingdoms as well as plants and animals. There are 12 human annexin subfamilies (A1–A11 and A13) that have been found to have various intra- and extracellular roles in a range of cellular processes (Gerke and Moss 2002; Gerke et al. 2005). The annexins are classified into five groups (A–E), and within each of these groups, individual annexins are identified numerically. Annexins in group A are human annexins, with group B referring to animal annexins without human orthologs, group C to fungi and moulds, group D to plants and group E to protists (Liemann and Huber 1997; Rand 2000; Hayes and Moss 2004; Rescher and Gerke 2004; Lim and Pervaiz 2007). At least one of the members can be found expressed in nearly every eukaryotic cell types. Almost all cells produce several kinds of annexins simultaniously and the expression levels are rather high. Why do they need annexins in plenty? It may be helpful in defining the question that the task of annexins is not a unique and annexins have to interact with various ligands both outside and inside of cells to act for multiple roles (Ponnampalam and Rogers 2006).

By definition, an annexin protein must be capable of binding in a Ca^{2+}-dependent manner to negatively charged phospholipids and has to contain a conserved structural element the so-called annexin repeat, a segment of some 70 amino acid residues. Molecular structures obtained for a number of annexins over the past decade helped to extend the similarities to the three-dimensional level. Moreover, they defined a hitherto unknown structural fold, the conserved annexin domain, which is built of four annexin repeats packed into a highly α-helical disk, and which now is considered to be a general membrane binding module. The annexin family has grown steadily in 1990s, and with the turn of the century, more than 160 unique annexin proteins have been discovered in more than 65 different species ranging from fungi and protists to plants and higher vertebrates (Fig. 21.1) (Morgan and Fernandez 1997; Morgan et al. 1999). For a detailed discussion of annexin properties, their structural organization, and intracellular as well as tissue distribution, the interested reader is referred to previous review (Gerke and Moss 2002).

The vertebrate A family includes the 13 annexins that make the family in mammals, but the number of annexins may vary in other classes of vertebrates as genes have been gained and lost. Ancient polyploidization events in bony fish, and more recent genome duplications in pseudotetraploid frogs (Xenopus), have duplicated many of the annexin genes. Thus, annexin A1 has undergone two successive duplications to yield up to four copies in some fish, amphibians and birds. Mammalian ANXA6 is a compound gene, probably derived from the fusion of duplicated ANXA5 and ANXA10 genes in early vertebrate evolution. Annexins A7, A8 and A10 have not yet been detected in fish, although genes similar to annexin A7 have been found in earlier-diverging species such as the sea urchin, the earthworm and

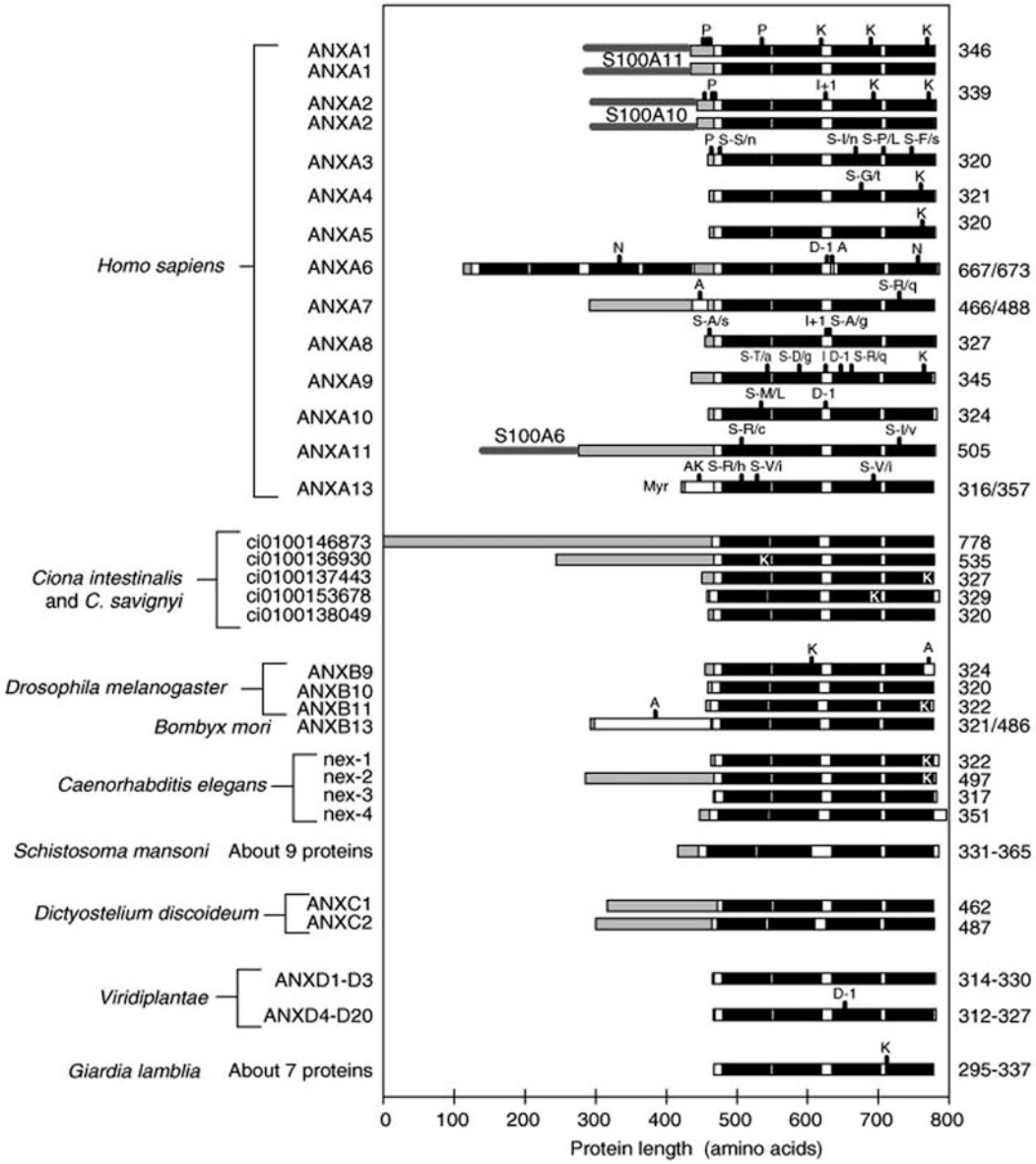

Fig. 21.1 Domain organization of representative annexin proteins: orthologs of the 12 human annexins shown in other vertebrates have the same structures, with strict conservation of the four repeats in the core region (*black*) and variation in length and sequence in the amino-terminal regions (*shaded*). Human ANXA1 and ANXA2 are shown as dimers, with the member of the S100 protein family that they interact with. Domain structures for other model organisms are derived from public data made available by the relevant genome-sequencing projects. Features: *S100Ax* sites for attachment of the indicated member of the S100 family of calcium-binding proteins, *P* known phosphorylation sites, *K*, KGD synapomorphy (a conserved, inherited characteristic of proteins), *I* codon insertions (+x denotes the number of codons inserted), *S-A/b* nonsynonymous coding polymorphisms (SNPs) with the amino acid in the major variant (*A*) and that in the minor variant (*b*), *N* putative nucleotide-binding sites, *D* codon deletions (−x denotes the number of codons deleted), *A* alternatively spliced exons, *Myr* myristoylation. The total length of each protein is indicated on the *right* (Printed from Moss and Morgan, Genome Biol. 5: 219 © 2004)

Hydra. The reasons for the tendency of annexin genes (or their chromosomal regions) to duplicate, their successful preservation, and the extent to which they contribute to vertebrate complexity are as yet unknown. The 12 human annexin genes range in size from 15 kb (*ANXA9*) to 96 kb (*ANXA10*) and are dispersed throughout the genome on chromosomes 1, 2, 4, 5, 8, 9, 10 and 15. Annexin genes from other vertebrates may vary slightly in size and chromosomal linkage, but orthologs are grossly similar in their sequence and splicing patterns (Moss and Morgan 2004).

21.1.3 Annexins in Tissues

Annexins are expressed in a wide range of organisms such as higher plants, slime molds, metazoans, insects, birds,

and mammals. The expression level and tissue distribution of annexins span a broad range, from abundant and ubiquitous (annexins A1, A2, A4, A5, A6, A7, A11) to selective (such as annexin A3 in neutrophils and annexin A8 in the placenta and skin) or restrictive (such as annexin A9 in the tongue, annexin A10 in the stomach and annexin A13 in the small intestine). Annexin A1, A2, A3, A4, A5 and A6 are present in cardiac tissue. Annexin A5 is present in both cardiomyocytes and non-myocytal cells of the heart. Annexin A5 mRNA levels were highest in the fibroblast-like cells, followed by the endothelial cells, and a weak signal in the cardiomyocytes (Jans et al. 1995; Matteo and Moravec 2000). Porcine heart expresses annexins A5 and A6 in large amounts, and annexins A3 and A4 in much smaller amount. Annexins A5 and A6 are involved in the regulation of membrane-related processes (Pula et al. 1990). Annexins play a role in the regulation of Ca^{2+} pumps and exchangers on the sarcolemma, and are altered in some cardiac disease states. The plasma membrane of the heart muscle cell and its underlying cytoskeleton are vitally important to the function of the heart. Two major annexin A6 binding proteins were identified as actin and annexin A6 itself. Annexin A6 bound to itself both in the presence and in the absence of calcium ions. Annexin A6 bound preferentially not only to the N terminal fragment (domains I-4, residues 1–352) but also to C-terminal fragments corresponding to domains V + VI and domains VII + VIII (Locate et al. 2008). During embryonic delopment, annexins 1, 2 and 4 have been identified in murine CNS with distinct patterns of temporal and spatial expression. Annexin A4 is the first annexin to be expressed on embryonic day E9.5 while annexin 1A is the last to be expressed (E21.5) (Hamre et al. 1995).

Annexin A1 is expressed by small sensory neurons of rat dorsal root ganglia (DRG), by most neurons of the spinal cord (SC), and by ependymal cells lining the central canal. Annexin A2 is expressed by most sensory neurons of the DRG but is primarily expressed in the SC by glial cells. Annexin A3 is expressed by most sensory neurons, regardless of size, by endothelial cells lining the blood vessels, and by the perineurium. In the SC, annexin A3 is primarily expressed by astrocytes. In the DRG and the SC, annexin A4 is primarily expressed by glial cells and at lower levels by neurons. In the DRG, annexin A5 is expressed in relatively high concentrations in small sensory neurons in contrast to the SC, where it is expressed mainly by ependymal cells and by small-diameter axons located in the superficial laminae of the dorsal horn areas. Annexin A6 is differentially expressed by sensory neurons of the DRG, being more concentrated in small neurons. In the SC, annexin 6 has the most striking distribution and concentrated subjacent to the plasma membrane of motor neurons and their processes. The differential localization pattern of annexins in cells of the SC and DRG could reflect their individual biological roles in Ca^{2+}-signal transduction within the CNS (Naciff et al. 1996). Annexin A3 is implicated in the microglial response to motor nerve injury. It is induced after hypoglossal nerve injury in rat, specifically in activated (axotomy-stimulated) microglia. A3 was the most prominent among annixins expressed in microglia. Results suggested that Annexin 3 may be a Ca^{2+}-dependent mediator between phospholipids and F-actin in microglia stimulated by peripheral nerve injury (Konishi et al. 2006).

21.1.4 Functions of Annexins

Annexins are generally cytosolic proteins, with pools of both a soluble form and a form stably or reversibly associated with components of the cytoskeleton or proteins that mediate interactions between the cell and the extracellular matrix (matricellular proteins). Although the broad themes in terms of cellular function have been uncovered, but the precise role of these proteins is unclear (Gerke and Moss 2002; Hill et al. 2008). Because of the ability of annexins to bind to and "annex" or aggregate membrane surfaces, they appear to participate in Ca^{2+}-regulated membrane dynamics. Thus they have been shown to be involved in exocytosis (Creutz 1992; Gerke and Moss 2002), membrane domain organization and ion channel activity regulation (Hill et al. 2008).

Some annexins such as A1 and A2, have been found in the nucleus (Tomas and Moss 2003; Eberhard et al. 2001) and in certain instances, annexins may be expressed at the cell surface, despite the absence of any secretory signal peptide; for example, annexin A1 translocates from the cytosol to the cell surface following exposure of cells to glucocorticoids (Solito et al. 1994), and annexin A2 is constitutively expressed at the surface of vascular endothelial cells where it functions in the regulation of blood clotting (Brownstein et al. 2001). The A5 and A6 knockout mice have subtler phenotypes and need further investigation (Brachvogel et al. 2003), and two independently derived A7 null mutant mouse strains are either embryonic lethal (Srivastava et al. 1999) or show changes in calcium homeostasis (Herr et al. 2001). The diversity of phenotype in the annexin knockout mice is consistent with these proteins having largely independent functions. Roles for annexins that have been established from studies using cultured cells are not always reflected in phenotypic abnormalities in the corresponding knockout mice, suggesting that functional redundancy may, in some instances, obscure the full range of functions of these multifunctional proteins. In mice that lack an overt phenotype, there is now the opportunity to test molecular theories of annexin function, such as the proposed calcium channel activity of A5.

The annexins have a wide range of biological functions related to their phospholipid/membrane-binding properties (Crompton et al. 1988), such as inhibition of coagulation, interactions with membranous and cytoskeletal elements, vesicular transport and exocytosis and endocytosis. Shifts in subcellular locations (from the cytosol to membrane) are observed on some intracellular annexins, suggesting the active movement of annexins corresponding to dynamic lipid vesicle transport. Since, the expression of some annexins depends on rate of cellular proliferation (Schlaepfer and Haigler 1990), it has been proposed that the cellular level of the annexins might be critical for the regulation of cell growth. However, this hypothesis has been questioned. Results show that cellular expression of annexins plays a general role in cell growth and support the concept that post-transcriptional mechanisms may control levels of annexin 1 and 7 (Raynal et al. 1997). They are anti-inflammatory proteins that inhibit phospholipase A2 activity in vitro by sequestering the substrate phospholipids from phospholipase A2 (Blackwell et al. 1980). Furthermore, the annexins exhibit anti-coagulant activity (Tait et al. 1989), calcium channel activity (Rojas et al. 1990), and cyclic phosphate phosphohydrolase activity (Ross et al. 1990). They function in the membrane fusion process, exocytosis, endocytosis, membrane-cytoskeleton interactions (Gerke et al. 2005) and regulation of calcium-dependent anion current activation (Chan et al. 1994).

Some annexins are capable of calcium-independent binding and several have roles in vesicle aggregation. Annexins A1, A2 and A11 function in cooperation with other calcium-binding proteins to form complexes while annexins A1, A2 and A5 interact with cytoskeletal proteins. Many annexins are involved in exocytic and endocytic pathways and some have roles in ion channel regulation (Gerke and Moss 2002). Extracellularly, annexin A1 has a role in controlling the inflammatory response while annexin A2 is present on the external surface of endothelial cells, where it may act as a receptor for ligands, including plasminogen and tissue plasminogen activator. Extracellular annexin A5 is thought to be involved in the anticoagulation process (Rand 2000; Hayes and Moss 2004) (see also Table 21.1).

21.2 Annexin Family Proteins and Lectin Activity

After being exported outside the cells, some annexins have been shown to function as receptors for extracellular proteins. There exists evidence that shows that annexins interact with glycoconjugates. Annexin A4 was first identified as a lectin binding to carbohydrate moieties of sialoglycoproteins and glycosaminoglycans in the presence of calcium; afterwards annexin 5 and 6 and some other annexins exhibited similar lectin activity. The localization sites of annexin A4 in major expression tissues, the kidney and pancreas, and the result of in vitro binding assay of several glycoconjugates suggest that annexin A4 is involved in the formation of apical sorting (secretory) vesicles due to the interaction with some GPI-anchored glycoproteins and proteoglycans. Exocrine-type neurotrophic activity is found in annexin 5 and involvement of some annexins in cell-adhesion (or inhibition of cell-adhesion) has been found. Future studies should aim at identifying endogenous glycoconjugate ligands recognized by annexins in a variety of cases and investigating the carbohydrate recognition mechanism. While annexin A4 binds glycosaminoglycans (GAGs) in a calcium-dependent manner (Kojima et al. 1996), annexin A5 bound to heparin and heparan sulfate column but not to chondroitin sulfate column. Annexin A5 binds to collagen and annexin A2 binds to tissue plasminogen activator, tenascin and heparin. Moreover, Annexin A6 was adsorbed to heparin and heparan sulfate columns in a calcium-independent manner and to chondroitin sulfate columns in a calcium-dependent manner. Binding of annexins to a wide variety of other proteins is also known (Moss and Morgan 2004) (Table 21.1). Thus, annexins A2, A4, A5, and A6 have different GAG binding properties and function as recognition elements for GAGs in extracellular space (Ishitsuka et al. 1998). Many annexins have posttranslational modifications, such as phosphorylation and myristoylation. Such modifications and surface remodeling of individual members presumably account for much of the subfamily specificity in annexin interactions (Moss and Morgan 2004).

21.3 Annexin A2 (p36)

Annexin A2/(p36) contains three distinct functional regions, the N-terminal region, the C-terminal region, and the core region. The core region of p36 contains Ca^{2+}- and phospholipid-binding sites, whereas C-terminal region contains 14-3-3 homology domain and the plasminogen-binding domain. The N terminus of annexin A2 (p36) contains two important regulatory domains, the L and P domains. The L domain consists of the first 14 residues of the N terminus that contains a high affinity binding site for the p11 protein. The P domain of p36 contains the phosphorylation sites for protein kinase C (Ser^{25}) and pp60src (Tyr23). The N-terminal L and P domains play regulatory roles; activation of the phosphorylation sites of annexin A2 tetramer results in an increase in the $A_{1/2(Ca2+)}$ for chromaffin granule aggregation and F-actin binding, whereas binding of the p11 subunit decreases the $A_{1/2(Ca2+)}$ for these activities. Annexin A2 is a receptor for the alternatively spliced segment of fibronectin type III domains in tenascin-C (Chung and Erickson 1994; Chung et al. 1996; Ling et al.

2004) and for plasminogen and tissue plasminogen activator (Hajjar et al. 1994).

21.3.1 Annexin 2 Tetramer (A2t)

Annexin A2 often co-expresses with another protein, p11 (S100A10), forming an (annexin A2)$_2$-(S100A10)$_2$ heterotetramer (Annexin 2 tetramer (A2t)). Monomeric and tetrameric annexin A2 share many of the same binding properties, e.g. the abilities to bind Ca^{2+}, phospholipid membranes, and certain GAGs (including heparin and heparan sulfate), albeit typically with some modification in affinity. The p11 component alone, despite belonging to the E-F hand family of Ca^{2+}-binding proteins, does not bind Ca^{2+} nor does it bind phospholipids or GAG. The association between annexin A2 and p11 does not require Ca^{2+} and utilizes the concave annexin molecular surface (reviewed in Shao et al. 2006).

Interaction of A2t with Heparin: The heterotetrameric complex formed by the binding of p11 to p36 (A2t) is the predominant form in most cells (Kassam et al. 1997). Interactions of heparin with Ca^{2+}- and A2t have been studied. A2t has been shown to be present at both the cytosolic and extracellular surfaces of the plasma membrane of many cells (Waisman 1995). Extracellular A2t has been proposed to function as a cell adhesion factor (Tressler and Nicolson 1992; Tressler et al. 1993), a receptor for plasminogen and tissue plasminogen activator (Cesarman et al. 1994), and a receptor for tenascin-C (Chung and Erickson 1994; Hajjar et al. 1994; Hubaishy et al. 1995). It is possible that heparin might be involved in the regulation of the interaction of A2t with these ligands. In the absence of Ca^{2+}, heparin induces a more moderate change in the conformation of A2t. Of interest was the heparin-induced increase in the β-sheet from ~21% to 27% and decrease in unordered structure from 22% to 18%. Hubaishy et al. (1995) reported that A2t bound to a heparin affinity column and that the phosphorylation of A2t on tyrosine residues blocked the heparin-binding activity of the protein. The study on interaction of A2t with heparin identified A2t as a specific, high affinity heparin-binding protein. Furthermore, the Ca^{2+}-dependent binding of heparin to A2t causes a dramatic conformational change in the protein. The p36 subunit of A2t contains a Cardin-Weintraub glycosaminoglycan recognition site (Cardin and Weintraub 1989) and that a peptide to this region of A2t binds heparin.

The interaction of A2t with heparin was also shown to be inhibited by tyrosine phosphorylation of A2t (Hubaishy et al. 1995). Since the role that heparin binding plays in the structure or function of A2t is unknown, the current study was aimed at the characterization of the interaction of heparin with A2t. Analysis of the CD spectra of A2t showed that the binding of heparin to A2t resulted in a profound change in the conformation of A2t. We also found that in the absence of Ca^{2+}, a small change in the conformation of A2t occurred upon heparin binding. It was also observed that A2t formed a large complex with heparin. A2t bound heparin with an apparent K_D of 32 ± 6 nM and a stoichiometry of 11 ± 0.9 mol of A2t/mol of heparin. A2t does not bind to disaccharides of heparin, but does bind to 3-kDa heparin that contains ~10 monosaccharides. The binding of ~11 molecules of A2t to a single 17-kDa heparin strand that contains ~50 monosaccharide units suggests that A2t requires ~4–5 monosaccharide units for binding.

Several consensus sequences have been identified among members of the heparin-binding family of proteins. For example, the heparin-binding sequence of the C-terminal region of fibronectin has been identified as WQPPRARI. The p36 subunit of A2t contains a Cardin-Weintraub heparin-binding consensus sequence. Furthermore, a peptide to this region of p36 subunit of A2t (300LKIRSEFKK-KYGKSLYY316) undergoes a conformational change upon heparin binding. These results therefore suggest that residues 300–316 of the p36 subunit of A2t are involved in heparin binding (Kassam et al. 1997).

Fucoidan, a sulfated fucopolysaccharide, mimics the fucosylated glycans of glycoproteins and has been used as a probe for investigating the role of membrane polysaccharides in cell − cell adhesion. A2t is bound to fucoidan with an apparent K_D of 1.24 ± 0.69 nM; the binding of fucoidan to A2t was Ca^{2+}-independent. Furthermore, in the presence but not the absence of Ca^{2+}, the binding of fucoidan to A2t decreased the α-helical content from 32% to 7%. A peptide corresponding to a region of the p36 subunit of A2t, F (306) − S(313), which contains a sequence for heparin binding, was shown to undergo a conformational change upon fucoidan binding. This suggests that heparin and fucoidan bound to this region of A2t. Thus, the binding of A2t to the carbohydrate conjugates of certain membrane glycoproteins have profound effects on the structure and biological activity of A2t (Sandra et al. 2000).

Ca^{2+}-dependent binding of A2t to heparin caused a large decrease in the α-helical content of A2t from ~44% to 31%, a small decrease in the β-sheet content from ~27% to 24%, and an increase in the unordered structure from 20% to 29%. The binding of heparin also decreased the Ca^{2+} concentration required for a half-maximal conformational change in A2t from 360 to 84 μM. Data suggests that in the presence of Ca^{2+}, heparin induces a large conformational change in A2t, resulting in a substantial change in the conformation of the protein. However, heparin can also interact with A2t in the absence of Ca^{2+} and, to a much smaller degree, affect the conformation of the protein (Kassam et al. 1997). A2t

appears to be a unique member of the heparin-binding proteins because A2t can discriminate between heparin and heparan sulfate ligands.

21.3.2 Crystal Analysis of Sugar-Annexin 2 Complex

Identification of annexin A2 as a heparin-binding protein (Kassam et al. 1997) raised the possibility that this protein-GAG interaction may participate in the regulation of thrombotic processes. To characterize the heparin-annexin A2 interaction and to determine the basis for its Ca^{2+} dependence, crystallographic studies were carried out on human annexin A2 in complex with heparin-derived oligosaccharides of varying lengths. Binding measurements in solution were also performed using surface plasmon resonance (SPR) (Shao et al. 2006). Crystallographic analysis revealed that the common heparin-binding site is situated at the convex face of domain IV of annexin A2. At this site, annexin A2 binds up to five sugar residues from the nonreducing end of the oligosaccharide. Unlike most heparin-binding consensus patterns, heparin binding at this site does not rely on arrays of basic residues; instead, main-chain and side-chain nitrogen atoms and two calcium ions play important roles in the binding. Especially significant is a novel calcium-binding site that forms upon heparin binding. Two sugar residues of the heparin derivatives provide oxygen ligands for this calcium ion. Comparison of all four structures shows that heparin binding does not elicit a significant conformational change in annexin A2. The combined data with surface plasmon resonance measurements provide a clear basis for the calcium dependence of heparin binding to annexin A2 (Shao et al. 2006).

21.3.3 Functions of Annexin A2

Dual Action of Annexin 2 Tetramer and Arachidonic Acid: Annexin 2 has been implicated in membrane fusion during the exocytosis of lamellar bodies from alveolar epithelial type II cells. Immunodepletion of Annexin 2 from type II cell cytosol reduced its fusion activity. The A2t induced the fusion of lamellar bodies with the plasma membrane in a dose-dependent manner. This fusion is Ca^{2+}-dependent and is highly specific to A2t because other annexins (1 and 2 monomer, 3, 4, 5, and 6) were unable to induce the fusion. The fusion between lamellar bodies with the plasma membrane is driven by the synergistic action of A2t and arachidonic acid (Chattopadhyay et al. 2003).

In an earlier report, though annexins 1–4 mediated liposome aggregation in the presence of 1 mM Ca^{2+}, only A2 tetramer had aggregation activity at 10 μM Ca^{2+}, where as A5 and A6 had negligible aggregation activity at Ca^{2+} concentrations up to 1 mM Ca^{2+}. Of six purified annexins (A1–A6) tested for their ability to reconstitute secretion from permeabilized cells, only Annexin A2 was effective. Annexin A2 was not involved in the exocytosis of lamellar bodies (Liu et al. 1996; Blanchard et al. 1996). The neuroblastoma cells are known to express annexins and confirmed in cell membrane. Following stimulated release of noradrenaline by K^+ depolarisation or by treatment with the ionophore A23187, results favoured for a general role in calcium signaling at discrete intracellular locations by annexins 2 and 5. The results did not support the specific involvement for Annexin 2 in membrane fusion at sites of vesicle exocytosis (Blanchard et al. 1996).

Antiphospholipid (aPL) antibodies recognize receptor-bound β2 glycoprotein I (β2GPI) on target cells, and induce an intracellular signaling and a procoagulant/proinflammatory phenotype that leads to thrombosis. Evidence indicates that annexin A2 binds β2GPI on target cells. Romay-Penabad et al. (2009) studied the effects of human aPL antibodies in A2-deficient ($A2^{-/-}$) mice. After IgG-APS (antiphospholipid syndrome) or 4C5 injections and vascular injury, mean thrombus size was significantly smaller and tissue factor activity was significantly less in $A2^{-/-}$ mice compared with $A2^{+/+}$ mice. The expression of VCAM-1 induced by IgG-APS or 4C5 in explanted $A2^{-/-}$ aorta was also significantly reduced compared with $A2^{+/+}$ mice. It was suggested that annexin A2 mediates the pathogenic effects of aPL antibodies in vivo and in vitro APS.

Soluble A2t activates human monocyte-derived macrophages (MDM), resulting in secretion of inflammatory mediators and enhanced phagocytosis. The modulation of macrophage function by A2t is mediated through TLR4, suggesting an important role for this stress-sensitive protein in the detection of danger to the host, whether from injury or invasion (Swisher et al. 2010). Up-regulation of annexin A2 in differentiated retinal pigment epithelial (RPE) cells may be required for development of phagocytic capability. Law et al. (2009) showed that annexin A2 is highly enriched on newly formed phagosomes in RPE cells and that siRNA-mediated depletion of annexin A2 results in impairment of photoreceptor outer segments (POS) internalization. Findings provide direct evidence that annexin A2 is necessary for the normal circadian phagocytosis of POS by RPE cells.

21.4 Annexin A4 (p33/41)

21.4.1 General Characteristics

Annexin A4 (also called endonexin, protein II, chromobindin 4, placental anticoagulant protein II, and PP4-X) is one of a family of proteins that interact with

phospholipids in the presence of calcium. Annexin A4 is a 36-kDa protein that can aggregate on the inner leaflet of cellular membranes. It has additional unique property of recognizing carbohydrates. Calcium-dependent phospholipid binding activities are common and characteristic properties of the annexin family proteins. Phospholipids are suggested to bind via hydrophilic head groups to annexins, and the phospholipid-binding region is proposed to be localized on the convex surface side where calcium-binding sites are located in the crystal structure of annexin A5. Annexin A4 has been proposed to be involved in exocytosis and in the coagulation process. These functions are related to the ability of the annexins to bind to acidic phospholipids. Annexin A4 strongly binds to either lipid at acidic pH. At neutral pH only weak binding to phosphatidic acid (PA) and no binding to phosphatidylserine (PS) occurs. But addition of Ca^{2+} leads to a strong binding to the lipids also at neutral pH. Binding of annexin A4 induces dehydration of the vesicle surface (Zschörnig et al. 2007). Annexin A4 shares 50–60% sequence homology to annexin 5 and was shown to bind heparin, but the binding of heparin to this protein was inhibited by a variety of carbohydrates including glucose, N-acetylneuraminic acid, heparan sulfate, and chondroitin sulfate (Kojima et al. 1996). In contrast, heparan sulfate or other glycosaminoglycans do not induce a conformational change in A2t (Becker et al. 1990).

Gene trap disruption of the first intron revealed that there were in fact three splice variants of annexin A4 with differing tissue expression (Li et al. 2003). The knockout animal lacked the major transcript, annexin A4a, which has a broad tissue distribution. However, two further transcripts, designated annexin A4b and Annexin A4c, were unaffected by the intron disruption in this region. Annexin A4b was shown to be expressed only in the digestive tract and annexin A4c exhibited a restricted expression pattern within solitary chemosensory cells. In nonstratified epithelia, they extend from the basement membrane to the lumen and appear to perform paracrine/endocrine functions (Li et al. 2003).

Annexin A4 is localized in the apical cytoplasmic region of pancreatic acinar cells where zymogen granules are concentrated. Since it is the major component of the zymogen granule membrane, the glycosylphosphatidylinositol-anchored glycoprotein GP-2 was suggested to play a role in apical sorting and secretion of zymogens. The major carbohydrate structures of porcine GP-2 were trisialo-triantennary and tetrasialo-tetra-antennary complex-type oligosaccharides. Annexin IV interacts with GP-2 in the presence of calcium and it recognizes the terminal sialic acid residues linked through α2-3 linkages to the carbohydrate of GP-2. Thus, GP-2 is an endogenous ligand of annexin IV in the exocrine pancreas (Tsujii-Hayashi et al. 2002).

21.4.2 Tissue Distribution

Annexin A4 is found at high levels in secretory epithelia in the lung, intestine, stomach, trachea, and kidney. It is thought to be a marker for polarized epithelial cells. Although the biological roles for annexin A4 remain largely unclear, it has been implicated in the regulation of calcium-activated epithelial chloride channels (Chan et al. 1994) and shown to have anti-inflammatory properties (Katoh 2000; Gotoh et al. 2005). Annexin A4 has also been shown to play a role in kidney organogenesis in *Xenopus laevis*, where ablation of the gene product results in abnormal development of pronephric tubules (Seville et al. 2002). Hill et al. (2003) showed that Annexin A4 can regulate passive membrane permeability to water and protons and can alter physical properties of the membranes by associating with them (Ponnampalam and Rogers 2006). Hill et al. (2008) demonstrated that the major transcript of annexin A4, annexin A4a, is present in the superficial and transitional epithelium of the bladder, where it is expressed throughout the urothelium. Umbrella cells have large numbers of unique elongated vesicles underlying the apical membrane. These "fusiform vesicles" are thought to play a key role in the bladder's ability to stretch by providing a large amount of membrane available for exocytosis and endocytosis. This allows umbrella cells to increase their apical surface area in response to filling and then to decrease it upon emptying (Hill et al. 2008). Given the biophysical properties and urothelial expression of annexin A4, it was hypothesized that this annexin could be important to the integrity or the regulation of bladder permeability barrier.

Bladder filling has been shown to activate a complex set of mechano-sensitive responses in umbrella cells, including ATP release and purinergic receptor-dependent membrane trafficking (Wang et al. 2005). Hydrostatically induced stretch has been shown to raise intracellular Ca^{2+} in the urothelium; furthermore, blocking Ca^{2+} release from intracellular stores inhibited exocytosis (Wang et al. 2005). Given the known membrane-organizing ability of annexins and sensitivity to $Ca^{2+,}$ annexin A4 might play a specific role in umbrella cell membrane trafficking (reviewed in Hill et al. 2008).

It was demonstrated in wild-type bladders that stretch induces a redistribution of annexin A4 within basal and intermediate cells to the cellular periphery. A genetically modified mouse model in which the protein is not expressed in renal epithelia (Li et al. 2003) revealed no alterations in normal bladder function or morphology in the annexin $A4a^{-/-}$ animals, suggesting that the role of annexin A4 in the bladder does not include barrier function or stretch-regulated intracellular trafficking. $Anx4^{-/-}$ mouse model shows no protein in the urothelium where as wild-type umbrella cells showed uniform cytoplasmic staining and

some association with the nuclear membrane. It was indicated that loss of anaxin A4 from the urothelium does not affect barrier function, membrane trafficking, or normal bladder-voiding behavior (Hill et al. 2008).

Microarray studies of human endometrium have shown that Annexin A4 mRNA is significantly up-regulated during the secretory phase of the menstrual cycle compared with the proliferative phase (Kao et al. 2002; Riesewijk et al. 2003; Ponnampalam et al. 2004; Mirkin et al. 2005). Anx4 mRNA is up-regulated during mid-secretory (MS) and late-secretory (LS) phases compared with proliferative phases during the menstrual cycle. Anx4 was localized to glandular and luminal epithelium and was present in high levels throughout the menstrual cycle except during early-secretory (ES) phase, when it was significantly reduced. Results suggest that, in proliferative explants, progesterone significantly increased the Anx4 mRNA and protein after 48 h in culture. Estrogen did not have any significant effects. Anx4 transcription and translation are regulated by progesterone and suggests that Anx4 may be important in regulating ion and water transport across the endometrial epithelium (Ponnampalam and Rogers 2006).

21.4.3 Characterization

Soluble monomers of annexin A4 trimerize in the cytoplasm bind to the membrane and then assemble into higher order structures at the membrane interface creating a crystallization cascade in 2D across a large cross-sectional area of membrane (Zanotti et al. 1998). In vitro cross-linking studies demonstrate that trimer, hexamer and higher aggregates of annexin form in the presence of Ca^{2+} and phospholipid-containing vesicles (Concha et al. 1992). Annexin A4 has a short amino-terminal of 12 amino acids, which is susceptible to phosphorylation by protein kinase C. Phosphorylation by protein kinase C causes the release of the N-terminal of annexin A4 and inhibits annexin A4's ability to aggregate on the membrane (Kaetzel et al. 2001). Annexin A4 has also been found in the cytoplasm (Zimmermann et al. 2004) and nucleus (Raynal et al. 1996), and it can be secreted (Masuda et al. 2004). Intranuclear annexin A4 has been shown to translocate to the cytoplasm because of an increase in intracellular calcium (Mohiti et al. 1995; Barwise and Walker 1996; Raynal et al. 1996), and during Fas-induced cell death (Gerner et al. 2000), however, the functional role of nuclear annexin A4 is currently not well known (reviewed in Ponnampalam and Rogers 2006).

Annexin (A4) possesses four repeat domains with one Ca^{2+}-binding site (CBS) in each domain. A4 binds the Na^+ ion in CBSs. One structure (1.58 A) bound Na^+ ion in CBS I, whereas another structure (1.35 A) bound the Na^+ ion in CBS II and CBS III. The Cα atoms of CBS III largely moved by coordination of Na^+ ion. In the Cα atoms of CBS I, however, little change resulted from Na^+-coordination. Only the side chain of Glu71 was moved by Na^+-coordination in CBS I. These results indicate that Annexin A4 binds not only Ca^{2+} but also Na^+ ion in CBS (Butsushita et al. 2009).

21.4.4 Pathophysiology

Annexins have been implicated in tumor progression. There is significant increase in expression in annexins A1, A2, A4 and A11 in primary tumors compared with normal colon. Expression of annexins A2, A4 and A11 was related with increasing tumor stage (Duncan et al. 2008). Annexin A4 is elevated in ovarian CCC tumors and is associated with chemoresistance in cultured ovarian cancer cells. Results demonstrate that Annexin A4 confers chemoresistance in ovarian cancer cells in part by enhancing drug efflux (Kim et al. 2009).

The presence of antiphospholipid (aPL) antibodies increases the risk for recurrent miscarriage (RM). Annexins bind to anionic phospholipids (PLs) preventing clotting on vascular phospholipid surfaces. Plasma levels of annexin 5 are significantly higher at the beginning of pregnancy, at the sixth and eighth week of pregnancy in women with aPL antibodies compared with those without aPL antibodies, where as there were no significant differences in plasma annexin A4 levels between women with and without aPL antibodies. These antibodies could displace annexin from anionic phospholipid surfaces of syncytiotrophoblasts (STBs) and hereby promote coagulation activation (Ulander et al. 2007).

Annexins have well characterized anti-inflammatory properties and Lipoxin A4 (Annexin A4) has been shown to exert protective effects in stomach. Suppression of aspirin-triggered lipoxin synthesis, through co-administration of a selective COX-2 inhibitor, results in a significant exacerbation of gastric injury. The gastroprotective effects of lipoxin A4 appear to be receptor mediated, and attributable to suppress leukocyte adherence to the vascular endothelium and to elevate gastroduodenal blood flow, and mediated via lipoxin-induced nitric oxide generation (Wallace et al. 2005).

21.4.5 Doublet p33/41 Protein

Ca^{2+}-dependent carbohydrate-binding proteins from bovine kidney under nonreducing conditions form doublet protein bands corresponding to 33 kDa (p33) and 41 kDa (p41) where as under reducing conditions, a single protein band (p33) was observed. The p33/41 is a lectin which binds to

sialoglycoproteins and glycosaminoglycans in a calcium-dependent manner. Amino acid sequences of p33/41 are highly homologous to those of calcium/phospholipid-binding annexin protein, annexin 4 (endonexin) especially in the consensus sequences. The p33/41 exhibited calcium/phospholipid-binding activity similar to annexin 4 (Kojima et al. 1994). The p33/41 cDNA encodes a protein of 319 amino acids with a molecular mass of 35,769 Da. The deduced amino acid sequence was identical to that of bovine annexin 4 except for one amino acid substitution. The recombinant protein revealed that p41 is a dimer of p33 cross-linked at Cys-198 via a disulfide bond. The recombinant protein bound to columns of heparin and fetuin glycopeptides in a calcium dependent manner and to phospholipid vesicles composed of phosphatidylserine (PS)/phosphatidylcholine (PC), phosphatidylethanolamine (PE)/PC or phosphatidylinositol (PI)/PC. Thus, p33/41 binds two types of ligands via different sites and that phospholipids modulate the carbohydrate binding activity of p33/21. The p33/41 does not contain any of the consensus sequences conserved in the CRDs of any animal lectins. Furthermore, consensus heparin-binding motifs (such as BXBXBXXXXB), identified glycosaminoglycan-binding sequences, or basic amino acid clusters capable of binding to acidic polysaccharides through simple ionic interaction were not observed within the primary sequence of p33/41, that suggested that p33/41 may contain a unique CRD. The p33/41 is highly concentrated in the apical plasma membrane of the epithelial cells in the renal proximal tubules, and integrated into the renal brush border membrane. Heparin does not compete with phospholipids in the binding to p33/21.

Chen et al. (1993) reported three-dimensional models of annexins (1, 2, 3, 5 and 7) constructed by homology modeling using the crystal structure of annexin 5 as a template, and showed that cysteines 198, 242, and 315 in the annexin 3 model do not appear to be exposed on the protein surface. A conformational change to expose cysteine 198 to the outside and to allow it to form an intermolecular disulfide bridge appears to occur upon dimer formation of annexin A4.

21.5 Annexin A5/Annexin V

Annexin A5/Annexin V is a protein of unknown biological function that undergoes Ca^{2+}-dependent binding to phospholipids located on the cytosolic face of the plasma membrane. Annexin 5 has been isolated as placental anticoagulant protein I, inhibitor of blood coagulation, vascular anticoagulant-α, endonexin II, lipocortin 5, placental protein 4, and anchorin CII. The function of the protein is unknown. However, based on in vitro experiments, annexin A5 has been proposed to play a role in the inhibition of blood coagulation by competing for phosphatidylserine binding sites with prothrombin and also to inhibit the activity of phospholipase A1. Annexin 5 is a Ca^{2+}-dependent membrane-binding protein that forms voltage-dependent Ca^{2+} channels in phospholipid bilayers and is structurally and functionally characterized. Data indicate that key amino acid residues act as selectivity filters and voltage sensors, thereby regulating the permeability of the channel pore to ions (Demange et al. 1994).

21.5.1 Gene Encoding Human Annexin A5

Characterization of three genomic clones for human annexin 5 revealed that annexin 5 spans at least 29 kb of the human genome and contains 13 exons ranging in length from 44 to 513 bp and 12 introns from 232 to 8 kb. The absence of a typical tata box and the presence of high G + C content and Sp1-binding sites in its promoter characterize it as a 'housekeeping' gene and account for its broad pattern of expression. Potential binding sites for Cis-regulatory elements identified in 5′-upstream region of annexin 5 are consistent with its known regulation by oncogenic and growth-related stimuli. Annexin 5, like its chick homologue, differs from the genes encoding annexins I, II and III in features of its promoter and in the size of its exons 1, 2 and 3 in ways that may impart individuality to its regulation and function (Fernández et al. 1994). The human gene encoding annexin 5 was localized to 4q26-q28. This localization overlaps but differs slightly from the previous assignment of annexin 5 to 4q28-q32 (Tait et al. 1991).

21.5.2 Interactions of Annexin A5

Annexin A5 is a collagen binding protein of the annexin family associated with plasma membranes of chondrocytes, osteoblasts, and many other cells. As a major constituent of cartilage-derived matrix vesicles it has been shown to bind to native type II and X collagen. In accordance with this observation, annexin A5 is localized in the extracellular matrix of calcifying cartilage in the fetal human growth plate, and that it was restricted to the chondrocyte surface in proliferating and resting cartilage. Furthermore, annexin A5 not only binds to native type II and X collagen, but also to chondrocalcin, the carboxy-terminal extension of type II procollagen, in a calcium-independent manner (Table 21.1). Pepsin digestion of type II collagen results in loss of annexin A5 binding. This confirms a notion that the telopeptide region of type II collagen carries annexin A5 binding sites (Kirsch and Pfaffle 1992; Rahman et al. 1997).

Collagen/Annexin A5 Interactions Regulate Chondrocyte Mineralization: Physiological mineralization in growth plate cartilage is highly regulated and restricted to terminally differentiated chondrocytes. Extracellular matrix components (collagens) of growth plate cartilage are directly involved in regulating the mineralization process. Findings showed that types II and X collagen interact with cell surface-expressed annexin A5. These interactions lead to stimulate annexin A5-mediated Ca^{2+} influx resulting in an increased $[Ca^{2+}]i$, and ultimately increased alkaline phosphatase activity and mineralization of growth plate chondrocytes. Thus, the interactions between collagen and annexin A5 regulate mineralization of growth plate cartilage. Because annexin A5 is up-regulated during pathological mineralization events of articular cartilage, it is possible that these interactions also regulate pathological mineralization (Kim and Kirsch 2008).

Annexin A5 Associates with the IFN-γ Receptor and Regulates IFN-γ Signaling: Many of the biological activities of IFN- γ are mediated through the IFN-γR3-linked Jak-Stat1α pathway. However, regulation of IFN-γ signaling is not fully understood, and not all responses to IFN-γ are Stat1α dependent. Annexin A5 is a putative IFN-γR binding protein. Through an inducible association with the R2 subunit of the IFN-γR, annexin A5 modulates cellular responses to IFN- γ by modulating signaling through the Jak-Stat1 pathway (Leon et al. 2006). Annexin A5 is a specific high-affinity inhibitor of PKC-mediated phosphorylation of annexin 1 and myosin light chain kinase substrates. It appears that inhibition occurred by direct interaction between annexin A5 and PKC (Schlaepfer et al. 1992). Annexin A5 has been shown to interact with kinase insert domain receptor and integrin β5 (Marina et al. 2003; Wen et al. 1999).

21.5.3 Molecular Structure of Annexin A5

Each annexin is composed of two principal domains: the divergent NH2-terminal "head" and the conserved COOH-terminal protein core. The latter harbors the Ca^{2+} and membrane binding sites and is responsible for mediating the canonical membrane binding properties. An annexin core comprises four (in annexin A6 eight) segments of internal and interannexin homology that are easily identified in a linear sequence alignment (Gerke and Moss 2002). It forms a highly α-helical and tightly packed disk with a slight curvature and two principle sides. The more convex side contains novel types of Ca^{2+} binding sites, the so-called type II and type III sites (Weng et al. 1993), and faces the membrane when an annexin is associated peripherally with phospholipids. The more concave side points away from the membrane and thus appears accessible for interactions with the NH2-terminal domain and/or possibly cytoplasmic binding partners (Fig. 21.2).

The first structure for an annexin core was solved by Huber et al. (1990) for annexin A5. More than ten crystal structures for annexin cores have been described showing a remarkable conservation of the overall three-dimensional fold (Huber et al. 1990; Liemann and Lewit-Bentley 1995; Swairjo and Seaton 1994). Human annexin A5 (PP4) has been analysed by crystallography. Two crystal forms of human annexin A5 have been refined at 2.3 A and 2.0 A resolution to R-values of 0.184 and 0.174, respectively, applying very tight stereochemical restraints with deviations from ideal geometry of 0.01 A and 2°. The polypeptide chain of 320 amino acid residues is folded into a planar cyclic arrangement of four repeats. The repeats have similar structures of five α-helical segments wound into a right-handed compact superhelix. Three calcium ion sites in repeats I, II and IV and two lanthanum ion sites in repeat I have been found in the R3 crystals. They are located at the convex face of the molecule opposite the N terminus. Repeat III has a different conformation at this site and no calcium bound. The calcium sites are similar to the phospholipase A2 calcium-binding site, suggesting analogy also in phospholipid interaction. The center of the molecule is formed by a channel of polar charged residues, which also harbors a chain of ordered water molecules conserved in the different crystal forms. Comparison with amino acid sequences of other annexins shows a high degree of similarity between them. Long insertions are found only at the N termini. Most conserved are the residues forming the metal-binding sites and the polar channel. Annexins A5 and A7 form voltage-gated calcium ion channels when bound to membranes in vitro (Huber et al. 1992).

The molecule has dimensions of 64 × 40 × 30 A3 and is folded into four domains of similar structure. Each domain consists of five α-helices wound into a right-handed superhelix yielding a globular structure of ~18 Å diameter. The domains have hydrophobic cores whose amino acid sequences are conserved between the domains and within the annexin family of proteins. The four domains are folded into an almost planar array by tight (hydrophobic) pair-wise packing of domains II and III and I and IV to generate modules (II–III) and (I–IV), respectively. The assembly is symmetric with three parallel approximate diads relating II to III, I to IV and the module (II–III) to (I–IV), respectively. The latter diad marks a channel through the centre of the molecule coated with charged amino acid residues. The protein has structural features of channel forming membrane proteins and a polar surface characteristic of soluble proteins. It is a member of the third class of amphipathic proteins different from soluble and membrane proteins (Huber et al. 1990; Voges et al. 1995).

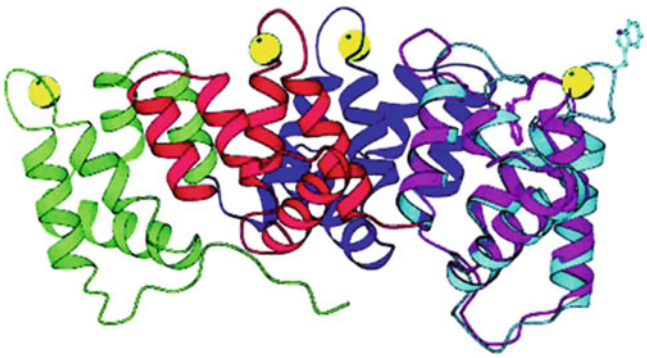

Fig. 21.2 Crystal structure of human annexin A5. The ribbon drawing illustrates the highly α-helical folding of the protein core that forms a slightly curved disk. Different colors were chosen to highlight the four annexin repeats that are given in *green* (repeat I), *blue* (repeat II), *red* (repeat III), and *violet*/*cyan* (repeat IV). The NH2-terminal domain appears unstructured and extends along the concave side of the molecule (*green*). The high and low Ca^{2+} forms are shown in a superposition revealing the conformational change in repeat III, which leads to an exposure of Trp-187 (*violet* for the low and *cyan* for the high Ca^{2+} form). Bound Ca^{2+} are depicted as *yellow spheres* (Printed with permission from Liemann and Lewit-Bentley 1995 © Elsevier)

Annexin 5 is known to form doublet bands exhibiting an apparent difference of 4 kDa on SDS-PAGE. Unlike annexin A4, the annexin 5 isoforms are not sensitive to reducing agents and occur as a result of only two amino acid substitutions between the isoforms (Learmonth et al. 1992; Bianchi et al. 1992). Emerging evidences suggest that both annexin 1 and annexin 5 can bind to many types of cell surfaces, and the identification of annexins as new receptors of tissue plasminogen activator in human endothelial cells (Hajjar et al. 1994) and of influenza virus in cultured cells (Otto et al. 1994) implies functional expression of annexins on the cell surface. Annexin 5, also identified as a collagen-type II binding protein (anchorin CII), was shown to be secreted and present in plasma, amniotic fluid, and post-culture medium (Kirsch and Pfaffle 1992).

21.5.4 Annexin A5-Mediated Pathogenic Mechanisms

Antiphospholipid syndrome: Annexin A5 forms a shield around negatively-charged phospholipid molecules. Without the shield, there is an increased quantity of phospholipid molecules on cell membranes, speeding up coagulation reactions and causing the blood-clotting characteristic of the antiphospholipid antibody syndrome. Antibodies directed against annexin A5 are the cause of a syndrome called the antiphospholipid syndrome. Annexin A5 binds to phospholipid bilayers, forming two-dimensional crystals that block the phospholipids from availability for coagulation enzyme reactions. Antiphospholipid (aPL) antibodies cause gaps in the ordered crystallization of Annexin A5 which expose phospholipids and thereby accelerate blood coagulation reactions. Recently, hydroxychloroquine, a synthetic antimalarial drug, could reverse this antibody-mediated process. In another translational application, Annexin A5 resistance may identify a subset of aPL syndrome patients for whom this is a mechanism for pregnancy losses and thrombosis. The elucidation of aPL-mediated mechanisms for thrombosis and pregnancy complications may open new paths towards addressing this disorder with targeted treatments and mechanistic assays (Rand et al. 2010).

Annexin A5 in prevention of atherothrombosis in SLE: It is becoming evident that atherosclerosis is an inflammatory disease, which is modulated autoimmunity in animal models. An interesting example of how autoimmune reactions can influence atherosclerosis and consequences thereafter is systemic lupus erythematosus (SLE)-associated cardiovascular disease (CVD). Antithrombotic effect exerted by Annexin A5 is thought to be mediated mainly by forming a mechanical shield over phospholipids (PLs) reducing availability of PLs for coagulation reactions. It may be hypothesized that Annexin A5 can be effective as a treatment to prevent plaque rupture and atherothrombosis not only in SLE, but also in the general population prone to CVD (Cederholm and Frostegård 2007).

21.5.5 A Novel Assay for Apoptosis

Annexin A5 is used as a probe in the annexin A5 affinity assay to detect cells that have expressed phosphatidylserine on the cell surface, a feature found in apoptosis as well as other forms of cell death (Koopman et al. 1994; Wen et al. 1999). Platelets also expose phosphatidylserine on their surface when activated, which serves as binding site for various coagulation factors. In early stages of apoptosis changes occur at the cell surface, which have remained difficult to recognize. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin A5 can be used as a sensitive probe for PS exposure upon the cell membrane. Translocation of PS to the external cell surface is not unique to apoptosis, but occurs also during cell necrosis. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very moment that necrosis occurs the cell membrane looses its integrity and becomes leaky. Therefore the measurement of Annexin A5 binding to the cell surface as

indicative for apoptosis has to be performed in conjunction with a dye exclusion test to establish integrity of the cell membrane. In comparison with other tests the Annexin A5 assay is sensitive and easy to perform. The Annexin A5 assay permits measurements of the kinetics of apoptotic death in relation to the cell cycle (Vermes et al. 1995).

21.5.6 Calcium-Induced Relocation of Annexins 4 and 5 in the Human Cells

Cultured human DCs, human osteoblasts and the osteosarcoma cell line MG-63 express annexins A1, A2, A4, A5 and A6. The bulk of these annexins is intracellular express. During endocytosis by DCs, there was a redistribution of annexin A5 which was found to colocalize with vesicles (Larsson et al. 1995). In non-confluent cells, annexin A4and annexin A5 are strongly present throughout the nucleus and are also present in the cytoplasm. On elevation of the intracellular calcium with the ionomycin, the intranuclear pools of annexin A4 and annexin A5 cells showed relocation to the nuclear membrane within seconds. Results support a role for annexins at cellular membranes in response to elevation of cytosolic calcium levels (Mohiti et al. 1995; Raynal et al. 1996). Studies are consistent with the role for annexins in mediating the calcium signal at the plasma membrane and within the nuclei of fibroblasts (Barwise and Walker 1996).

21.6 Annexin A6 (Annexin VI)

21.6.1 Structure

Annexin A6 participates in the formation of a reversible, membrane-cytoskeleton complex in smooth muscle cells through association of protein kinase C (Babiychuk et al. 1999; Schmitz-Peiffer et al. 1998). In contrast to other annexins which have a structural motif of four repeats in the central core region, annexin A6 has eight repeats (Raynal and Pollard 1994). Analysis of the crystal structure of annexin A6 also indicated that it is uniquely organized into two lobes, the N-terminal half (from repeat one to four) and the C-terminal half (from repeat five to eight) of the molecule (Kawasaki et al. 1996; Benz et al. 1996), and each lobe has convex and concave sides and a hydrophilic pore surrounded by the four repeats that might be involved in GAG interactions. Bovine brain annexin A6 is bound to chondroitin sulfate in a Ca^{2+}-dependent manner (Ishitsuka et al. 1998). Furthermore, exon 21 was alternatively spliced, giving rise to two annexin 6 isoforms that differ with respect to a six amino-acid insertion at the starting site of repeat seven (Smith et al. 1994). Results suggest that both lobes of annexin A6, the N- and C-terminal halves of the molecule, are necessary for specific attachment of the annexin-6-expressing cells to CS chains. Calcium-free recombinant human annexin A6 consists of two similar halves closely resembling annexin A1 connected by an alpha-helical segment and arranged perpendicular to each other. The calcium and membrane binding sites assigned by structural homology are therefore not located in the same plane. Analysis of the membrane-bound form of annexin A6 by electron microscopy shows the two halves of the molecule coplanar with the membrane, but oriented differently to the crystal structure, suggesting a flexible arrangement. Ion channel activity has been found for annexin 6 and the half molecules by electrophysiological experiments (Benz et al. 1996).

21.6.2 Functions

Though annexin A6 is a cytoplasmic protein, it functions as a receptor for CS chains. Some reports have described the extracellular expression of annexins on the outer plasma membrane (Kirsch and Pfaffle 1992; Yeatman et al. 1993; Chung and Erickson 1994; Tressler et al. 1994). Since, a significant amount of annexin A6 is exposed on the external cell surface membrane, it is likely that annexin A6 functions as a receptor for CS chains and is involved in the anti-adhesive activity of CS proteoglycans.

During cell-substratum adhesion, cells undergo attachment, spreading and form stress fibers and focal adhesion; these are sequential steps requiring different molecular mechanisms (Sage and Bornstein 1991; Murphy-Ullrich 1995; Murphy-Ullrich 2001). Chondroitin sulfate (CS) proteoglycan PG-M/versican has an inhibitory effect on cell-substratum adhesion. CS chains are needed for this activity, and the immobilization of CS chains on substrata is essential (Yamagata et al. 1989). Takagi et al. (2002) isolated a 68 kDa protein as a candidate receptor for CS chains and identified that protein as annexin 6. Moreover, taking advantage of A431 cells that do not express annexin A6 and transfecting them with exogenous annexin A6, it was demonstrated that annexin 6 is directly involved in the attachment of cells to CS chains and is expressed on cell surfaces. Taken together, it was concluded that annexin A6 is a receptor for CS chains or that it, at least, binds to CS on the cell surface in the presence of Ca^{2+}.

Annexins A2 and A6, which contain KFERQ-like sequences, are degraded more rapidly in response to serum withdrawal, while annexins A5 and A11, without such sequences are degraded at the same rate in the presence and absence of serum. Using isolated lysosomes, only the annexins containing KFERQ-like sequences are degraded by chaperone mediated-autophagy. These results provide evidence for the importance of KFERQ motifs in substrates of chaperone-mediated autophagy (Cuervo et al. 2000).

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Part VIII

C-Type Lectins: Collectins

C-Type Lectins Family

22

Anita Gupta and G.S. Gupta

22.1 C-Type Lectins Family

22.1.1 The C-Type Lectins (CLEC)

Historically the C-Type lectins (CLEC) or C-type lectin receptors (CLR) form a family of Ca^{2+} dependent carbohydrate binding proteins which have a common sequence motif of 115–130 amino acid residues, referred to as the carbohydrate recognition domain (CRD). The C-type designation is from their requirement for calcium for binding. The C-type lectin superfamily is a large group of proteins which is characterized as having at least one carbohydrate recognition domain (CRD), which has been found in more than 1,000 proteins, and it represents a ligand-binding motif that is not necessarily restricted to binding sugars (Drickamer 1999). Proteins that contain C-type lectin domains have a diverse range of functions including cell-cell adhesion, immune response to pathogens and apoptosis. However, many C-type lectins actually lack calcium- and carbohydrate-binding elements and thereby have been termed C-type lectin-like proteins. The CRD has four cysteines that are perfectly conserved and involved in two disulfide bonds. The CRD has been found in various kinds of proteins such as hepatic asialoglycoprotein receptor, lymphocyte IgE receptor, mannose binding protein, selectins (Drickamer 1999; Lasky et al. 1989; Zeng and Weigel 1996) and proteoglycan core protein. Their functions include complement activation, endocytosis, cell recognition, defense mechanism, and morphogenesis (Drickamer 1999; Weis et al. 1998). Many evolutionarily related sequences belonging to the C-type lectins do not show overall sequence similarity. However, they have been classified on the basis of four cysteine residues, being involved in disulfide bridging, which are the trademark of this domain type. The framework surrounding this domain type can be very different from its C-terminal location in the collectins. The monomeric and membrane bound selectins have an N-terminal C-type lectin domain that mediates adhesion between certain cell types through carbohydrate binding. Other proteins, such as phospholipase A2 receptor and the macrophage mannose receptor, contain multiple copies of C-type lectin domains within a single polypeptide. Proteins possessing C-type lectin-like domain (CTLD) type are not necessarily lectins. Type II antifreeze protein present in some arctic fish and the mammalian pancreatic stone protein bind to and inhibit the growth of ice and calcium carbonate crystals, respectively (Håkansson and Reid 2000) and hence are not exactly CLEC but form a part of CTLD.

C-type lectins are either produced as transmembrane proteins or secreted as soluble proteins. Examples of soluble C-type lectins include members of the collectins family, such as the lung surfactant proteins A (SP-A) and SP-D (Wintergest et al. 1989), which are secreted at the luminal surface of pulmonary epithelial cells, and the mannose-binding protein (MBP), a collectin present in the plasma (Kawasaki et al. 1983). Transmembrane C-type lectins can be divided into two groups, depending on the orientation of their amino (N)-terminus. These are type I and type II C-type lectins depending on their N-terminus pointing outwards or inwards into the cytoplasm of the cell respectively. Examples of transmembrane C-type lectins are the selectins (Ley and Kansas 2004), the mannose receptor (MMR) family (East and Isacke 2002), and the dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) (Geijtenbeek et al. 2002).

22.1.2 C-Type Lectin Like Domain (CLRD/CTLD)

Use of terms of "C-type lectin", "C-type lectin domain" (CLRD), "C-type lectin-like domain" (also abbreviated as CLRD), often used interchangeably and use of CRD in the literature, have been clarified by Zelensky and Gready (2005). With the large number of CLR sequences and structures now available, studies indicate that the implications of the CRD domain are broad and vary widely in function. In metazoans, most proteins with a CLR are not lectins. Moreover, proteins use the C-type lectin fold to bind other proteins, lipids, inorganic molecules (e.g., Ca_2CO_3), or

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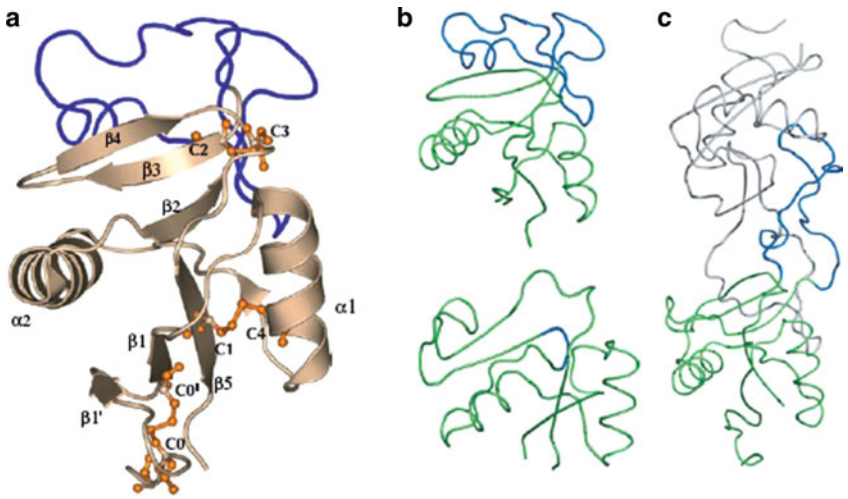

Fig. 22.1 CTLD structure. (**a**) Cartoon representation of a typical CTLD structure (PDB 1K9I). The long loop region is shown in *blue*. Cystine bridges are shown as *orange sticks*. The cystine bridge specific for long form CTLDs (C0-C0') is also shown. (**b**), (**c**), and (**d**): **Variation of the long loop region structure**. Three common forms of the CTLD long loop region are shown. Panels (B: PDB 1K9I) and (C: PDB 1UUH) show canonical CTLDs in which long loop region is tightly packed (**b**) or flipped out to form a domain-swapping dimer (D: PDB 1C3A). A compact CTLD from human CD44 Link domain is shown in panel (**c**). The core domain and long loop region are colored *green and blue*, respectively (Reviewed by Zelensky and Gready 2005)

even ice (e.g., the antifreeze glycoproteins). An increasing number of studies show that "atypical" C-type lectin-like proteins are involved in regulatory processes pertaining to various aspects of the immune system. Examples include the NK cell inhibitory receptor Ly49A, a C-type lectin-like protein, which is shown to complex with the MHC class I ligand (Correa and Raulet 1995), and the C-type lectin-like protein mast cell function-associated antigen which is involved in the inhibition of IgE-Fc γRI mediated degranulation of mast cell granules (Guthmann et al. 1995). Yet glycan binding by C-type lectins is always Ca^{2+}-dependent because of specific amino acid residues that coordinate Ca^{2+} and bind the hydroxyl groups of sugars (Cummings and McEver 2009). To resolve the contradiction, a more general term C-type lectin like domain (CLRD) was introduced to distinguish a group of Ca^{2+}-independent carbohydrate-binding animal proteins from the Ca^{2+}-dependent C-type of animal lectins (CLR/CLEC). The usage of this term is however, somewhat ambiguous, as it is used both as a general name for the group of domains with sequence similarity to C-type lectin CRDs (regardless of the carbohydrate-binding properties), and as a name of the subset of such domains that do not bind carbohydrates, with the subset that does bind carbohydrates being called C-type CRDs. Also both 'C-type CRD' and 'C-type lectin domain' terms are still being used in relation to the C-type lectin homologues that do not bind carbohydrate, and the group of proteins containing the domain is still often called the 'C-type lectin family' or 'C-type lectins', although most of them are not in fact lectins. The abbreviation CRD is used in a more general meaning of 'carbohydrate-recognition domain', which encompasses domains from different lectin groups. Occasionally CRD is also used to designate the short amino-acid motifs (i.e. amino-acid domain) within CLRDs that directly interact with Ca^{2+} and carbohydrate (Zelensky and Gready 2005). In this book authors will use the term C-type lectin domain or C-type lectin-like domain (CTLD/CLRD) interchangeably in its broadest definition to refer to protein domains that are homologous to the CRDs of the C-type lectins, or which have structure resembling the structure of the prototypic C-type lectin CRD or as used by different researchers in their work. More over, due to contradictions (Zelensky and Gready 2005) and uncertainties which may arise in future studies our sequence of chapters is not based on structure databases as in the SCOP; instead chapters are linked more to cell functions or cell biology.

22.1.3 The CLRD Fold

The CLRD fold has a double-loop structure (Fig. 22.1a). The overall domain is a loop, with its N- and C-terminal β strands (β1, β5) coming close together to form an antiparallel β-sheet. The second loop, which is called the long loop region, lies within the domain; it enters and exits the core domain at the same location. Four cysteines (C1–C4), which are the most conserved CLRD residues, form disulfide bridges at the bases of the loops: C1 and C4 link β5 and α1 (the whole domain loop) and C2 and C3 link β3 and β5 (the long loop region). The rest of the chain forms two flanking α helices (α1 and α2) and the second ('top') β-sheet, formed by strands β2, β3 and β1. The long loop region is involved in

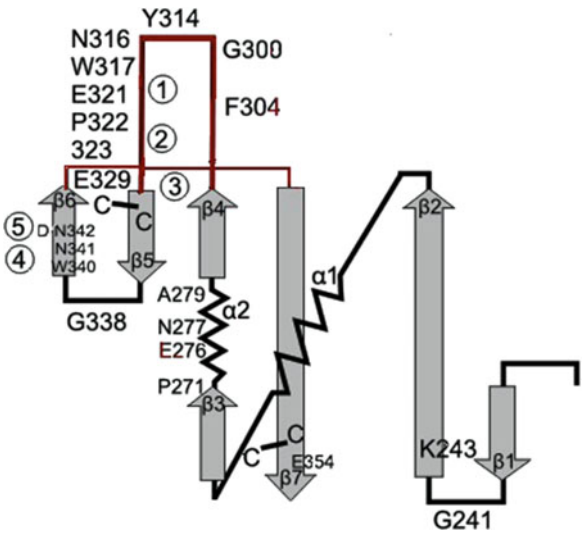

Fig. 22.2 Two-dimensional representation of the C-type lectin fold. The β-strands and α-helices have been numbered β1–β7 and α1–α2, respectively. Secondary structure elements comprise the following residues: (SP-D numbering); α1, 254–264; α2, 273–288; β1, 236–240; β2, 243–253; β3, 267–269; β4, 291–297; β5, 331–335; β6, 339–343; β7, 347–355. The amino acids that are coordinated to the carbohydrate binding calcium ion are denoted with *encircled numbers 1–5*. The conserved residues are indicated with their amino acid one letter symbol. The second calcium ligand is normally an asparagine but is an arginine or alanine in the SP-A sequences. The fifth calcium ligand is an aspartic acid residue in all sequences except dog SP-A, where it is an asparagine (Adapted by permission from Håkansson and Reid 2000 © John Wiley and Sons)

Ca^{2+}-dependent carbohydrate binding, and in domain-swapping dimerization of some CLRDs (Fig. 22.1a), which occurs via a unique mechanism (Mizuno et al. 1997; Feinberg et al. 2000; Mizuno et al. 2001; Hirotsu et al. 2001; Liu and Eisenberg 2002). As suggsted, the CLRD structure is mainly composed of two antiparallel β-sheets; one of these is four-stranded and the other five stranded. The four-stranded β-sheet is found at the N-terminal part of the domain and is flanked by two helices. In trimeric collectins, this sheet is interacting with the α-helical coiled-coil. The five-stranded β-sheet is rather distorted and more remotely located away from the trimer center. This β-sheet, together with some of the loop structure, makes up the carbohydrate binding ligand site. Two of the β-strands (β2 and β7) are relatively long and participate in both sheets. One of the disulfide bridges anchors helix α1 to strand β7 of the four-stranded β-sheet, the other ties together the two most peripheral strands (β5 and β6) of the five-stranded β-sheet. Since the calcium binding amino acid ligands are found at or close to these two strands, this disulfide bridge probably plays an important role in stabilizing the structure around the functional carbohydrate binding site (Fig. 22.2). For conserved positions involved in CLRD fold maintenance and their structural roles readers are referred elsewhere (Zelensky and Gready 2003). In addition to four conserved cysteines, one other sequence feature and the highly conserved 'WIGL' motif is located on the β2 strand and serves as a useful landmark for sequence analysis.

The crystal structure of the CRD of a rat mannose-binding protein, determined as the holmium-(Ho^{3+}) substituted complex by multi-wavelength anomalous dispersion (MAD) phasing, reveals an unusual fold consisting of two distinct regions, one of which contains extensive nonregular secondary structure stabilized by two holmium ions. The structure explains the conservation of 32 residues in all C-type CRDs, suggesting that the fold seen is common to these domains (Weis et al. 1991). Structurally, CLRDs can be divided into two groups: canonical CLRDs having a long loop region, and compact CLRDs that lack it. The second group includes Link or protein tandem repeat (PTR) domains (Brissett and Perkins 1996; Kohda et al. 1996) and bacterial CLRDs (Hamburger et al. 1999; Kelly et al. 1999). Link domain or PTR is a special variety of CLRD, which lacks the long loop region. The major function of Link domains is binding hyaluronan. Although proteins containing it have different domain architecture, their number is small, and they have not been divided into subgroups. Group I CLRDcps contain both canonical and Link-type CLRDs. Other Link-domain containing proteins have four types of domain architecture. Domain composition of Link proteins is similar to that of the N-terminal part of Group I (lecticans). Another family usually included in the CLRD superfamily is that of endostatin (Hohenester et al. 1998). However, endostatin fold is not a suitable example of a CLRD and hence not to be considered further.

The 3D structure of the lectin domain is known to atomic resolution from X-ray analyses of rat and human mannan-binding protein fragments (Weis et al. 1991; Sheriff et al. 1994; Weis and Drickamer 1994), a human lung surfactant protein D fragment (Håkansson et al. 1999), a human E-selectin fragment (Graves et al. 1994), pancreatic stone protein (Bertrand et al. 1996), tetranectin (Nielsen et al. 1997), snake venom factor IX0X binding protein (Mizuno et al. 1997), tunicate C-type lectin (Poget et al. 1999), and from an NMR study of sea raven antifreeze protein (Gronwald et al. 1998). The fold of the C-type lectin domain in some the C-type lectins is shown in Fig. 22.3.

22.1.3.1 Naming of Secondary Structure Elements

Although the CTLD fold is well conserved among its known representatives, there is no general agreement on the numbering of CTLD secondary structural elements in the literature. The secondary structure element numbering scheme in first solved CTLD structure (rat MBP-A by Weis et al. 1991) included five strands, two helices and four loops. However, this description turned out to be insufficient, as MBP lacks some secondary structure elements that are present in long-form CTLD structures, while other small strands were not defined. Other reports describing the structures of CTLDs that have a different number of secondary structure

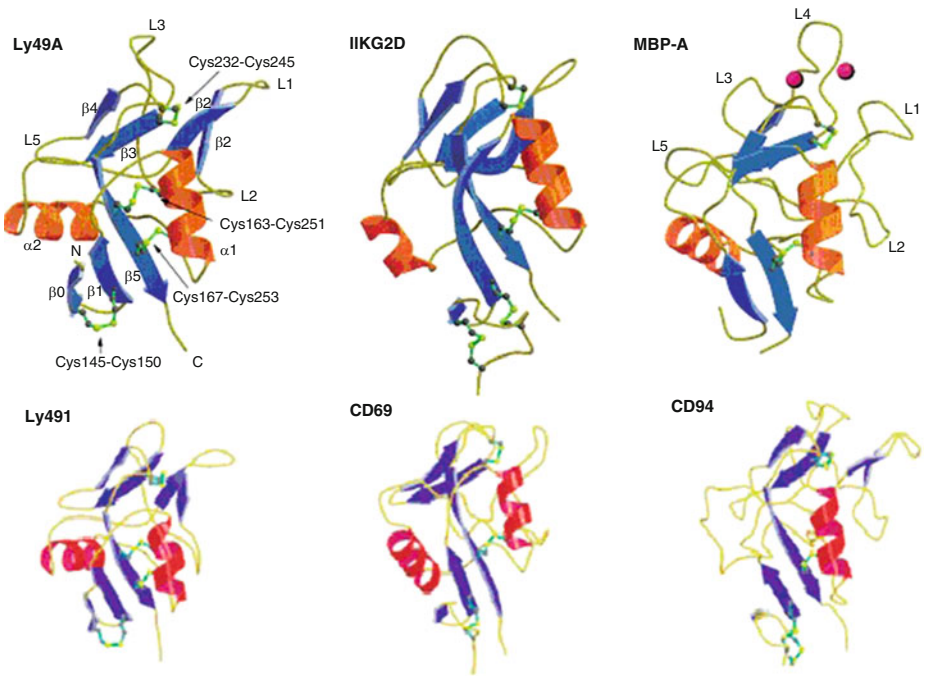

Fig. 22.3 Anatomy of some of the C-type lectin-like domains. Ribbon diagrams of Ly49A (PDB: ID 1QO3) (Tormo et al. 1999), Ly49I (1JA3) (Dimasi et al. 2002), NKG2D (1HQ8) (Wolan et al. 2001), MBP-A (1BCH) (Weis et al. 1991), CD94 (1B6E) (Boyington et al. 1999) and CD69 (1FM5) (Natarajan et al. 2000). The secondary structural elements are colored as follows: β strands *blue*, α-helices *red*, and loop regions *gold*. The disulphide bonds are shown in *green* as ball-and-stick representation. The Ca^{2+} ions bound to MBP-A are drawn as *magenta spheres* (Reviewed in Natarajan et al. 2002)

elements than MBP-A either introduced their own numbering: β strands 1–6 in asialoglycoprotein receptor (ASGPR) by Meier et al. (2000); six β strands in Link module, with labeling not consistent with ASGPR or MBP-A (Kohda et al. 1996); β1–β7 in NKG2D (Wolan et al. 2001); β1–β8 in EMBP (Swaminathan et al. 2001), or extended the secondary structure element naming scheme used for MBP-A (Ly49A secondary structure element numbering is consistent with that in MBP-A) (Tormo et al. 1999).

22.1.3.2 Ca^{2+}-Binding Sites in CLRD/CTLD

Four Ca^{2+}-binding sites recur in CTLD structures from different groups (Chap. 1). The site occupancy depends on the particular CTLD sequence and on the crystallization conditions; in different known structures zero, one, two or three sites are occupied. Sites 1, 2 and 3 are located in the upper lobe of the structure, while site 4 is involved in salt bridge formation between α2 and the β1/β5 sheet. Sites 1 and 2 were observed in the structure of rat MBP-A complexed with holmium, which was the first CTLD structure determined. Site 3 was first observed in the MBP-A complex with Ca^{2+} and oligomannose asparaginyl-oligosaccharide. It is located very close to site 1 and all the side chains coordinating Ca^{2+} in site 3 are involved in site 1 formation. Since biochemical data indicate that MBP-A binds only two calcium atoms (Loeb and Drickamer 1988), Ca^{2+}-binding site 3 is considered a crystallographic artifact. However, in many CTLD structures where site 1 is occupied, a metal ion is also found in site 3; examples include the structures of DC-SIGN and DC-SIGNR (Feinberg et al. 2001), invertebrate C-type lectin CEL-I (Sugawara et al. 2004), lung surfactant protein D (Hakansson et al. 1999) and the CTLD of rat aggrecan (Lundell et al. 2004). It is interesting to note that molecular dynamics simulations of the MBP-A/mannose complex suggested that Ca^{2+} binding site 3 is involved in the binding interaction (Harte and Bajorath 1994; Zelensky and Gready 2005). Residues with carbonyl side chains involved in Ca^{2+} coordination in site 2 form two characteristic motifs in the CTLD sequence, and together with the calcium atom are directly involved in monosaccharide binding. Despite their spatial proximity, from the evolutionary and structural points of view Ca^{2+}-binding sites 1 and 2 should be considered as independent. Crystallographic studies of rat MBP-A CTLD crystallized at a low metal ion concentration (0.325 mM Ho^{3+} instead of 20 mM as used to obtain the CTLD complexed with mannose) have shown that site 1 has higher affinity for Ca^{2+} as it remains occupied and Ca^{2+}-coordination geometry is retained while site 2 loses its metal ion (Ng et al. 1998). Unlike many other functions of the CTLD, Ca^{2+}-dependent carbohydrate binding is found across the whole phylogenetic distribution of the family, from sponges to human, and thus is likely to be the ancestral function (Weis and Drickamer 1996).

22.1.4 Ligand Binding

CTLDs selectively bind a wide variety of ligands. As the superfamily name suggests, carbohydrates are primary ligands for CTLDs and the binding is Ca^{2+}-dependent (Weis and Drickamer 1996). However, the fold has been shown to specifically bind proteins (Natarajan et al. 2002), lipids (Sano et al. 1998) and inorganic compounds including $CaCO_3$ and ice. In several cases the domain is multivalent and may bind both protein and sugar (Zelensky and Gready 2005). Carbohydrate binding is, however, a fundamental function of the superfamily and the best studied one. The first characterized vertebrate CTLDs were Ca^{2+}-dependent lectins, and most of the functionally characterized CTLDs from lower organisms were isolated because of their sugar-binding activity. Although as the number of CTLD sequences grows it becomes clearer that the majority of them do not possess lectin properties, CTLDs are still regarded as a lectin family (Zelensky and Gready 2004).

Four Link protein-encoding genes have been identified in mammals, each physically linked with one of the lectican genes; this suggests that lecticans and Link proteins are of a common evolutionary origin (Spicer et al. 2003). Link proteins and lecticans are also functionally associated: cartilage Link proteins bind both aggrecan and hyaluronan stabilizing the proteoglycan/glycosaminoglycan network (Faltz et al. 1979). CD44 and its recently identified close homologue Lyve-1 are type I transmembrane molecules and cell surface receptors for hyaluronan (Banerji et al. 1999). Tumor necrosis factor-inducible protein (TSG-6) is a soluble protein with a CUB and a Link domain (Lee et al. 1992; Wisniewski and Vilcek 1997). The structure of the latter in free and hyaluronan-bound states has been determined by NMR (Blundell et al. 2003). Stabilin-1 and -2 (also known as FEEL-1/-2) (Tamura et al. 2003; Adachi and Tsujimoto 2002) are scavenger receptors which have the capacity to internalize conventional scavenger ligands such as low density lipoprotein, bacteria and advanced glycation end products. Unlike Stabilin-2, Stabilin-1 does not bind hyaluronan or other glycosaminoglycan (Prevo et al. 2004).

22.1.5 C-Type Lectin-Like Domains in Model Organisms

An important approach to understanding the roles of mammalian carbohydrate-binding proteins is to examine the functions of orthologs in simpler organisms. Because of the advanced state of the developmental, genetic and genomic study of *Caenorhabditis elegans* and *Drosophila melanogaster*, these organisms are particularly attractive models. Homologs of six classes of lectins have been identified using methods of sequence comparisons combined with knowledge of how these proteins interact with glycan ligands.

With regard to C-type lectin-like domains, relative to genome size there are far more CTLDs in the model invertebrates than in mammals. However, the *C elegans* and *Drosophila* proteins that contain CTLDs are quite distinct from the mammalian proteins that contain CTLDs. These differences are evident in the sequences of the CTLDs and the domain organization of the proteins in which they are found. Only a small subset of invertebrate CTLDs contain the constellation of amino acid residues that form Ca^{2+} and sugar-binding sites in mammalian C-type carbohydrate-recognition domains (Drickamer and Dodd 1999; Dodd and Drickamer 2001).

22.1.5.1 Evolution of the C-Type Lectin-Like Domain

The CTLDs of higher eukaryotes are protein modules originally identified as carbohydrate-recognition domains (CRDs) in a family of Ca^{2+}-dependent animal lectins. Less closely related but still definitely homologous CTLDs have been identified in a variety of proteins that do not appear to have carbohydrate-binding activity. All of the domains in the CTLD group show distinct evidence of sequence similarity and are thus believed to have descended from a common ancestor by a process of divergent evolution.

22.2 Classification of CLRD/CTLD-Containing Proteins

The C-type lectin superfamily is a large group of proteins that are characterized by the presence of one or more CLRDs. They can be divided into 17 subgroups based on additional non-lectin domains and gene structure, the two independent sets of criteria. The two approaches give essentially the same results, indicating that members of each group are derived from a common ancestor, which had already acquired the domain architecture that is characteristic of the group. Initially classified into seven subgroups (I to VII) based on the order of various protein domains in each protein: (I) Lecticans or hyalectans, (II) asialoglycoprotein receptors, (III) collectins, (IV) selectins, (V) NK cell receptors, (VI) endocytic receptors, and (VII) lectins as product of regenerating genes (*Reg*) (Drickamer 1993; McGreal et al. 2004). This classification was subsequently updated in 2002, leading to seven additional groups (VIII to XIV) (Drickamer and Fadden 2002) based on structural organization. A further

three subgroups were added (XV to XVII) by Zelensky and Gready (2005). More than 100 different proteins encoded in the human genome contain the CTLD. Most of these groups have a single CTLD, but the macrophage mannose receptor (group VI) has eight CTLDs. The domain architecture of the CTLDcps in different groups is shown in Fig. 22.4. The monomeric and membrane bound selectins have an N-terminal C-type lectin domain that mediates adhesion between certain cell types through carbohydrate binding. Groups VII (REG), IX (tetranectin), XI (attractin), XIII (DGCR2; DiGeorge syndrome critical region gene 2), XV (BIMLEC), and XVII (CBCP) have no known glycan ligands. The section of animal lectins genomics resource includes information on the structure and function of proteins in each group, as well as annotated sequence alignments, and a comprehensive database for all human and mouse CTLD-containing proteins (Table 22.1).

Despite the presence of a highly conserved domain, C-type lectins are functionally diverse and have been implicated in various processes including cell adhesion, tissue integration and remodeling, platelet activation, complement activation, pathogen recognition, endocytosis (Table 22.1). From a functional perspective, we know most about collectins, endocytic receptors, myeloid lectins, and selectins, and these groups are discussed in detail in chapters to follow. However, proteins possessing CTLDs type are not necessarily lectins.

22.3 Disulfide Bonds in Lectins and Secondary Structure

22.3.1 Arrangement of Disulfide Bonds in CTLDs

Drickamer and Dodd (1999) summarized positions of six different disulfide bonds in CTLDs (Fig. 22.5). Chemical evidence for the presence of each of these bonds, except number 4, has been provided in at least one CTLD (Fuhlendorff et al. 1987; Usami et al. 1993). The positions of disulfide bonds designated 1, 2, and 3 have been demonstrated by x-ray crystallography as well (Weis et al. 1991; Nielsen et al. 1997), while homology modeling of CTLDs containing disulfide bonds 5 and 7 shows that they could readily be accommodated into the C-type lectin fold. The patterns of cysteine residues in the CTLDs from *C. elegans* are consistent with the presence of disulfide bonds in each of the arrangements shown in Fig. 22.5 except for bond type 4. No additional pairs of cysteine residues within the CTLDs are consistently evident for any of the subgroups, indicating that the cysteine residues are mostly involved in disulfide bonds of the types already characterized in vertebrate homologues.

CTLDs lacking one of a pair of cysteine residues almost invariably also lack the cysteine side chain to which the first residue would be linked. Like the CTLDs from other organisms, those from *C.elegans* each contain a subset of the possible disulfide bonds. CTLDs in a given subgroup generally show the same disulfide bonds, although a few domains contain extra unique pairs of cysteine residues that might form disulfides. Thus, the similarity in disulfide bond structure in each subgroup reflects the overall similarity in sequence of the CTLDs (Drickamer and Dodd 1999). CEL-I from the sea cucumber, *Cucumaria echinata* is composed of two identical subunits held by a single disulfide bond. A subunit of CEL-I is composed of 140 amino acid residues. Two intrachain (Cys3–Cys14 and Cys31–Cys135) and one interchain (Cys36) disulfide bonds were also identified from an analysis of the cystine-containing peptides obtained from the intact protein (Hatakeyama et al. 2002; Yamanishi et al. 2007).

22.3.2 Functional Role of Disulfides in CTLD of Vertebrates

Single cysteine residues appear in several of these groups at positions 7 and 8 as well as at a unique position 9. The turn between β-strands 3 and 4 is exposed on the surface of the domain, so it is expected that cysteine residues at position 9 would be accessible for formation of disulfide bonds. It is possible that such bonds could form with other cysteine residues within the same polypeptide but outside the CTLDs. However, no likely pairing partner is evident for any of these residues, suggesting that they are more likely to form interchain disulfide bonds. Homo- and hetero-dimer formation through cysteine residues at positions 7 and 8 has been particularly well documented in snake venom proteins containing CTLDs (Usami et al. 1993). The botrocetin, which promotes platelet agglutination in the presence of von Willebrand factor, from venom of the snake Bothrops jararaca is a heterodimer composed of the α subunit and the β subunit held together by a disulfide bond. Seven disulfide bonds link half-cystine residues 2–13, 30–128, and 103–120 of the α subunit; 2–13, 30–121, and 98–113 of the β subunit; and 80 of the α subunit to 75 of the β subunit. In terms of amino acid sequence and disulfide bond location, two-chain botrocetin is homologous to echinoidin (a sea urchin lectin) and other C-type lectins (Usami et al. 1993). The disulfide bond pattern of Trimeresurus stejnegeri lectin (TSL), a member of the C-type lectin family, showed four intrachain disulfide bonds: Cys3–Cys14, Cys31–Cys131, Cys38–Cys133 and Cys106–Cys123, and two interchain linkages, Cys2–Cys2 and Cys86–Cys86 (Zeng et al. 2001). The antifreeze polypeptide (AFP) from the sea raven, Hemitripterus americanus, a member of the cystine-rich class of blood antifreeze proteins,

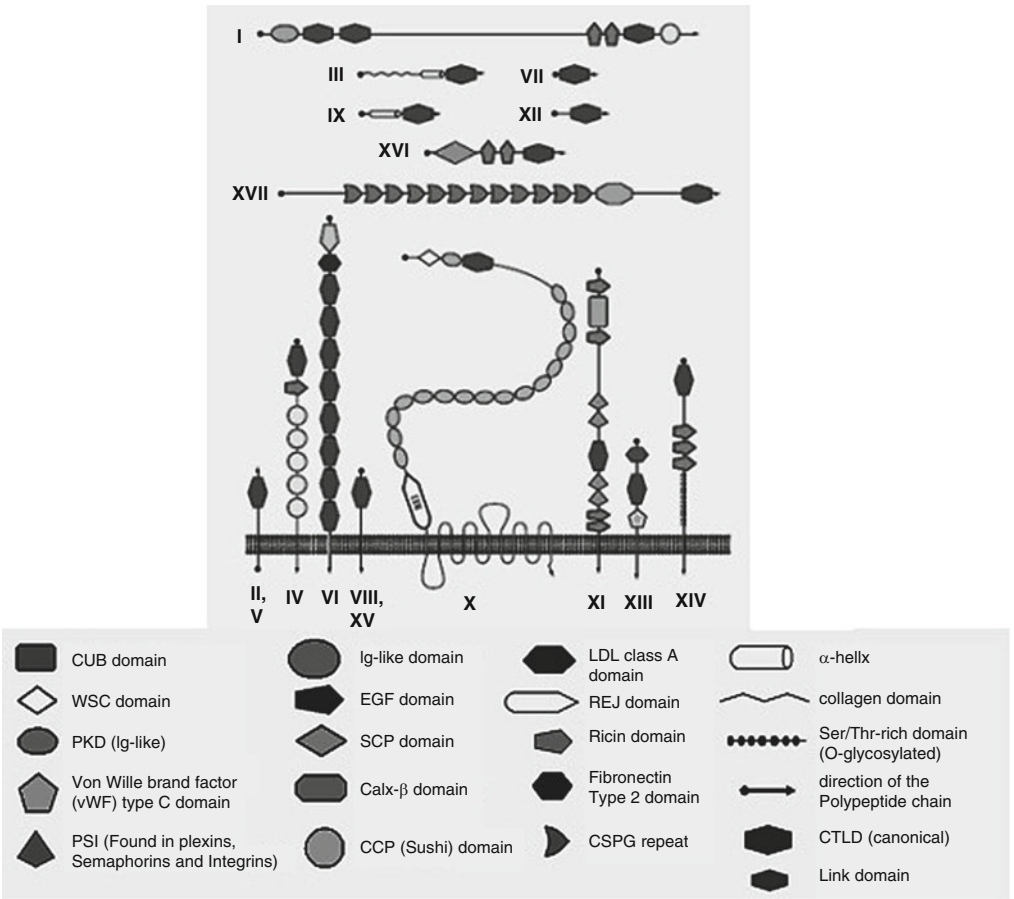

Fig. 22.4 **Domain architecture of vertebrate CTLD**, with mammalian homologues, from different groups. Group numbers are indicated next to the domain charts. *I* lecticans, *II* the ASGR group, *III* collectins, *IV* selectins, *V* NK receptors, *VI* the macrophage mannose receptor group, *VII* REG proteins, *VIII* the chondrolectin group, *IX* the tetranectin group, *X* polycystin 1, *XI* attractin, *XII* EMBP, *XIII* DGCR2, *XIV* the thrombomodulin group, *XV* Bimlec, *XVI* SEEC, *XVII* CBCP (Adapted with permission from Zelensky and Gready 2005 © John Wiley and Sons)

contains 129 residues with 10 half-cystine residues and all 10 half-cystine residues appeared to be involved in disulfide bond formation. The disulfide bonds are linked at Cys7 to Cys18, Cys35 to Cys125, and Cys89 to Cys122. Similarities in covalent structure suggest that the sea raven AFP, pancreatic stone protein, and several lectin-binding proteins comprise a family of proteins which may possess a common fold (Ng and Hew 1992).

Functional rat or human asialoglycoprotein receptors (ASGP-Rs), the galactose-specific C-type lectins, are hetero-oligomeric integral membrane glycoproteins. Rat ASGP-R contains three subunits, designated rat hepatic lectins (RHL) 1, 2, and 3; human ASGP-R contains two subunits, HHL1 and HHL2. Both receptors are covalently modified by fatty acylation (Zeng et al. 1996). Unfolded forms of the HHL2 subunit of the human ASGP-Rs are degraded in the ER, whereas folded forms of the protein can mature to the cell surface (Wikström and Lodish 1993). Deacylation ofASGP-Rs with hydroxylamine results in the spontaneous formation of dimers through reversible disulfide bonds, indicating that deacylation concomitantly generates free thiol groups. Results also show that Cys57 within the transmembrane domain of HHL1 is not normally palmitoylated. Thus, Cys35 in RHL1, Cys54 in RHL2 and RHL3, and Cys36 in HHL1 are fatty acylated, where as Cys57 in HHL1 and probably Cys56 in RHL1 are not palmitoylated (Zeng et al. 1996). Eosinophil granule major basic protein 2 (MBP2 or major basic protein homolog) is a paralog of major basic protein (MBP1) and, similar to MBP1, is cytotoxic and cytostimulatory in vitro. MBP2, a small protein of 13.4 kDa molecular weight, contains ten cysteine residues. Mass spectrometry shows two cystine disulfide linkages (Cys20–Cys115 and Cys92–Cys107) and six cysteine residues with free sulfhydryl groups (Cys2, Cys23, Cys42, Cys43, Cys68, and Cys96). MBP2, similar to MBP1, has conserved motifs in common with C-type

Table 22.1 Classification of C-type lectins with associated domains

Group	Name	Associated domains
I	Lecticans (Hyalectans)	EGF, Sushi, Ig and Link domains
II	Asialoglycoprotein and DC receptors	None
III	Collectins	None
IV	Selectins	Sushi and EGF domains
V	NK – cell receptors	None
VI	Multi-CTLD endocytic receptors	FnII and Ricin domains
VII	Reg group of lectins	None
VIII	Chondrolectin, Layilin	None
IX	Tetranectin	None
X	Polycystin	WSC, REJ, PKD domains
XI	Attractin	PSI, EGF and CUB domains
XII	Eosinophil major basic protein (EMBP)	None
XIII	DGCR2	None
XIV	Thrombomodulin	EGF domains
XV	Bimlec	None
XVI	SEEC	SCP and EGF domains
XVII	CBCP/Frem1/QBRICK	CSPG repeats and CalX-β domains

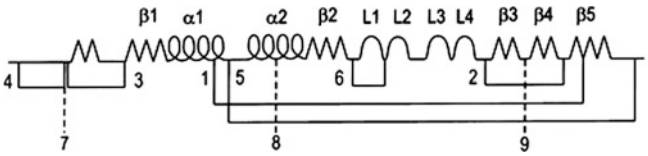

Fig. 22.5 Disulfide bonds in CTLDs. Secondary structure shared by most CTLDs is summarized, with *coils* representing α-helices, *jagged lines* denoting β-strands and loops shown as *curved segments*. The number of these elements corresponds to the secondary structure organisation of rat serum mannose-binding protein (Weis et al. 1991). Potential disulfide bonds within the CTLD are numbered 1 through 6 and cysteines that participate in interchain disulfide bonds are numbered 7 through 9 (Adapted with permission from Drickamer and Dodd 1999 © Oxford University Press)

lectins. The disulfide bond locations are conserved among human MBP1, MBP2 and C-type lectins (Wagner et al. 2007).

The biological functions of rat surfactant protein-A (SP-A), an oligomer composed of 18 polypeptide subunits are dependent on intact disulfide bonds. Reducible and collagenase-reversible covalent linkages of as many as six or more subunits in the molecule indicate the presence of at least two NH2-terminal interchain disulfide bonds. However, the reported primary structure of rat SP-A predicts that only Cys6 in this region is available for interchain disulfide formation. Direct evidence for a second disulfide bridge was obtained by analyses of a set of three mutant SP-As with telescoping deletions from the reported NH2-terminus. Two of the truncated recombinant proteins formed reducible dimers despite deletion of the domain containing Cys6. A novel post translational modification results in naturally occurring cysteinyl isoforms of rat SP-A which are essential for multimer formation (Elhalwagi et al. 1997). Pulmonary SP-D is assembled predominantly as dodecamers consisting of four homotrimeric subunits each. Association of these subunits is stabilized by interchain disulfide bonds involving two conserved amino-terminal cysteine residues (Cys-15 and Cys-20). Mutant recombinant rat SP-D lacking these residues (RrSP-Dser15/20) is secreted in cell culture as trimeric subunits rather than as dodecamers. Disulfide cross-linked SP-D oligomers are required for the regulation of surfactant phospholipid homeostasis and the prevention of emphysema and foamy macrophages in vivo (Zhang et al. 2001).

22.4 Collectins

22.4.1 Collectins: A Group of Collagenous Type of Lectins

Collectins are a small family of secreted oligomeric glycoproteins that contain in common an NH2-terminal collagen-like domain and a COOH-terminal lectin (carbohydrate binding) domain (collagenous + lectin domains = collectin), and found in both lung and serum. The name collectin is derived from the words "collagen" and "lectin." Collectins are C-type lectins that contain a collagen-like domain and usually assemble in large oligomeric complexes containing 9–27 subunits. They belong to C-type lectins family and have an important function in innate immunity, recognizing and binding to microorganisms via sugar arrays on the microbial surface. To date, nine different collectins have been identified: mannan-binding lectin (MBL), surfactant protein A (SP-A), surfactant protein D (SP-D), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), conglutinin, collectin of 43 kDa (CL-43) and collectin of 46 kDa (CL-46), and collectin from kidney (CL-K1). The collectins MBP, conglutinin, CL-43, CL-46, CL-K1, SP-A, and SP-D are soluble, whereas CL-L1 and CL-P1 are membrane proteins. Some collectins, such as MBP and SP-A, organize into a "bouquet," and others, such as bovine conglutinin and SP-D, organize into a "cruciform" shape. One of the best-studied serum collectins is MBP (Chaps. 23–25).

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Collectins: Mannan-Binding Protein as a Model Lectin

23

Anita Gupta

23.1 Collectins

A group of collagenous type of lectins: Collectins are a small family of secreted oligomeric glycoproteins that contain in common an NH2-terminal collagen-like domain and a COOH-terminal lectin (carbohydrate binding) domain (collagenous + lectin domains = collectin), and found in both lung and serum. The name collectin is derived from the words "collagen" and "lectin." Collectins are C-type lectins that contain a collagen-like domain and usually assemble in large oligomeric complexes containing 9–27 subunits. They belong to C-type lectins family and have an important function in innate immunity, recognizing and binding to microorganisms via sugar arrays on the microbial surface. To date, nine different collectins have been identified: mannan-binding lectin (MBL), surfactant protein A (SP-A), surfactant protein D (SP-D), collectin liver 1 (CL-L1), collectin placenta 1 (CL-P1), conglutinin, collectin of 43 kDa (CL-43) and collectin of 46 kDa (CL-46), and collectin from kidney (CL-K1). The collectins MBP, conglutinin, CL-43, CL-46, CL-K1, SP-A, and SP-D are soluble, whereas CL-L1 and CL-P1 are membrane proteins. Some collectins, such as MBP and SP-A, organize into a "bouquet," and others, such as bovine conglutinin and SP-D, organize into a "cruciform" shape. One of the best-studied serum collectins is MBP. Bovine CL-43 is structurally one of the simplest collectins, consisting of only three polypeptides, each of which contains a terminal CTLD. Rats have two serum MBPs designated A and C, sometimes called mannan-binding proteins. Humans appear to have only a single MBP corresponding to the rat MBP-A. The collectins can be classed into two distinct group, with MBP and SP-A being hexamers and SP-D, conglutinin and collectin-43 (CL-43) being tetramers, with proteins in the latter group also having significantly larger dimensions with respect to the length of their collagen-like 'stalks'. The structural and functional relationships of this group of collectins have been reviewed (Håkansson and Reid 2000; Hansen and Holmskov 2002; and others).

Each polypeptide chain in collectins consists of four regions: a relatively short N-terminal region, a collagen like region, an α-helical coiled-coil, and a C-terminal lectin domain. With the exception of the N-terminal region, these regions or domain types are also found in molecules other than collectins (Fig. 22.1). The presence of a collagen like region in these molecules imposes a trimeric structure. This is a prerequisite for their proper function; the carbohydrate affinity of a single collectin carbohydrate recognition domain (CRD) is weak but the trimeric organization permits a trivalent and hence stronger interaction between collectin and carbohydrate-containing target surface. In most of the collectins, these trimers are further assembled into larger entities, which enables them to cross-link several target particles and perhaps also to interact simultaneously with target and with host cells (Håkansson and Reid 2000).

Lung surfactant protein D (SP-D) and bovine conglutinin are X-shaped molecules consisting of four trimers extending pairwise from the hub with the lectin domains at the tip of the collagenous arms (Crouch et al. 1994). These dodecamers have been shown to associate further into even larger complexes, with an increased capacity to aggregate bacteria. Surfactant protein A (SP-A) is the major protein component of lung surfactant complex and exhibits a reduced and denatured molecular mass of 35 kDa in humans. SP-A has been shown to have a flower-bouquet-like octadecameric structure similar to that of complement C1q. SP-A interacts with lipids and specifically binds to dipalmitoylphosphatidylcholine (DPPC). Lung SP-A (Voss et al. 1988) and most mannan binding proteins form bouquet-like complexes with up to six trimers. The degree of oligomerization differs between the different proteins, but may also vary within the molecular population of a single preparation. Only the larger complexes, i.e., pentamers or hexamers, give a full biological

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response in terms of macrophage stimulation and complement activation. Comparisons of hybrid peptide chains of rat MBP-A and MBP-C and site-directed mutagenesis led to the conclusion that oligomerization depends on the N-terminal region and that the most N-terminal of its cysteines (Cys6) is indispensable for the formation of oligomers of trimers. The other two cysteines (Cys13 and Cys18) are involved in intratrimer disulfide bridge formation (Wallis and Drickamer 1999). There are a several reviews, which described the biological, physiological, and functional properties of the collectins (Håkansson and Reid 2000; Weis and Drickamer 1996; Lu 1997; Crouch 1998). Håkansson and Reid, (2000) focussed on the structural aspects of the mannan-binding proteins and the lung surfactant proteins.

23.1.1 N-Terminal Region

The N-terminal region of collectins is defined as the segment N-terminal to the first collagenous triple-helix residue. This relatively cysteine-rich region stabilizes the trimers through disulfide bridging (Crouch et al. 1994; Holmskov et al. 1995) and links them together in the collectin oligomers. There seems to be no overall homology between the collectins in this part of the molecule. The only general trend (to which canine SP-A, Rhesus monkey MBP-C, and CL-L1 do not conform) seems to be two cysteine residues separated in sequence by 4–5 amino acids, and perhaps also that the amino acid preceding the second of these cysteines is hydrophobic in most of the collectins. Most of the other amino acids separating these two cysteine residues are relatively hydrophobic in MBP and SP-D but hydrophilic in SP-A. The collectins can nonetheless be divided into different groups with related N-terminal regions. The N-terminal sequences of SP-D, conglutinin, and collectin-43 are clearly related and of similar length, i.e., 25–28 amino acids, and include two cysteine residues. The N-terminal region of SP-A is much shorter and, due to variation in signal peptidase cleavage, is not homogenous; there are two isoforms with seven or ten residues containing one or two cysteines, respectively (Elhalwagi et al. 1997). The third group consists of mannan-binding proteins A and C, which have distinct, yet related, N-terminal regions, each approximately 20 amino acids long. The hepatic forms rat MBP-C and mouse MBP-C have only two cysteines while the other mannan-binding proteins have three, although the sequence of rhesus monkey MBP-C deviates from the similarity displayed by the other sequences. Human MBP has three cysteines despite its closer evolutionary relationship to rat MBP-C and forms larger oligomers in serum than it does in the liver (Kurata et al. 1994). The fourth group, so far represented only by human CL-1, has a much longer N-terminal sequence with only one cysteine.

23.1.2 Collagenous Region

Information on collagen structure is mainly derived from fiber diffraction studies (Beck and Brodsky 1998), and the crystallographic structures of model compounds (Kramer et al. 1998, 1999). A collagen structure can be recognized from the amino acid sequence with its characteristic Gly-X-Y repetitive pattern, where X and Y can be any amino acid but are frequently prolines or hydroxyprolines. Each of the three chains forms a left-handed polyproline II like helix and then chains are coiled around each other in a right-handed manner with the glycine residues in the interior of the superhelix. Interchain hydrogen bonds between N-H groups of glycine and the C_5O groups of the amino acid in X position stabilize the structure. On account of this and absence of intrachain hydrogen bonds, the collagen helix can exist as a trimer. The helical parameters are sequence dependent and differ between imino acid rich and amino acid rich regions (Kramer et al. 1999). The collagen triple helix is surrounded by water molecules that interact with most polar groups, which are exposed to the solvent, either by their side chains or carbonyl groups. Accordingly, the triple helix in the collectins is rich in charged amino acids. The triple helix is most stable due to its high tensile strength, stability, and relative resistance to proteolysis. In addition, a triple helical region can also mediate binding interactions with other macromolecules as shown for C1q and the macrophage scavenger receptor. The collagenous region in MBP contains a binding site for its associated serine proteases (MASP-1 and MASP-2) through which the complement cascade of reactions is triggered (Thiel et al. 1997).

Collectins bind to their macrophage receptor through the collagenous region (Malhotra et al. 1993). The length of collagenous region differs between the collagens; the X- shaped SP-D and bovine collectin have the longest triple-helical regions, and SP-A and MBP the shortest. Most of the collectin prolines in Y position are hydroxylated. Hydroxylated and glycosylated lysines have been demonstrated in SP-D, MBP, collectin 43 and conglutinin (Håkansson and Reid 2000). SP-A oligomerizes as an octadecamer, which forms a flower bouquet-like structure. The collagen-like domain of human SP-A consists of 23 Gly-X-Y repeats with an interruption near the midpoint of this domain. This interruption causes a kink, but its role remains largely unknown. In SP-A, there appears to be a kink in the collagenous rod in this part of the helix, as judged by electron microscopy. The collagen triple helices aggregate laterally at their N-terminal ends, but there is no evidence for covalent crosslinking. At the site of the Gly-X-Y interruption, the helices make a 60° bend and diverge (Voss et al. 1988) in a manner familiar from studies on the C1q structure (Lu et al. 1993). Oligomerization of MBP-A is in part promoted by the N-terminal part of its collagenous region (Wallis and Drickamer 1999), but this region is not indispensable for SP-A oligomer formation (McCormack et al. 1997).

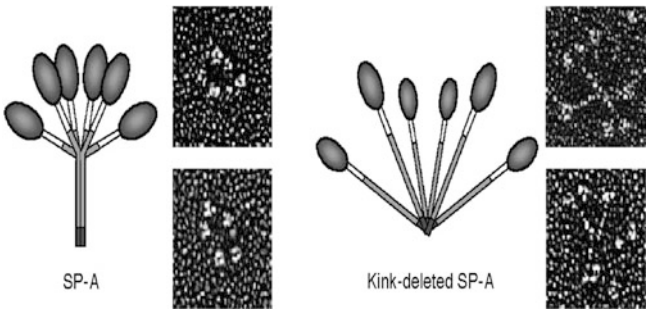

Fig. 23.1 Interruption of Gly-X-Y repeats in the SP-A is critical for the formation of a flower bouquet-like octadecamer (Adapted by permission from Uemura et al. 2006 © American Chemical Society)

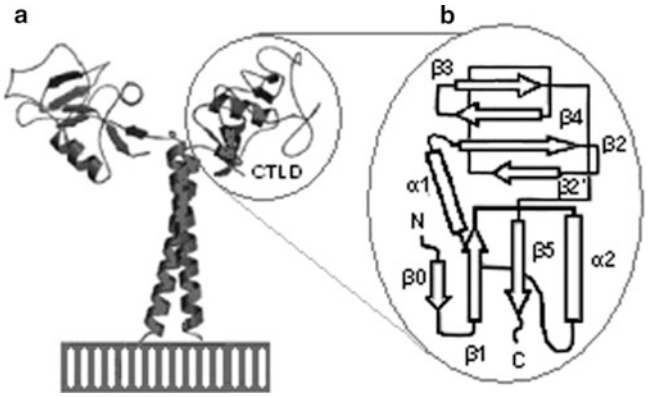

Fig. 23.2 (**a**) **Ribbon representation of the maltose-binding protein (MBP-A;** PDB ID: 1KMB), a CTLD family prototype. (**b**) The topology diagram of CTLD receptor family. This secondary structure organization is well conserved in all known CTLD of identified three-dimensional. In diagrams, *C* and *N* represent the carboxyl- and N-terminus ends of the protein, respectively. For simplicity, the Ca^{2+} ions in the MBP-A ribbon representation are omitted (Adapted by permission from Sawicki et al. 2001© John Wiley and Sons)

To define the importance of the kink region of SP-A, two mutated proteins were constructed to disrupt the interruption of Gly-X-Y repeats. Results indicate that the interruption of Gly-X-Y repeats in the SP-A molecule is critical for the formation of a flower bouquet-like octadecamer and contributes to SP-A's capacity to aggregate phospholipid liposomes (Uemura et al. 2006; Fig. 23.1).

23.1.3 C-Type Lectin Domain

MBP-A is a prototype member of Ca^{2+} dependent lectin in CTLD family (Fig. 23.2a). Collectins have a common sequence motif of 115–130 amino acid residues as carbohydrate recognition domain (CRD) and has been described in Chap. 22. Despite the diversity of ligand recognized by CTLD, members of this family exhibit a highly conserved structure (Fig. 23.2b). The core structure consists of two α-helixes (α1 and β2) and two anti-parallel β-sheets formed by β strands (β0, β1, and β5) and (β2, β2', β3, and β4). The secondary structure elements composing the CTLD core region are conserved among the determined structure (Sawicki et al. 2001), whereas considerable variation is observed in the sequence and structure of the loops connecting the secondary structure elements. It has been demonstrated that the sequence and structure variability of these loops confer ligand specificity (Torgersen et al. 1998).

A comparison of the amino acid sequences of 22 different extracellular collectins yields 18 conserved amino acids in addition to the four cysteines. Perhaps most important from a structural point of view is a hydrophobic cluster containing the conserved residues Phe^{304}, Tyr^{314}, Trp^{317}, Pro^{322}, Trp^{340}, in addition to the semi-conserved Val^{332}. They hold together the carbohydrate binding region consisting of strand β6 and the stretch between the two strands β4 and β5. Trp^{340} is also hydrogen bonded to the conserved Asn^{277} of helix α2. Asn^{277} is in turn hydrogen bonded to the main-chain carbonyl group of Gly^{338}, as is the conserved residue Asn^{316}. Glycine 338 appears to play a key role in the loop structure preceding the β6 strand. Its main-chain conformation is not compatible with the presence of a side chain, which is also true for the other two conserved glycines, Gly^{241} and Gly^{309}, and for most of the nonconserved SP-D glycines as well. Glu^{276}, Asn^{277}, and Ala^{279} are on the nonexposed side of helix α2, which is preceded by the conserved Pro^{271}. Asn^{277}, as mentioned, is hydrogen bonded to both Gly^{338} and Trp^{340}, and Glu^{276} is hydrogen bonded to the main-chain amino group of the nonconserved. Studies indicate a slower evolutionary pace in and around the β5 and β6 strands, i.e., the carbohydrate binding site. In addition to the carbohydrate binding calcium ion, two more calcium ions have been found in crystallographic studies of MBP and SP-D (Weis et al. 1998; Weis and Drickamer 1994; Håkansson et al. 1999). One of these is found 8 Å away from the carbohydrate binding calcium and is complexed by three aspartic acid and one glutamic acid residues. These residues are either conserved or only conservatively replaced in MBPs, SP-D, conglutinin, and Cl-43, but not in SP-A (Håkansson and Reid 2000; Weis and Drickamer 1994).

The electrostatic potential along the surface of collectins displays a large positively charged area within the cavity between the three lectin domains. This surface charge can be observed both for the mannan-binding proteins and SP-D, but is not present on the C-type lectins that are not collectins, e.g., in tetranectin or on the surface of E-selectin. In SP-D,

this charge results from the presence of nonconserved lysine residues. The charged area may be involved in interactions with negative charges on the surface of its targets, e.g., LPS moiety of microbial carbohydrates or the phospholipids of the pulmonary surfactant. The calcium ions in these proteins do influence the surface charge, and the apo protein is characterized by large negatively charged areas on the central and peripheral parts of the lectin domains. Thus, the calcium ions might have a functional role other than carbohydrate binding, e.g., they may maintain a certain electrostatic potential pattern on the surface of the molecule. In this context, it is interesting to note that SP-D was found to bind to its putative receptor gp340 in a manner that was calcium dependent but not inhibited by the presence of maltose (Holmskov et al. 1997; Håkansson and Reid 2000).

The C-type lectin domains in collectins are attached to collagen regions via α-coiled neck regions. Collectins form trimers that may assemble into larger oligomers. Each polypeptide chain consists of four regions: a relatively short N-terminal region, a collagen like region, an α-helical coiled-coil, and the lectin domain. Primary structure data are available for many proteins, while the most important features of the collagen-like region can be derived from its homology with collagen. Carbohydrate binding has been structurally characterized in several complexes between MBP and carbohydrate and between SP-D and carbohydrate; all indicate that the major interaction between carbohydrate and collectin is the binding of two adjacent carbohydrate hydroxyl groups to a collectin calcium ion. In addition, these hydroxyl groups form hydrogen bond to some of the calcium amino acid ligands. While each collectin trimer contains three such carbohydrate binding sites, deviation from overall threefold-symmetry has been demonstrated for SP-D, which may influence its binding properties. The protein surface between the three binding sites is positively charged in both MBP and SP-D (Crouch 1998; Håkansson and Reid 2000).

23.1.4 Comparative Genetics of Collagenous Lectins

MBLs and ficolins (FCNs) are structurally related to C1q, but activate the lectin complement pathway via interaction with MBL-associated serine proteases (MASPs). MBLs, FCNs, and other collagenous lectins also bind to some host macromolecules and contribute to their removal. While there is evidence that some lectins and the lectin complement pathway are conserved in vertebrates, many differences in collagenous lectins have been observed among humans, rodents, and other vertebrates. For example, humans have only one MBL but three FCNs, whereas most other species express two FCNs and two MBLs. Bovidae express CG and other SP-D-related collectins that are not found in monogastric species. Some dysfunctions of human MBL are due to single nucleotide polymorphisms (SNPs) that affect its expression or structure and thereby increase susceptibility to some infections. Collagenous lectins have well-established roles in innate immunity to various microorganisms. So it is possible that some lectin genotypes or induced phenotypes influence resistance to some infectious or inflammatory diseases in animals (Lillie et al. 2005).

23.1.5 Generalized Functions of Collectins

While MBL and SP-D interact with mannose, glucose, L-fucose, ManNAc, GlcNAc as their ligands, SP-A has affinity for glucose, mannose, maltose, and inositol (Kerrigan and Brown 2009). The collectins function in "innate" immunity and act before the induction of an antibody-mediated response. Collectins stimulate in vitro phagocytosis by recognizing surface glycans on pathogens, they promote chemotaxis, and they stimulate the production of cytokines and reactive oxygen species by immune cells. Lung surfactant lipids have the ability to suppress a number of immune cell functions such as proliferation, and this suppression of the immune response is further augmented by SP-A. Although SP-A and SP-D were originally found in the lung, they are also expressed in the intestine. Their function is to enhance adhesion and phagocytosis of microorganisms by agglutination and opsonization. The genes for MBL, SP-A and SP-D have been mapped to human chromosome 10, with at least two expressed SP-A genes (SP-AI and SP-AII) forming a cluster with an SP-A pseudogene. Somatic cell hybrid mapping places the human SP-A and SP-D genes at 10q22-q23 while MBL is localised at 10q21. The close evolutionary relationship between the collectins is further emphasized by a common pattern of exons in their genomic structures and the presence of a gene cluster on chromosome 10 in humans that contains the genes known for the human collectins. Studies on the structure/function relationships within the collectins could provide insight into the properties of a growing number of proteins also containing collagenous regions such as C1q, the hibernation protein, the α- and β-ficolins, as well as the membrane acetylcholinesterase and macrophage scavenger receptor.

All defense collagens (C1q, SP-A, MBL) have shown a qualitatively and quantitatively similar enhancement of monocyte phagocytosis of targets that are suboptimally opsonized with IgG or CR type 1 ligands, C4b and C3b (Wright 2005). The six amino acid sequence required for this functional stimulation has been identified within the collagen-like domain (Arora et al. 2001). This rapid enhancement of

phagocytic activity is triggered when the defense collagen is bound to the particle to be ingested. Interaction of C1q with its cell-surface receptor on neutrophils induces the activation of respiratory burst. However, this action did not occur with MBP and SP-A, proteins that also contain collagen-like domains (Goodman and Tenner 1992). Interaction of C1q with its cell-surface receptor on neutrophils induces the activation of respiratory burst. This action did not occur with MBP and SP-A, proteins that also contain collagen-like domains (Goodman and Tenner 1992).

23.2 Human Mannan-Binding Protein

Mannose-binding lectin (MBL) is a Group III C-type lectin belonging to the collectins (Holmskov et al. 2003), which are a group of soluble oligomeric proteins containing collagenous regions and CTLDs. MBL is secreted into the blood stream as a large multimeric complex and is primarily produced by the liver, although other sites of production, such as the intestine, have been proposed (Uemura et al. 2002). Ca^{2+}-dependent MBLs belong to the family of animal lectins isolated from the liver and serum of rabbits, humans and rodents. Mannan-binding protein was discovered as a rabbit lectin binding to mannan (Kawasaki et al. 1978). Later the term mannose-binding protein (MBP) was introduced, unfortunately implying a more selective reactivity than is characteristic for this protein. The burst of investigations on MBP deficiency in serum (MBL) and susceptibility to infectious diseases was roused by the seminal demonstration of low MBL levels in children deficient in opsonizing activity and suffering from unexplained sensitivity to infections (Super et al. 1989). Initially, investigations of causal relationship between MBL and disease susceptibility relied on quantification of MBL in serum or plasma (or in some cases, of opsonizing activity). However, the finding of genetic influence on the MBL level opened up for determining the MBL status by genotyping (Holmskov et al. 2003; Kerrigan and Brown 2009; Nuytinck and Shapiro 2004; Worthley et al. 2006).

Sheriff et al. (1994) confirmed that human MBP is a hexamer of trimers with each subunit consisting of an amino-terminal region rich in cysteine, 19 collagen repeats, a 'neck', and a carbohydrate recognition domain that requires calcium to bind ligand. A 148-residue peptide, consisting of the 'neck' and CRDs forms trimers in solution and in crystals. The structure of this trimeric peptide has been determined in two different crystal forms. The 'neck' forms a triple α-helical coiled-coil. Each α-helix interacts with a neighbouring carbohydrate recognition domain. The spatial arrangement of the carbohydrate recognition domains suggests how MBP trimers form the basic recognition unit for branched oligosaccharides on microorganisms (Sheriff et al. 1994) (Fig. 23.3).

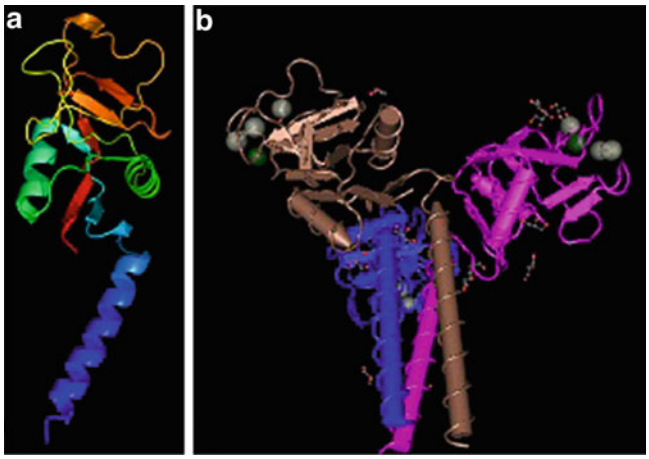

Fig. 23.3 (**a**) Monomeric structure of human MBP (PDB ID: 1HUP) (Sheriff et al. 1994). (**b**). Trimeric structure of a C-type mannose-binding protein from rat (PDB ID: 1RTM) (Weis and Drickamer 1994 © Elsevier)

23.2.1 Characterization of Serum MBL

23.2.1.1 The Protein

Serum MBL contains both a collagen-like domain and a carbohydrate-recognition domain (CRD). The overall polypeptide structure of MBL is similar to that of the other collectins (SP-A, SP-D, conglutinin, CL-43, liver collectin 1, and CL-46). It includes a short, cysteine-rich N-terminal stretch (aa 1–21), a collagen like region (aa 22–81) with one interruption (aa 43–44) that causes the collagen-like structure to bend, a neck region (aa 82–115), and a carbohydrate recognition domain (aa 116–228) (Drickamer and Taylor 1993). This domain confers the carbohydrate specificity of MBL and is stabilized by two disulfide bonds. Due to collagen-like domain, MBL forms homotrimers, designated the MBL subunit. The collagen-like structure is stabilized by the presence of hydroxyprolines and glycosylated hydroxylysines (Ma et al. 1997). The subunit structures assemble from C to N terminus. The neck region initiates the folding (Childs et al. 1990), and the collagen-like region zips toward the N terminus, creating trimeric subunits. The structure is finally stabilized by intrasubunit disulfide bonds in the N-terminal region (Hansen and Holmskov 1998). The oligomer structure of MBL is similar to the structure of C1q, the primary component of the classical pathway of complement (Ikeda et al. 1987), where the bouquet- like forms arise from the formation of intersubunit disulfide bonds in the N-terminal region (Hoppe and Reid 1994). The elucidation of the structure of MBL is

complicated by the fact that the polypeptide chain of MBL is very heterogeneous. In addition to several post-translational modifications, there are three well documented mutations in the collagen-like region (Garred et al. 1992; Lipscombe et al. 1992; Madsen et al. 1994). These mutations all lead to amino acid substitutions, which distort the collagen-like region and inhibit the correct formation of the oligomer forms of MBL. In addition to the heterogeneity of the polypeptide chain, promoter polymorphisms (Madsen et al. 1995) result in highly variable amounts of MBL in the blood.

Human MBP is a homooligomer composed of 32-kDa subunits. Each subunit has an NH_2-terminal region containing cysteines involved in interchain disulfide bond formation, a collagen-like domain containing hydroxyproline and hydroxylysine, a neck region, and a carbohydrate-recognition domain with an amino acid sequence highly homologous to other Ca^{2+}-dependent lectins. Three subunits form a structural unit, and an intact MBP consists of two to six structural units. The carbohydrate-recognition domain is specific for mannooligosaccharide structures on pathogenic organisms, whereas the collagen-like domain is believed to be responsible for interactions with other effector proteins involved in host defense. Cattle possess three serum C-type lectins capable of recognizing mannan in a calcium-dependent manner (Srinivasan et al. 1999).

23.2.1.2 Two Major Forms of MBL in Human Serum

MBP is mainly synthesized in the liver and occurs naturally in two forms, serum MBP (S-MBP) and intracellular MBP (I-MBP). S-MBP activates complement in association with MBP-associated serine proteases via the lectin pathway. The first precise analysis of the major human MBL oligomers was performed by Teillet et al. (2005). Two major MBL forms are present in human serum with mass values of 228,098 + 170 Da (MBL-I) and 304,899 + 229 Da (MBL-II) for the native proteins, whereas reduction of both species yielded a single chain with an average mass of 25,340 + 18 Da. This demonstrates that MBL-I and -II contain 9 and 12 disulfide-linked chains, respectively, and therefore are trimers and tetramers of the structural unit. As shown by surface plasmon resonance spectroscopy, trimeric and tetrameric MBL bound to immobilized mannose-BSA and N-acetylglucosamine-BSA with comparable K_D values (2.2 and 0.55 nM and 1.2 and 0.96 nM, respectively). However, tetrameric MBL exhibited significantly higher maximal binding capacity and lower dissociation rate constants for both carbohydrates. As shown by gel filtration, both MBL species formed 1:2 complexes with MASP-3 or MAp19. The oligomerization state of MBL has a direct effect on its carbohydrate-binding properties, but no influence on the interaction with the MASPs (Teillet et al. 2005). The serum MBP shares similarity with mammalian and chicken hepatic lectins in primary structure of carbohydrate-recognition domain, as well as in the ligand-binding mode: a high affinity (K_D ~ nM) is generated by clustering of approximately 30 terminal target sugar residues on a macromolecule, such as bovine serum albumin, although the individual monosaccharides have low affinity (K_D 0.1–1 mM). On the other hand, MBP does not manifest any significant affinity enhancement toward small, di- and trivalent ligands, in contrast to the hepatic lectins whose affinity toward divalent ligands of comparable structures increased from 100- to 1,000-fold. Such differences may be explained on the basis of different subunit organization between the hepatic lectins and MBP (Lee et al. 1992). Inhibition assays suggested that rat serum MBP has a small binding site which is probably of the trough-type. The 3- and 4-OH of the target sugar are indispensable, while the 6-OH is not required. These characteristics are shared by the rat hepatic lectin and chicken hepatic lectin, both of which are C-type lectins containing carbohydrate-recognition domains highly homologous to that of MBP. Apparently, the related primary structures of these lectins give rise to similar gross architecture of their binding sites, despite the fact that each exhibits different sugar binding specificities (Lee et al. 1991).

23.2.1.3 Intracellular MBP (I-MBP)

The I-MBP shows distinct accumulation in cytoplasmic granules, and is predominantly localized in the endoplasmic reticulum (ER) and involved in COPII vesicle-mediated ER-to-Golgi transport. However, the subcellular localization of either a mutant (C236S/C244S) I-MBP, which lacks carbohydrate-binding activity, or the wild-type I-MBP in tunicamycin-treated cells shows an equally diffuse cytoplasmic distribution, suggesting that the unique accumulation of I-MBP in the ER and COPII vesicles is mediated by an N-glycan-lectin interaction. The binding of I-MBP with glycoprotein intermediates occurs in the ER, which is carbohydrate- and pH-dependent, and is affected by glucose-trimmed high-mannose-type oligosaccharides. These results indicated that I-MBP may function as a cargo transport lectin facilitating ER-to-Golgi traffic in glycoprotein quality control (Nonaka et al. 2007).

23.2.1.4 Ligands for MBL

Among the cells tested, only lymphocytes from thymus of BALB/c mice express endogenous ligands for MBL on their surface, while those from bone marrow, spleen, mesenteric lymph nodes and peripheral blood all being negative. Interestingly, among the thymocytes, only the immature thymocytes with $CD4^+CD8^+CD3$ low phenotype expressed the MBL ligands, which decreased on cell maturation. The major cell surface glycoprotein bearing MBL ligands was identified as CD45RO, which is a transmembrane protein with tyrosine phosphatase activity. The MBL ligands on

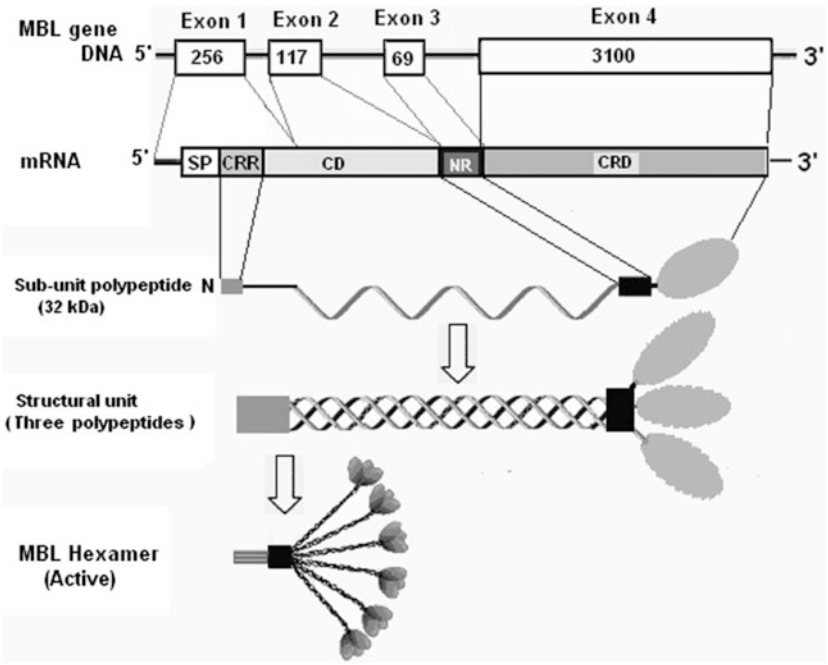

Fig. 23.4 The human MBL gene with the corresponding mRNA and protein domains. The lengths of the four exons and three introns are indicated by the number of base pairs in the box of the figure. The mRNA encodes for the various protein domains shown as signal peptide (*SP*), cysteine rich region (*CRR*), collagen domain (*CD*), neck region (*NR*) (coiled-coil), and carbohydrate recognition domain (*CRD*) in the figure. The peptides self associates into a homo- trimer (structural subunit). Each peptide contains a lectin domain (*gray*) to bind the specific, microbial carbohydrate motifs. Functional MBL circulates in higher-order multimers: (tetramers, pentamers and hexamers)

thymic CD45 contain high mannose type or hybrid type N-linked oligosaccharides (Uemura et al. 1996).

The MBL recognizes a wide array of pathogens independently of specific antibody, and initiates the lectin pathway of complement activation. The MBL binds to neutral carbohydrates on microbial surfaces and recognises carbohydrates such as mannose, glucose, L-fucose, N-acetyl-mannosamine (ManNAc), and N-acetyl-glucosamine (GlcNAc). Oligomerisation of MBL enables high avidity binding to repetitive carbohydrate ligands, such as those present on a variety of microbial surfaces, including *E. coli*, *Klebisella aerogenes*, *Neisseria meningitides*, *Staphylococcus aureus*, *S. pneumonia*, *A. fumigatus and C. albicans* (Kerrigan and Brown 2009; Ng et al. 2002) and Trichinella spirali (Gruden-Movsesijan et al. 2003).

23.2.2 Gene Structure of MBP

The MBP molecule comprises a signal peptide, a cysteine-rich domain, a collagen-like domain, a 'neck' region and a carbohydrate-binding domain. Each domain is encoded by a separate exon. The NH2 terminus of human MBP is rich in cysteines that mediate interchain disulphide bonds and stabilize the second collagen-like region. This is followed by a short intervening region, and the carbohydrate recognition domain is found in the COOH-terminal region. The genomic organization (Fig. 23.4) lends support to the hypothesis that the gene arose during evolution by a process of exon shuffling. Several consensus sequences that may be involved in controlling the expression of human serum MBP have been identified in the promoter region of the gene. The consensus sequences are consistent with the suggestion that this mammalian serum lectin is regulated as an acute-phase protein synthesized by the liver (Taylor et al. 1989). Analysis of the human MBP gene reveals that the coding region is interrupted by three introns, and all four exons appear to encode a distinct domain of the protein. It appears that the human MBP gene has evolved by recombination of an ancestral nonfibrillar collagen gene with a gene that encodes carbohydrate recognition, and is therefore similar to the human surfactant SP-A gene and the rat MBP gene. The gene for MBP is located on the long arm of chromosome 10 at 10q11.2-q21, a region that is included in the assignment for the gene for multiple endocrine neoplasia type 2A (Sastry et al. 1989; Schuffenecker 1991). In order to elucidate the mechanism underlying the wide intra- and interracial variety in MBP level in serum, Naito et al. (1999) studied the transcriptional regulation of human MBP. The 5′ RACE analysis of Hep G2 RNA indicated the presence of an exon, designated as "exon 0," upstream of exon 1 and thus, two MBP mRNAs with different sizes of 5′-noncoding regions were detected: the longer transcript starting at exon 0 and the shorter one at exon 1. Promoter analysis revealed that the transcript starting from exon 1 predominates over the one that starts from exon 0. The NH2-terminal residue of rat liver MBP, glutamic acid, is preceded by a predominantly hydrophobic stretch of 18 amino acids, which was assumed

to be a signal peptide. Near the NH2-terminal, there was a collagen-like domain, which consisted of 19 repeats of the sequence Gly-X-Y. Here, X and Y were frequently proline and lysine. Three proline and lysine residues were hydroxylated, and one of the latter appeared to link to galactose. Computer analysis of several lectins for sequence homology suggested that the COOH-terminal quarter of the MBP is associated with the calcium binding as well as carbohydrate recognition (Oka et al. 1987) (Fig. 23.4).

23.2.2.1 Evolutionary Relationship to the Asialoglycoprotein Receptor

cDNAs encoding MBP-A from rat liver is encoded by four exons separated by three introns. The NH2-terminal, collagen-like portion of the protein is encoded by the first two exons. These exons resemble the exons found in the genes for nonfibrillar collagens in that the intron which divides them is inserted between the first two bases of a glycine codon and the exons do not have the 54- or 108-bp lengths characteristic of fibrillar collagen genes. The carbohydrate-binding portion of MBP-A is encoded by the remaining two exons. This portion of the protein is homologous to the CRD of the hepatic asialoglycoprotein receptor, which is encoded by four exons. It appears that the three COOH-terminal exons of the asialoglycoprotein receptor gene have been fused into a single exon in the MBP-A gene. The organization of the MBP-A gene is very similar to the arrangement of the gene encoding the highly homologous pulmonary surfactant apoprotein, although one of the intron positions is shifted by a single amino acid (Drickamer and McCreary 1987).

23.2.2.2 MBL Genotypes and MBL Levels

Low serum concentrations of MBP are associated with three independent mutations in codons 52, 54, and 57 (Turner 2003) of exon 1, resulting in amino acid replacement of Arg-52 to Cys, Gly-54 to Asp, and Gly-57 to Glu, respectively, all of which occurred in the collagen-like domain. These replacements appear to inhibit oligomerization of the structural unit of the molecule and consequently abolish the ability to initiate complement activation without impairing the original lectin-binding specificity to oligosaccharide ligands. Information from increasing literature suggests that MBL deficiency, which mainly results from the three relatively common single point mutations in exon 1 of the gene, predisposes both to infection by extracellular pathogens and to autoimmune disease. In addition, the protein also modulates disease severity, at least in part through a complex, dose-dependent influence on cytokine production. The mechanisms and signaling pathways involved in such processes remain to be elucidated (Jack et al. 2001). The prevalence of mutations in the MBL gene is about 10%, but in Africa South of Sahara it is as high as 30% (Juul-Madsen et al. 2003).

MBL2 coding alleles associated with low blood levels are present in up to 40% of Caucasoids, with up to 8% having genotypes associated with profound reduction in circulating MBL levels. Low-producing MBL2 variants and low MBL levels are associated with increased susceptibility to and severity of a variety of infective illnesses, particularly when immunity is already compromised – for example, in infants and young children, patients with cystic fibrosis, and after chemotherapy and transplantation. These observations suggest that administration of recombinant or purified MBL may be of benefit in clinical settings where MBL deficiency is associated with a high burden of infection (Worthley et al. 2005).

23.2.3 Regulation of MBP Gene

Suppression by Glucocorticoid: In order to elucidate the mechanism underlying the wide intra- and interracial variety in MBP level in serum, Naito et al. (1999) studied the transcriptional regulation of human MBP. In addition, a hepatocyto-specific nuclear factor, (HNF)-3, which controls the expression of hepatocyte specific genes, up-regulates the transcription of human MBP from exon 1, while a glucocorticoid, which is known to up-regulate acute phase proteins, markedly suppresses MBP transcription. In addition, polymorphism also occurs in the promoter region at two positions (Madsen et al. 1995). Functional promoter analysis indicated that three haplotype variants as to these positions, HY, LY, and LX, exhibit high, medium and low promoter activity, respectively (Naito et al. 1999).

Upregulation by Interleukins: The MBP mRNA expression in human hepatoma cell line HuH-7 is increased by IL-6, dexamethasone, and heat shock, decreased by IL-1, and unaffected by IFNγ, TNFα and TGFβ. The binding of IL-2 to its receptor (IL-2Rβ) induces IL-2Rβ phosphorylation by the tyrosine kinase associated with the T-cell receptor (TCR) complex. This mechanism is due to the putative lectin activity of IL-2 (Cebo et al. 2002), which is a calcium-independent lectin specific for oligomannosidic N-glycans with five and six mannose residues. This lectin activity is preserved after binding of IL-2 to IL-2Rβ. IL-2 behaves as a bifunctional molecule that associates IL-2Rβ with specific glycoprotein ligands of the TCR complex including a glycosylated form of CD3 (Zanetta et al. 1996). Thus, the action of cytokines appears to be mediated by specific transcription factors.

Effect of Growth Hormone: Studies in animals and humans indicate that growth hormone (GH) and insulin-like growth factor-I (IGF-I) modulate immune function. In normal patients, GH therapy increases the level of MBL, and the treatment of acromegalics with pegvisomant decreases the levels of MBL. The effect on MBL was thought to be due to a specific action of GH, since IGF-I treatment did not affect MBL level. However, GH or hormone replacement therapy (HRT) in Turner syndrome (TS) influences the serum levels of MBL and other proteins participating in the innate immune defense such as surfactant protein D (SP-D) and vitamin D binding protein (DBP). The treatment with GH significantly increases MBL and SP-D levels in TS patients, while HRT marginally decreased DBP. Whether the present findings suggest a link between the endocrine and the immune system needs further examination (Gravholt et al. 2004).

Thyroid Hormone Regulates MBL Levels: Studies have indicated the existence of causal links between the endocrine and immune systems and cardiovascular disease. In all hyperthyroid patients, MBL levels are increased – median (range), 1,886 ng/ml before treatment and decreased to 954 ng/ml from normal levels of 1,081 ng/ml after treatment. Administration of thyroid hormones to healthy persons induced mild hyperthyroidism and increased MBL levels significantly to 1,714 ng/ml. Since MBL is part of the inflammatory complement system, the modulation by thyroid hormone, of complement activation may play a role in the pathogenesis of a number of key components of thyroid diseases (Riis et al. 2005).

23.2.4 Structure-Function Relations

MBL has an oligomeric structure (400–700 kDa), built of subunits that contain three identical peptide chains of 32 kDa each. Although MBL can form several oligomeric forms, there are indications that dimers and trimers are not biologically active and at least a tetramer form is needed for activation of complement. MBL in the blood is complexed with another protein, a serine protease called MASP-2 (MBL-associated serine protease). The crystal structure of the CRD of a rat mannose-binding protein, determined as the holmium-substituted complex, reveals an unusual fold consisting of two distinct regions, one of which contains extensive nonregular secondary structure stabilized by two holmium ions. The structure explains the conservation of 32 residues in all C-type carbohydrate-recognition domains, suggesting that the fold seen is common to these domains (Weis et al. 1991, 1998).

The basic structural unit is a triple helix of MBL peptides, which aggregate into complement-fixing higher-order structures (tetramers, pentamers and hexamers). MBP forms a trimeric helical structure via interactions of the collagenous tails that are stabilized by disulfide bonds in the cysteine-rich amino terminal region. These trimers aggregate to generate three or six trimers in a "bouquet" organization (Fig. 23.1). Each CRD in the trimer is separated by approximately 53 Å, which is critical to the function of the lectin. This is because each individual CRD has a relatively low affinity and low specificity for glycan ligands and can bind to glycans rich in *N*-acetylglucosamine, *N*-acetylmannosamine, fucose, and glucose. The spacing between CRDs provides regulation and enhances the potential interactions with extended mannan-containing glycoconjugates, especially those on bacteria, yeast, and parasites. Weis and Drickamer (1999) determined crystal structure at 1.8 A Bragg spacings of a trimeric fragment of MBP-A, containing the CRD and the neck domain that links the carboxy-terminal CRD to the collagen-like portion of the intact molecule. The neck consists of a parallel triple-stranded coiled coil of α-helices linked by four residues to the CRD (Chang et al. 1994). The isolated neck peptide does not form stable helices in aqueous solution. The carbohydrate-binding sites lie at the distal end of the trimer and are separated from each other by 53 A. The carbohydrate-binding sites in MBP-A are too far apart for a single trimer to bind multivalently to a typical mammalian high-mannose oligosaccharide. Thus MBPs can recognize pathogens selectively by binding avidly only to the widely spaced, repetitive sugar arrays on pathogenic cell surfaces. Sequence alignments revealed that other C-type lectins might have a similar oligomeric structure, but differences in their detailed organization will have an important role in determining their interactions with oligosaccharides.

Phagocytic Interaction Site on MBL: The phagocytic activity induced by MBL and other molecules that contain a collagen-like region contiguous with a pattern recognition domain is mediated by C1qR(P). The specific interaction site was identified through two mutants, one of which has five GXY triplets deleted below the kink region of MBL and the other one having only two of the GXY triplets deleted below the kink. These mutants, which failed to enhance phagocytosis, suggested the importance of a specific sequence GEKGEP in stimulating phagocytic activity. Similar sequences were detected in other defense collagens, implicating the consensus motif GE(K/Q/R)GEP as critical in mediating the enhancement of phagocytosis through C1qR(P) (Arora et al. 2001).

MASP-Binding Sites in MBP: Mutations in the collagen-like domain of serum MBP interfere with the ability of the protein to initiate complement fixation through MASPs. Studies with truncated and modified MBPs and synthetic peptides demonstrated that MASPs bind on the C-terminal side of the

hinge region formed by an interruption in the Gly-X-Y repeat pattern of the collagen-like domain. The binding sites for MASP-2 and for MASP-1 and -3 overlap but are not identical. The two most common naturally occurring mutations in MBP result in substitution of acidic amino acids for glycine residues in Gly-X-Y triplets on the N-terminal side of the hinge. Studies showed that the triple helical structure of the collagen-like domain is largely intact in the mutant proteins, but it is more easily unfolded than in wild-type MBP. Thus, the effect of the mutations is to destabilize the collagen-like domain, indirectly disrupting the binding sites for MASPs (Wallis et al. 2004).

23.2.5 Functions of MBL

23.2.5.1 Functions as an Opsonin

MBL has also been proposed to function directly as an opsonin by binding to carbohydrates on pathogens and then interacting with MBL receptors on phagocytic cells, promoting microbial uptake and stimulating immune responses (Fig. 23.5). This was described by Kuhlman et al. who observed that binding of MBL to *Salmonella montevideo* resulted in an MBL-dependent uptake by monocytes (Kuhlman et al. 1989). Thus MBL can interact directly with receptor(s) on the surface of monocytes and several potential MBL receptors have been proposed, although their likelihood is debated in the literature. Calreticulin has emerged as the main candidate, but further studies are required to confirm its interaction with MBL and its role in the phagocytosis of pathogens. Ip et al. (2008) has shown that MBL modifies cytokine responses through cooperation with TLR2/6 in the phagosome. Although the stimulation of the inflammatory response was not caused by enhanced phagocytosis, bacterial engulfment was required. This study demonstrates the importance of phagocytosis in providing the appropriate cellular environment to facilitate cooperation between molecules.

MBL has the capacity to modify the efficiency of uptake and the expression of other phagocytic receptors. Activation of the complement system via MBL-associated serine proteases (MASPs) (Kerrigan and Brown 2009; Dahl et al. 2001; Stover et al. 1999; Thiel et al. 1997), results in deposition of complement on the microbial surface that can lead to uptake via complement receptors (Neth et al. 2002). However, inhibition of bacterial growth associated with the MBL-MASP activation of complement has also been observed, without any enhancement of phagocytosis (Ip and Lau 2004). This indicates that the specific responses induced by MBL may be dependent on the nature of the microbial target. MBL can also influence expression of other PRRs, as demonstrated by the ability of MBL to augment the uptake of *S. aureus* through the up-regulation of scavenger receptor A (SR-A) (Ono et al. 2006) (Fig. 23.5).

Recognition of Pathogens: MBL has a major protective effect through activation of the complement system via MBL-associated serine proteases (MASPs). This can cause the lysis of Gram-negative bacteria and also opsonize a wide spectrum of potential pathogens for phagocytosis. MBL may also influence phagocytosis in the absence of complement activation through an interaction with one or more collectin receptors. This may also be the basis for a direct effect of the protein on inflammatory responses. MBL forms individual complexes with MBL-associated serine proteases (MASP)-1, -2, -3 and a truncated form of MASP-2 (MAp19) and triggers the lectin pathway of complement through MASP-2 activation. MBL, like C1q, is a six-headed molecule that forms a complex with two protease zymogens, which in the case of the MBL complex are MASP-1 and MASP-2. MASP-1 and MASP-2 are closely homologous to C1r and C1s, and all four enzymes are likely to have evolved from gene duplication of a common precursor. When the MBL complex binds to a pathogen surface, MASP-1 and MASP-2 are activated to cleave C4 and C2. Thus the MBL pathway initiates complement activation in the same way as the classical pathway, forming a C3 convertase from C2b bound to C4b. Humans deficient in MBL experience a substantial increase in infections during early childhood, indicating the importance of the MBL pathway for host defense.

The Mannan-Binding Lectin Pathway Is Homologous to the Classical Pathway: MBP has been shown to have complement-dependent bactericidal activity; for example, *Escherichia coli* strains *K12* and *B*, which have exposed *N*-acetylglucosamine and L-glycero-D-mannoheptose, respectively, are killed by MBP with the help of complement. MBP serves as a direct opsonin and mediates binding and uptake of bacteria that express a mannose-rich O-polysaccharide by monocytes and neutrophils. MBP functions as a β-inhibitor of the influenza virus and protects cells from HIV infection by binding to gp120, a high mannose-type oligosaccharide-containing envelope glycoprotein on HIV. MBL can alter the function of microbial structures, such as gp120 of HIV, to prevent infection. In addition, the α-mannosidase inhibitor 1-deoxymannojirimycin-treated baby hamster kidney (BHK) cells, which have high mannose-type oligosaccharide exposed on their surfaces, can be killed by MBP with the help of complement. MBP activates complement through interactions with complement subcomponents C1r/C1s or two novel C1r/C1s-like serine proteases, MBP-associated serine proteases (MASP-1 and MASP-2). The MBP-mediated complement activation is named the MBP pathway. The protein may also interact with the components of other cascade systems such as the clotting system, which will have a role in microbial pathogenesis. An understanding of these basic mechanisms will be vital if we are to use purified or recombinant MBL in therapeutic applications (Jack et al. 2001).

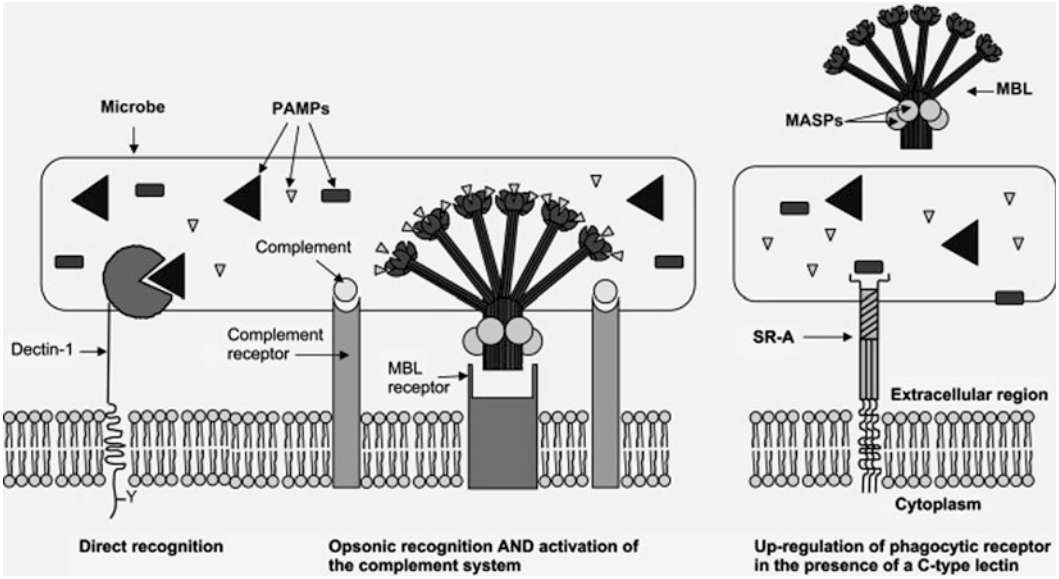

Fig. 23.5 Direct recognition, opsonisation, complement activation and receptor up-regulation by C-type lectins. Phagocytic C-type lectins can directly recognise PAMPs on the surface of microbes and mediate phagocytosis (e.g. Dectin-1). Alternatively, soluble C-type lectins can interact directly with pathogens to promote opsonisation of the microbe (e.g. MBL) which can subsequently be phagocytosed via specific receptors. In addition, some C-type lectins can activate complement leading to its deposition on the microbial surface and phagocytosis mediated by complement receptors (e.g. MBL associated MASPS are activated on binding to pathogens which in turn cleave complement components and activate the complement system). Finally, C-type lectins can cause up-regulation of other phagocytic receptors, independently of their binding to the microbe [e.g. MBL up-regulation of scavenger receptor A (*SR-A*)] (Adapted with permission from Kerrigan and Brown 2009 © Elsevier)

The MBL binds through multiple lectin domains to the repeating sugar arrays that decorate many microbial surfaces, and is then able to activate the complement system through a MBL-associated protease-2. For selected Gram-negative organisms, such as *Salmonella* and *Neisseria*, the relative roles of lipopolysaccharide (LPS) structure and capsule are important in binding; the LPS is of major importance. Studies on clinically relevant organisms showed that MBL binding leads to activation of purified C4, suggesting that the bound lectin is capable of initiating opsonophagocytosis and/or bacterial lysis.

Normal mammalian cells such as circulating blood cells are usually covered with complex oligosaccharides terminated with sialic acids and do not bind MBP. On the other hand, malignant transformations or viral infections modify the oligosaccharide structures on cell surfaces, and some tumor tissues have been shown to bind to MBP. Ma et al. (1999) showed that MBP recognizes and binds specifically to oligosaccharide ligands expressed on the surfaces of a human colorectal carcinoma. Interestingly, the recombinant vaccinia virus carrying human MBP gene was demonstrated to possess a potent growth-inhibiting activity against human colorectal carcinoma cells transplanted in nude mice and a significant prolongation of life span of tumor-bearing mice. Unexpectedly, the mutant MBP, which had essentially no complement-activating activity, was nearly as active as wild-type MBP. These results indicated that MBP has a cytotoxic activity, which was termed as MBP-dependent cell-mediated cytotoxicity (MDCC).

23.2.5.2 Functions in Antibody-Independent Pathway of Complement System

The complement system comprises a complex array of enzymes and non-enzymatic proteins that is essential for the operation of innate as well as the adaptive immune defense. The complement system is activated in three ways: by the classical pathway which is initiated by antibody-antigen complexes, by the alternative pathway initiated by certain structures on microbial surfaces, and by an antibody-independent pathway that is initiated by the binding of mannan-binding lectin to carbohydrates. The MBL is structurally related to the complement C1 subcomponent, C1q, and seems to activate the complement system through an associated serine protease known as MASP-1 or p100, which is similar to C1r and C1s of the classical pathway. The MBL binds to specific carbohydrate structures

found on the surface of a range of microorganisms, including bacteria, yeasts, parasitic protozoa and viruses, and exhibits antimicrobial activity by killing that is mediated by the terminal, lytic complement components or by promoting phagocytosis. The level of MBL in blood plasma is genetically determined, and deficiency is associated with frequent infections in childhood, and possibly also in adults. A new MBL-associated serine protease (MASP-2) which shows a striking homology with the reported MASP-1 and the two C1q-associated serine proteases C1r and C1s has also been identified. Thus complement activation through MBL, like the classical pathway, involves two serine proteases and may antedate the development of the specific immune system of vertebrates (Thiel et al. 1997).

IgA, an important mediator of mucosal immunity, activates the complement system via the lectin pathway. Results indicate a dose-dependent binding of MBL to polymeric, but not monomeric IgA coated in microtiter plates. This interaction involves the carbohydrate recognition domain of MBL. MBL binding to IgA results in complement activation, which is proposed to lead to a synergistic action of MBL and IgA in antimicrobial defense. These results may explain glomerular complement deposition in IgA nephropathy (Roos et al. 2001).

23.2.5.3 Modulation of Associated Serine Proteases

MBP neutralizes invading microorganisms by binding to cell surface carbohydrates and activating MBP-associated serine proteases-1, -2, and -3 (MASPs). MASP-2 subsequently cleaves complement components C2 and C4 to activate the complement cascade. The MBP modulates MASP-2 activity in two ways. First, MBP stimulates MASP-2 auto-activation by increasing the rate of autocatalysis when MBP.MASP-2- complexes bind to a glycan-coated surface. Second, MBP occludes accessory C4-binding sites on MASP-2 until activation occurs. Once these sites become exposed, MASP-2 binds to C4 while separate structural changes create a functional catalytic site able to cleave C23. Only activated MASP-2 binds to C2, suggesting that this substrate interacts only near the catalytic site and not at accessory sites. MASP-1 cleaves C2 almost as efficiently as MASP-2 does, but it does not cleave C23. Thus MASP-1 probably enhances complement activation triggered by MBP.MASP-2 complexes, but it cannot initiate activation itself (Chen and Wallis 2004). MASP-1 probably enhances complement activation triggered by MBP. MASP-2 complexes, but it cannot initiate activation itself.

In the human lectin pathway, MASP-1 and MASP-2 are involved in the proteolysis of C4, C2 and C3. The human MBL-MASP complex contains a new 22 kDa protein [small MBL-associated protein (sMAP)] bound to MASP-1. The nucleotide sequence of sMAP cDNA revealed that it is a truncated form of MASP-2, consisting of the first two domains (i.e. the first internal repeat and the EGF-like domain) with four different C-terminal amino acids. sMAP mRNAs are expressed in liver by alternative polyadenylation of the MASP-2 gene, in which a sMAP-specific exon containing an in-frame stop codon and a polyadenylation signal is used. The involvement of sMAP in the MBL-MASP complex suggests that the activation mechanism of the lectin pathway is more complicated than that of the classical pathway (Takahashi et al. 1999).

23.2.5.4 Ra-Reactive Factor: A Complex of MBL and the Serine Protease

The Ra-reactive factor (RaRF) is a complement dependent antimicrobial factor that reacts with numerous microorganisms such as viruses, bacteria, fungi and protozoa. The RaRF consists of a complement-activating component (CRaRF) called P 100 component (MASP) and a polysaccharide-binding (mannose-binding) component (Matsushita et al. 1992). The cDNA for RaRF-P100 from the human liver contains an ORF of 2,097 nt encoding a protein of 699 aa residues in the cloned cDNA of 4,489 nt. This protein exhibits 87.4% amino acid homology with mouse P100, and 36.4% and 37.1% homologies with that of the C1r and C1s subcomponents of human complement, respectively. The P100, together with the C1r and C1s, forms a unique protein family having the same module/domain constitution (Takada et al. 1993; Takayama et al. 1999).

The polysaccharide-binding component of RaRF consists of two different 28-kDa polypeptides, P28a and P28b. Partial amino acid sequences of P28a and P28b indicated that these polypeptides are similar to MBP-C and MBP-A (liver and serum MBP respectively) (Drickamer et al. 1986; Oka et al. 1987). The primary structures of P28a and P28b deduced from cDNAs are homologous to one another. They have three domains, a short NH2-terminal domain, a collagen-like domain, and a domain homologous to regions of some carbohydrate-binding proteins, as in rat MBPs. The P28a and P28b polypeptides are the products of two unique mouse genes which are expressed in hepatic cells (Kuge et al. 1992).

23.3 MBP/MBL from Other Species

23.3.1 Rodents

Rat liver MBP contains two distinct but homologous polypeptides. Each polypeptide consists of three regions: (a) an NH2-terminal segment of 18–19 amino acids which is rich in cysteine and appears to be involved in the formation of interchain disulfide bonds which stabilize dimeric and trimeric forms of the protein, (b) a collagen-like domain consisting of 18–20 repeats of the sequence Gly-X-Y and containing 4-hydroxyproline residues in several of the Y

positions, and (c) a COOH-terminal carbohydrate-binding domain of 148–150 amino acids. The sequences of the COOH-terminal domains are highly homologous to the sequence of the COOH-terminal carbohydrate-recognition portion of the chicken liver receptor for N-acetylglucosamine-terminated glycoproteins and the rat liver asialoglycoprotein receptor. Each protein is preceded by a cleaved, NH2-terminal signal sequence, consistent with the finding that this protein is found in serum as well as in the liver. The entire structure of the mannose-binding proteins is homologous to dog pulmonary surfactant apoprotein (Drickamer et al. 1986). Rat liver MBP is specific for mannose and N-acetylglucosamine and is encoded by two species of mRNA of 1.4 and 3.5 kb respectively. The sequence of the open reading frame of 3.5 kb mRNA was completely identical to that of the 1.4 kb mRNA. Each mRNA species arises from one gene, with the differences in size most easily being accounted for by differential utilization of the polyadenylation sites of one transcript (Wada et al. 1990).

Mouse *Mbl1* and *Mbl2* have five and six exons, respectively. The structure of the mouse Mbl genes is similar to that of the rat and human MBP genes and shows homology to the other collectin genes, with the entire carbohydrate recognition domain being encoded in a single exon and all introns being in phase 1. The MBP encoded by mouse Mbl1 with three cysteines in the first coding exon, like the rat Mbl1 and human MBL, is capable of a higher degree of multimerization and has apparent ability to fix complement in the absence of antibody or C1q. However, the structural features of other exons, that is, the larger size of collagen domain region in the first coding exon (64 bp in Mbl2 vs 46 bp in Mbl1) and the smaller size of the exon encoding the trimerization domain (69 bp in Mbl2 vs 75 bp in Mbl1) revealed that the single human MBL gene is closely related to rodent Mbl2 rather than rodent Mbl1. In contrast to the evolution of bovine surfactant protein-D – which duplicated in bovidae after divergence from humans, MBP gene most likely duplicated prior to human-rodent divergence, and that the human homolog to *Mbl1* was perhaps lost during evolution (Sastry et al. 1995). The deduced amino acid sequence of the mouse MBP, as with rat and the human forms, have an NH2 terminus that is rich in cysteine, which stabilizes a collagen α-helix followed by a carboxyl- terminal carbohydrate binding domain. Though, the mouse MBP-A mRNA, as with the human, is induced like the acute phase reactant serum amyloid P protein, yet the expression of mouse MBP-C mRNA is not regulated above its low baseline level. The expression of both MBP-A and -C mRNA is restricted to the liver under basal and stress conditions (Sastry et al. 1991).

23.3.2 Primates MBL

The nucleotide and amino-acidic sequences of MBL2 among primates are highly homologous, underlining the importance of this molecule in the defense system against pathogen invasions. In particular, in the collagen-like domain that confers the characteristic structure to MBL2 protein, the identity among primates is really high. In the carbohydrate recognition domain, primates' group-specific amino-acidic mutations did not result in changes of the structure or function of this MBL2 domain. Results indicate that MBL2 is well conserved in agreement with its important role in the immune system. However, same 'plasticity' of the MBL2 human gene was not observed in non-human primates, where a frequency of more than 1% of nucleotide variations was described in the coding and promoter regions (Verga Falzacappa et al. 2004).

A bovine cDNA encodes a protein of 249 aa residues with a signal peptide of 19 aa. This MBP has the ability to activate complement and expressed only in liver. The bovine MBP is likely to be a homologous to human MBP and bears homology to rat and mouse MBP-C which are localized in liver cells rather than to rat and mouse MBP-A found in serum (Kawai et al. 1997). C-type lectins in alligator liver, termed alligator hepatic lectin (AHL) is specific for mannose/L-fucose and contained equal amounts of 21- and 23-kDa bands on SDS (Lee et al. 1994).

23.4 Similarity Between C1Q and Collectins/Defense Collagens

23.4.1 Structural Similarities

The C1q is the first component of classical pathway of complement activation that links the adaptive humoral immune response to the complement system by binding to antibodies complexed with antigens. C1q can, however, also bind directly to the surface of certain pathogens and thus trigger complement activation in the absence of antibody. Collectins family of macromolecules is characterized by a conserved, collagen-like region of repeating Gly-X-Y triplets contiguous with a non-collagen-like sequence. The structural similarity between the collectins and C1q has been demonstrated: They all contain multiple polypeptides which are organised into subunits containing triple-helical stalks throughout their collagen-like regions and globular 'heads' in C-terminal regions. Four, or six, of these structures are associated via distinct, short, N-terminal regions to form oligomeric

molecules seen in electron microscope. The overall structural similarity between C1q and collectins, however, does not extend to similarity in amino acid sequences over the C-terminal regions. The C-terminal regions of C1q, unlike those of collectins, do not contain conserved residues found in CRDs present in C-type lectins. Instead, C1q has a high degree of homology to collagen sequences (Type VIII and X) and this is consistent with the fact that, unlike collectins, C1q binds to protein motifs in IgG, or IgM, rather than to carbohydrate structures. Also, despite showing interruptions in their collagen-like regions, collectins do not always display a 'bend' in their collagen-like 'stalks' similar to that which is seen in C1q. Therefore, C1q may be more closely related to collagens than to collectins.

23.4.2 Functional Similarities

The C1q and MBL function to eliminate invading microorganisms by activating the classical pathway (C1q) or the lectin pathway (MBL and ficolin) (Ma et al. 2004) of the complement system by transmitting a signal from the recognition domains of the globular heads to their collagen-like domains, which autoactivates their associated serine proteases ($C1r_2s_2$ or mannan-binding lectin-associated serine proteases (Sim and Tsiftsoglou 2004).

Defense collagens modulate the cytokine expression, specifically by monocyte cytokine production by phagocyte cells under conditions in which phagocytosis is enhanced. Under conditions in which phagocytosis is enhanced, C1q and MBL modulate cytokine production and contribute signals to human peripheral blood mononuclear cells, leading to the suppression of LPS-induced pro-inflammatory cytokines, IL-1α and IL-1β, and an increase in the secretion of cytokines IL-10, IL-1 receptor antagonist, monocyte chemoattractant protein-1, and IL-6. Thus, defense collagen-mediated suppression of a pro-inflammatory response may be an important step in the avoidance of autoimmunity during the clearance of apoptotic cells (Fraser et al. 2006). Understanding the mechanisms involved in defense collagen and other soluble pattern recognition receptor modulation of the immune response may provide important novel insights into therapeutic targets for infectious and/or autoimmune diseases and additionally may identify avenues for more effective vaccine design (Fraser and Tenner 2008; Paidassi et al. 2007).

It is proposed that C1q, MBL, and other opsonins prevent autoimmunity and maintain self-tolerance by supporting the efficient clearance of apoptotic material, as well as by actively modulating phagocyte function. In absence of danger, defense collagens appear to recognise and remove apoptotic cells (Nauta et al. 2003). C1q and MBL have been shown to bind directly to apoptotic cell surfaces and apoptotic cell blebs via their globular heads (Navratil et al. 2001). Interaction of the collagen-like tails with the phagocyte surface triggers apoptotic cell ingestion via macropinocytosis (Ogden et al. 2001). Indeed, deficiencies and/or knock-out mouse studies have highlighted critical roles for C1q, SP-D, and MBL and other soluble pattern recognition receptors in the clearance of apoptotic bodies and protection from autoimmune diseases along with mediating protection from specific infections (Botto et al. 1998; Gabriaud et al. 2003; Stuart et al. 2005), in which mice exhibit impaired clearance of apoptotic cells. Deficiency of C1q is a risk factor for the development of autoimmunity in humans and mice (Botto and Walport 2002; Mitchell et al. 2002; Miura-Shimura et al. 2002).

23.4.3 Receptors for Defense Collagens

A number of putative MBL-binding proteins/receptors have been proposed including cC1qR/Crt (Klickstein et al. 1997), C1qRp (Tenner et al. 1995; Stuart et al. 1997) and CR1 (Ghiran et al. 2000). However, it is unclear whether MBL is acting as a direct opsonin or is merely enhancing other complement pathways and/or antibody-mediated phagocytosis. Membrane receptors for the soluble 'defense collagens' – naturally occurring chimeric molecules that contain a recognition domain contiguous with a collagen-like triple helical domain and play a role in protecting the host from pathogens entering the blood, lung and other tissues – have been isolated and are being characterized. These receptors are key to understanding the mechanisms by which defense collagens influence cellular responses in clearance of cellular debris or to initiate the responses that lead to the destruction of microbial pathogens.

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Pulmonary SP-A: Forms and Functions

24

Anita Gupta and Rajesh K. Gupta

24.1 Pulmonary Surfactant Proteins

24.1.1 Pulmonary Surfactant

Pulmonary surfactant is a complex mixture of lipids and proteins, and is synthesized and secreted by alveolar type II epithelial cells and bronchiolar Clara cells. It acts to keep alveoli from collapsing during the expiratory phase of the respiratory cycle. After its secretion, lung surfactant forms a lattice structure on the alveolar surface, known as tubular myelin. Surfactant proteins (SP)-A, B, C and D make up to 10% of the total surfactant. SP-B and SP-C are relatively small hydrophobic proteins, and are involved in the reduction of surface-tension at the air-liquid interface. SP-A and SP-D, on the other hand, are large oligomeric, hydrophilic proteins that belong to the collagenous Ca^{2+}- dependent C-type lectin family (known as "Collectins"), and play an important role in host defense and in the recycling and transport of lung surfactant. There is increasing evidence that surfactant-associated proteins A and -D (SP-A and SP-D, respectively) contribute to the host defense against inhaled microorganisms. Based on their ability to recognize pathogens and to regulate the host defense, SP-A and SP-D have been recently categorized as "Secretory Pathogen Recognition Receptors". In nut-shell, the four lung-specific surfactant-associated proteins: SP-A, SP-B, SP-C, and SP-D serve a number of different roles, including enhancement of surface-active properties of surfactant glycerophospholipids, surfactant phospholipid reutilization, and immune defense within the alveolus. The basic structures of SP-A and SP-D include a triple-helical collagen region and a C-terminal homotrimeric lectin or CRD. The trimeric CRDs can recognize carbohydrate or charge patterns on microbes, allergens and dying cells, while the collagen region can interact with receptor molecules present on a variety of immune cells in order to initiate clearance mechanisms. Gene knock-out mice models of lung hypersensitivity and infection, and functional characterization of cell surface receptors have revealed the diverse roles of SP-A and SP-D in control of lung inflammation (Kishore et al. 2005)

24.1.2 Pulmonary Surfactant Protein A

24.1.2.1 Intracellular and Intraalveolar Localization

Although SP-A is secreted by type II pneumocytes as a component of pulmonary surfactant, its secretion pathway as well as its subcellular localization in the human lung are uncertain. In adult human lungs, only type II pneumocytes could be identified as SP-A positive cells within the parenchymal region. SP-A was localized mainly in small vesicles and multivesicular bodies close to the apical plasma membrane. Only few lamellar bodies were weakly labeled at their outer membranes. The strongest SP-A activity was found over tubular myelin figures. Labeling for SP-A was also found in close association with the surface film and unilamellar vesicles. Results supported that SP-A is mainly secreted into the alveolar space via an alternative pathway that largely bypasses the lamellar bodies. After secretion, the outer membranes of unwinding lamellar bodies become enriched with SP-A when tubular myelin formation is initiated. SP-A may also be involved in the transition of tubular myelin into the surface film (Ochs et al. 2002). Immunogold labeling showed that SP-A occurs predominantly at the corners of the tubular myelin lattice. Being an integral component of the lamellar body, peripheral compartment and secreted surfactant membranes support the concept that lysozyme may participate in the structural organization of lung surfactant (Haller et al. 1992).

24.1.2.2 Lamellar Bodies

Lamellar bodies of identical periodicity and ultrastructural geometry are present in lung (type II pneumocytes), serosal mesothelium (peritoneum, pleura, and pericardium), and

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joints (type A and type B synoviocytes) (Dobbie 1996). SP-A was detected in the cytoplasm of type II cells as asymmetrically distributed punctate fluorescent bodies that resembled lamellar bodies. Most of the SP-A was located within bodies of the type II cell. Although, lamellar bodies are enriched in SP-A, yet it is insufficient for structural transformation to tubular myelin and surface film formation in vitro. SP-A is secreted from type II cells primarily by a pathway separate from lamellar bodies. Surfactant secretion by lung type II cells occurs when lamellar bodies fuse with plasma membrane and surfactant is released into alveolar lumen. The fetal lung secretes significant quantities of surfactant during late gestation to initiate respiration at birth. A pathway of extracellular routing of SP-A prior to its accumulation in lamellar bodies in cultured type II cells has been suggested (Jain et al. 2005).

24.2 Structural Properties of SP-A

24.2.1 Human SP-A: Domain Structure

SP-A, the major surfactant protein, is a C-type lectin that activates macrophages in the lung alveolus and plays an important role in immune defense. SP-A is synthesized primarily by type II pneumonocytes and is developmentally regulated in fetal lung in concert with surfactant glycerophospholipid synthesis (Boggaram and Mendelson 1988). In humans and baboons, SP-A is encoded by two highly similar genes, *SP-A1* and *SP-A2* (Katyal et al. 1992; McCormick et al. 1994), whereas, in rabbits (Chen et al. 1992), rats (Fisher et al. 1987; Smith et al. 1995) and mice (Korfhagen et al. 1996), SP-A is encoded by a single-copy gene. Genomic analysis of human cellular DNA with SP-A cDNA demonstrated the presence of multiple hybridizing fragments that are not accounted for by available SP-A gene sequences. The functional SP-A gene is present in human chromosome 10. The functional SP-A gene and the pseudogene are syntenic (Korfhagen et al. 1996).

The lung and serum SP-A and SP-D are assembled as oligomers of trimeric subunits. Each subunit consists of four major domains: a short cysteine-containing NH_2-terminal cross-linking domain (N); a triple helical collagen domain of variable length; a trimeric coiled-coil linking domain (L; also referred to as the neck); and a carboxyl-terminal, C-type lectin CRD (Fig. 24.1a, b). Interactions between the amino-terminal domains of SP-D subunits have been shown to be stabilized by interchain disulfide bonds (Brown-Augsburger et al. 1996; Crouch et al. 1994), and similar mechanisms stabilize the oligomerization of SP-A and most other collectins. The primary protein component of human SP-A is a 32 kDa glycoprotein. A cDNA clone encoding for rat pulmonary SP-A encodes the sequence of 56 amino acids at the N-terminus. Isolated rat alveolar type II cells contain two species of mRNA for this protein (Fisher et al. 1987). Structural details of SP-D have been discussed in Chap. 25.

The primary structure of mature SP-A is conserved among different mammals with some differences. It consists of four structural domains: (1) an NH_2-terminal segment involved in intermolecular disulfide bond formation; (2) a collagen-like domain characterized by 23 Gly-*X*-*Y* repeats with an interruption near the midpoint of the domain; (3) α-helical coiled-coil domain, which constitutes the neck region between the collagen and the globular domain; and (4) a COOH-terminal globular domain involved in phospholipid binding and in Ca^{2+}-dependent binding of oligosaccharides (McCormack 1998; Head et al. 2003). SP-A is modified after translation (cleavage of the signal peptide, proline hydroxylation, and Asn^{187}-linked glycosylation). Unlike SP-As from other mammalian species, baboon and human SP-As consist of two polypeptide chains, SP-A1 and SP-A2. The major differences between mature SP-A1 and SP-A2 are in the collagen domain. Electron micrographs show that SP-A like MBP assembles as six trimers arrayed in parallel and in register, resembling a bouquet of tulips (Voss et al. 1988), whereas SP-D is cruciform, formed by radial arrangements of four trimers (Crouch et al. 1994) (Fig. 24.1a, b). Properties of SP-A are dependent on the presence of calcium. Each SP-A monomer binds two to three calcium ions in conditions chosen as similar to those found in the alveolar lumen. The higher affinity site for calcium is located in the non-collagenous carboxy-terminal end of SP-A that contains a CRD homologous to other C-type lectins. The binding of calcium to this region of SP-A causes a conformational change (Haagsman et al. 1990; Sohma et al. 1993).

24.2.1.1 SP-A is Assembled as Large Oligomer

Larger aggregates of SP-A are formed by means of disulfide bond formation within a short N-terminal segment containing two cysteine residues. More variability in the degree of oligomerization was observed with recombinant human SP-A than with natural canine SP-A. Collagenase digestion suggested that the full assembly of protein subunits was dependent on an intact collagen-like domain. Cysteines in noncollagen domain of SP-A form intrachain bonds between residues 135–226 and 204–224. The CD spectra of both recombinant and natural SP-A were consistent with the presence of a collagen-like triple helix. As determined by the change in ellipticity at 205 nm, thermal transition temperatures of canine, natural human, and recombinant SP-A were 51.5°C, 52.3°C, and 42.0°C, respectively (Haagsman et al. 1989). SP-A exists as fully assembled complexes with 18 polypeptide chains, but it is also consistently found in smaller oligomeric forms. This is true for both the water- and lipid-soluble fractions of SP-A. Hydroxyproline residues are present in SP-A in a region

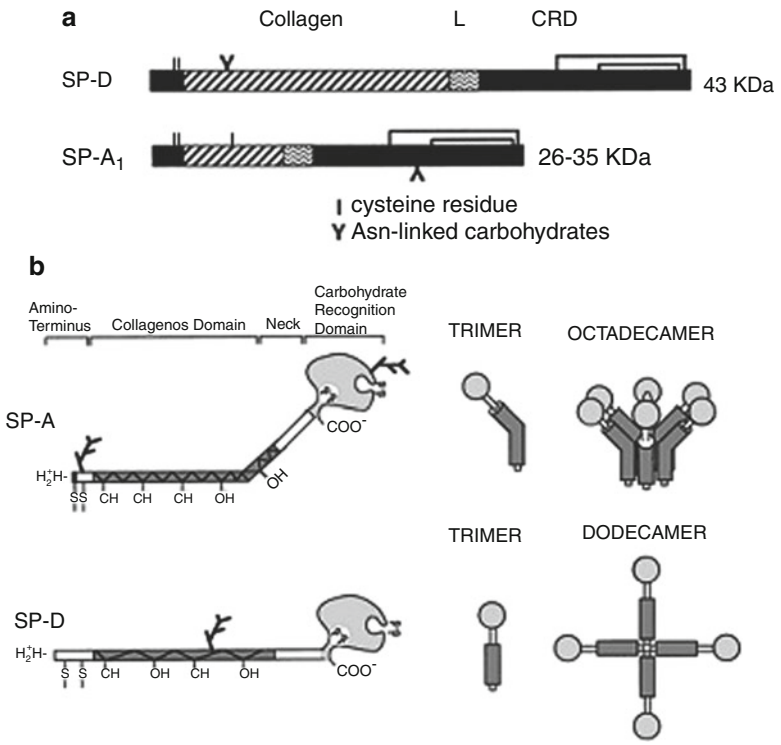

Fig. 24.1 Structural organization of SP-A and SP-D. (**a**) Each subunit of SP-A and SP-D consists of four major domains: a short cysteine-containing NH_2-terminal cross-linking domain (*N*); a triple helical collagen domain of variable length; a trimeric coiled-coil linking domain (*L*; also referred to as the neck); and a carboxyl-terminal, C-type lectin carbohydrate recognition domain (*CRD*) Differences in monomer size result from differences in the length of the collagen domain (*diagonal lines*). The Asn-linked oligosaccharide in SP-D is located in the collagen domain, whereas the Asn-linked sugar in human SP-A is located within the CRD (Crouch 1998). (**b**) Monomers form trimers that, in turn, form higher ordered oligomers of 18 (6 × 3) units for SP-A and 12 (4 × 3) units for SP-D. Molecules are not drawn to scale. SP-D is significantly larger than SP-A (Wright 1997) (Adapted with permission from Mason et al. 1998 © The American Physiological Society)

with a collagen-like sequence. SP-A treated with tunicamycin to block N-glycosylation and with 2,2-dipyridyl to inhibit the hydroxylation of proline residues suggests that hydroxylation of proline residues is required for perfect oligomerization of SP-A and for thermal stability in interaction with lipid (García-Verdugo et al. 2003; McCormack 1998; Palaniyar et al. 2001).

24.2.1.2 The Collagen-Like Domain of Human SP-A

A collagen-like domain of human SP-A consists of 23 Gly-X-Y repeats with an interruption near the midpoint of this domain. McCormack et al. (1999) suggested that : (1) SP-A trimerization does not require the collagen-like region or interchain disulfide linkage; (2) the N-terminal portion of collagen-like domain in SP-A is required for specific inhibition of surfactant secretion but not for binding to liposomes or for enhanced uptake of phospholipids into type II cells; (3) N-terminal interchain disulfide linkage in SP-A can functionally replace the N-terminal segment for lipid binding, receptor binding, and enhancement of lipid uptake, and (4) the N-terminal segment is required for the association of trimeric subunits into higher oligomers, for phospholipid aggregation, and for specific inhibition of surfactant secretion and cannot be replaced by disulfide linkage alone for these activities. To define the function of the kink region of SP-A, two mutated proteins were constructed to disrupt the interruption of Gly-X-Y repeats: SP-ADEL, which lacks the Pro47-Cys48-Pro49-Pro50 sequence at the interruption, and SP-AINS, in which two glycines were introduced to insert Gly-X-Y repeats (Gly-Pro47-Cys48-Gly-Pro49-Pro50). EM revealed that both mutants form octadecamers that lack a bend in the collagenous domain. The interruption of Gly-X-Y repeats in the SP-A molecule is critical for the formation of a flower bouquet-like octadecamer and contributes to SP-A's capacity to aggregate phospholipid liposomes (Uemura et al. 2006). The C-terminal non-collagenous domain of SP-A is essential for its correct folding and assembly, as judged by the secretion of various deletion mutants expressed in COS cells. Results suggest that three prefolded non-collagenous domains register and act as a nucleation center for the folding of the collagenous triple helix which

proceeds in a zipper-like fashion towards the N-terminus (Spissinger et al. 1991).

The mutant SP-A1(δAVC,C6S) was thermally less stable for collagen structure with increased susceptibility to trypsin degradation. The supratrimeric assembly of human SP-A is essential for collagen triple helix stability at physiological temperatures, protection against proteases, protein self-association, and SP-A-induced ligand aggregation. The supratrimeric assembly is not essential for the binding of SP-A to ligands and anti-inflammatory effects of SP-A (Sánchez-Barbero et al. 2005, 2007).

24.2.2 Structural Biology of Rat SP-A

24.2.2.1 Chemical Modification of SP-A Alters Binding Affinity to Alveolar Type II Cells

Alkylation of SP-A with excess of iodoacetamide yielded forms of SP-A that did not inhibit surfactant lipid secretion and did not compete with ^{125}I-SP-A for cell surface binding. Kuroki et al. (1988b) concluded that: (1) cell surface binding activity of rat SP-A is directly related to its capacity to inhibit surfactant lipid secretion; (2) the lectin activity of SP-A against mannose ligands does not appear to be essential for cell surface binding; (3) the human SP-A derived from individuals with alveolar proteinosis exhibits different binding characteristics from rat SP-A.

24.2.2.2 Amino Terminal Processing of SP-A Results in Cysteinyl Isoforms

Triple-helix formation from three polypeptide chains requires previous trimerization of COOH-terminal globular domains by a trimeric α-helical coiled-coil (Head et al. 2003; McCormack et al. 1997a; Elhalwagi et al. 1997). Octadecamers appear to be formed by lateral association of the NH_2-terminal half of six triple-helical stems, forming a microfibrillar end piece stabilized by disulfide bonds at the NH_2-terminal region (Head et al. 2003) (Fig. 24.2). In rat SP-A two cysteine residues are involved in multimer formation: a cysteine in the 6 position of the NH_2-terminal segment (McCormack et al. 1997b) and another cysteine in the position −1, which is the last position of the signal peptide, one amino acid before the NH_2 terminus (Elhalwagi et al. 1997). Human SP-A, as well as rhSP-A1 and rhSP-A2 also have considerable NH_2-terminal heterogeneity, in which about 50–75% of human SP-A molecules contain Cys^{-1} isoforms (Elhalwagi et al. 1997; Wang et al. 2004; Garcia-Verdugo et al. 2003). In human SP-A1, four cysteine residues are potentially involved in the arrangement of the disulfide bonding: two cysteine residues in the NH_2-terminal segment (Cys^{-1} and Cys^{6}), another in the middle of the collagen-like sequence within the Pro-Cys-Pro-Pro interruption (Cys^{48}), and a fourth cysteine at position 65 (Cys^{65}) in the collagen domain near the neck. Cys^{65} is substituted by Arg^{65} in the human SP-A2 polypeptide chain (Floros and Hoover 1998). The biological functions of rat SP-A are dependent on intact disulfide bonds. Reducible and collagenase-reversible covalent linkages of as many as six or more subunits in the molecule indicate the presence of at least two NH2-terminal interchain disulfide bonds. Primary structure of rat SP-A predicts that only Cys^{6} in this region is available for interchain disulfide formation. However, direct evidence for a second disulfide bridge was obtained by analyses of a set of mutant SP-As with deletions from the reported NH2-terminus. Thus, a post- translational modification results in naturally occurring rat SP-A cysteinyl isoforms, which are essential for multimer formation (Elhalwagi et al. 1997)

The role of the intermolecular bond at Cys^{6} and the collagen-like domain (Gly8-Pro80) in the interactions of SP-A with phospholipids and alveolar type II cells were investigated using mutant forms of protein. McCormack et al. (1997b) suggested that: (1) the Cys^{6} interchain disulfide bond of SP-A is required for aggregation of liposomes and for potent inhibition of surfactant secretion. (2) The collagen-like region is required for competition with ^{125}I-SP-A for receptor occupancy and specific inhibition of surfactant secretion in the presence of competing sugars. (3) Both the NH2-terminal disulfide and the collagen-like region are required to enhance the association of phospholipid vesicles with type II cells Yet, Zhang et al. (1998) suggested that neither Cys^{-1}-dependent multimerization nor the longer SP-A isoform is absolutely required for oligomeric association of trimeric SP-A subunits, SP-A/phospholipid interactions, or the regulation of surfactant secretion or uptake from type II cells by rat SP-A.

24.2.2.3 Glu^{195} and Arg^{197} Are Essential for Receptor Binding

The binding of SP-A to its high affinity receptor on alveolar type II cells is thought to be dependent on a CRD, while the interaction with lipids is attributed to the hydrophobic neck region of the molecule. To explore the role of the CRD in the interactions of SP-A with type II cells and lipids, McCormack et al. (1994) introduced mutations into the cDNA to encode for the substitutions Glu^{195}—>Gln and Arg^{197}—>Asp (SP-Ahyp, Gln^{195},Asp^{197}) and expressed the mutant protein in insect cells. Wild type SP-A produced in insect cells does not contain hydroxyproline (SP-Ahyp), but like rat SP-A it binds to carbohydrate affinity columns, lipids, and the SP-A receptor and is a potent inhibitor of the secretion of surfactant from type II cells. Study indicated that the binding of SP-A to its receptor and the inhibition of surfactant secretion are critically dependent on the carbohydrate binding specificity of CRD. Furthermore, phospholipid aggregation and augmentation of phospholipid uptake into

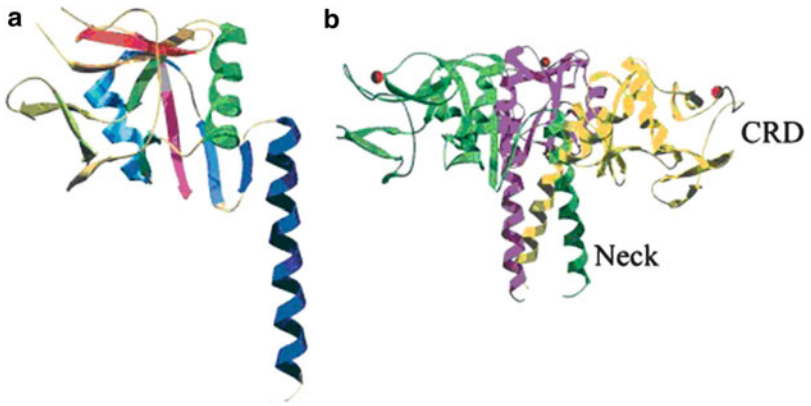

Fig. 24.2 Structure of CRD and neck domain (Δ1–80/N187S) of rat SP-A. A recombinant rat SP-A containing only the amphipathic linking domain and the CRD and lacking the consensus for asparagine-linked glycosylation (ΔN1-P80,N187S) was synthesized in insect cells using baculovirus vectors and purified on affinity column. (**a**) Ribbon diagram of (Δ1–80/N187S) monomer with secondary structure. (**b**) the (Δ1–80/N187S) trimer with each monomer colored differently and calcium ions in primary site are shown as *red spheres* (Adapted by permission from Head et al. 2003 © The American Society for Biochemistry and Molecular Biology)

type II cells are mediated by the C-terminal region of SP-A by a mechanism that is distinct from phospholipid binding.

Rat serum MBP-A is structurally analogous to SP-A and functionally equivalent to SP-A. MBP-A does not possess the ability to interact with lipids and type II cells. Honma et al. (1997) investigated the SP-A region involved in binding lipids and interacting with type II cells by using chimeric proteins with MBP-A. The binding of chimeras to DPPC and GalCer with activity comparable to recombinant SP-A suggested that MBP-A region of Glu185-Ala221 could functionally replace the homologous SP-A region of Glu195-Phe228 without loss of interaction with lipids and type II cells (Honma et al. 1997).

24.2.3 3-D Structure of SP-A Trimer

The crystal structure of the trimeric CRD and neck domain of SP-A was solved to 2.1-A resolution. The crystal structure was derived from a fragment, which encompasses the extended, helical neck region (residues 81–108) and the globular CRD (residues 111–228) of the SP-A monomer. The fragment forms a stable non-covalent trimer in solution (McCormack et al. 1999). The secondary structure of the CRD includes three α-helices and 11 short (3–7 residues) strands of β-sheet (Fig. 24.2b). The overall fold of the CRD in SP-A is similar to that of SP-D and MBP. The main structural differences are restricted to the region that includes surface loops and calcium binding sites. The neck region consists of a single amphipathic α-helix (residues 84–108) culminating in a reverse turn (residues 108–111) that leads into the first β-sheet of the CRD domain. The neck regions of three monomers are intertwined around a three-fold rotational symmetry axis to form the coiled-coil trimer (Fig. 24.2b). The trimer is held together primarily by means of hydrophobic face of the amphipathic helix from the neck of each monomer. This hydrophobic face is composed of side chains of Leu^{87}, Leu^{91}, Ile^{94}, Ile^{98}, Thr^{101}, Met^{102}, and Leu^{105}. The reverse turn is situated around the three-fold symmetry axis such that the polar side chains of Gln^{108} and Ser^{110} form H-bonds with their counterparts in the other two monomers (Fig. 24.2a). Three intermolecular salt bridges linking Glu^{90} and Lys^{95} of adjacent monomers provide additional stabilization. Intramolecular contacts between the neck and CRD are minimal in the SP-A monomer.

In the SP-A trimer, the interface between domains does not have the appearance of a freely mobile hinge region as it does in the monomer. Major contacts between the two domains are primarily intermolecular and mostly hydrophilic. In one such contact, the carboxylate group of the C terminus, Phe^{228}, of one monomer forms a salt bridge and an H-bond with the side chains of neck residues His^{96} and Gln^{100}, respectively, of another monomer. All three residues are invariant in mammalian SP-A sequences. The neck and CRD domains are oriented nearly perpendicularly to each other in SP-A, in contrast to both SP-D and MBP, where the interdomain angles are greater (i.e. "T" versus "Y" shapes, respectively).

Two metal binding sites were identified, one in the highly conserved lectin site and the other 8.5 A away. The interdomain CRD-neck angle is significantly less in SP-A than in SP-D, and MBL. This conformational difference may endow the SP-A trimer with a more extensive hydrophobic surface capable of binding lipophilic membrane components. The appearance of this surface suggests a putative binding region for membrane-derived SP-A ligands such as phosphatidylcholine and lipid A, the endotoxic lipid component of

bacterial lipopolysaccharide that mediates the potentially lethal effects of Gram-negative bacterial infection (Head et al. 2003).

Three-dimensional (3D) structure of SP-A in association with a lipid ligand (DPPC: egg phosphatidylcholine lipid monolayers) was determined using single particle electron crystallography and computational 3D reconstruction in combination with molecular modeling. TEM showed that SP-A subunits readily formed trimers and interacted with lipid monolayers, exclusively via the globular domains. The plane of the putative lipid-protein interface was relatively flat and perpendicular to the hydrophobic neck region; the cleft region in the middle of the trimer had no apparent charge clusters. Amino acid residues that were known to affect lipid interactions, Glu^{195} and Arg^{197}, were located at the protein-lipid interface. The molecular model indicated that the hydrophobic neck region of the SP-A did not interact with lipid monolayers but was instead involved in intratrimeric subunit interactions. The glycosylation site of SP-A was located at the side of each subunit, suggesting that the covalently linked carbohydrate moiety probably occupies the spaces between the adjacent globular domains, a location that would not sterically interfere with ligand binding (Palaniyar et al. 2000).

24.2.4 Bovine SP-A

24.2.4.1 Self-Aggregation of SP-A Depends on Ca^{2+} and Other Factors

Bovine SP-A forms extended fibers in the presence of calcium. On phosphatidylcholine or especially DPPC monolayers, SP-A at roughly 0.005 mg/ml formed large numbers of fibers and elaborate fibrous networks. The weak protein-protein interactions amongst free SP-As could be stabilized by phospholipids. In the presence of glycolipid GM1-ganglioside, SP-A's globular headgroup regions appeared enlarged and only small non-fibrous clusters were observed (Palaniyar et al. 1998b).

The importance of cations, particularly calcium, on SP-A function is well known and self-aggregation of bovine SP-A is Ca^{2+}-dependent. The concentration Ca^{2+} needed for half-maximal self-association ($K_{aCa}{}^{2+}$) depended on the presence of salts. Ca^{2+}-dependent SP-A aggregates formed in absence of NaCl are structurally different from those formed in its presence. Self-aggregation of SP-A can be pH and temperature-dependent and influenced by the presence of salts, which reduced the extent of self-association of the protein (Ruano et al. 2000). The CD spectra of bovine SP-A at various pH values indicated that α-helical content progressively decreased and that of β-sheet increased as the pH was reduced (Ruano et al. 1998). The quaternary organisation of bovine SP-A is altered by the presence of cations, especially calcium and sodium. There is a transition concentration, unique for each cation, at which a conformational switch occurs. The fact that the transition concentrations of cations are within physiological range suggests that cation-mediated conformational changes of SP-A could be operative in vivo (Ridsdale et al. 1999). The domains of SP-A that mediate lipid binding have been mapped. It was demonstrated that (1) lipid binding and pH-dependent liposome aggregation are mediated by CRD of SP-A, (2) distinct but overlapping domains within the CRD are required for pH- and Ca^{2+}-dependent liposome aggregation, and (3) conserved acidic and polar residues of the carbohydrate binding site of SP-A are essential for interactions with type II cells (McCormack et al. 1997c).

24.2.4.2 Substitution of His^{197} Creates Specific Lipid Uptake Defects

Mutagenesis of Glu^{195} and Arg^{197} of SP-A has implicated both residues as critical participants in the interaction of the molecule with alveolar type II cells and phospholipids. Substitution of Ala, Lys, His, Asp, and Asn for Arg at position 197 in SP-A action revealed that Ala^{197} retained complete activity in the SP-A functions and hence Arg^{197} is not essential for SP-A function. The Lys^{197} mutation displayed all functions of the wild type protein but exhibited a twofold increase in lipid uptake activity. The His^{197} mutation displayed all SP-A functions studied except for lipid uptake. The His^{197} mutation clearly demonstrated that lipid aggregation alone by SP-A is insufficient to promote lipid uptake by type II cells. Thus, specific interactions between type II cells and SP-A are involved in the phospholipid uptake processes (Pattanajitvilai et al. 1998)

Formation of Folds and Vesicles by DPPC Monolayers: Depositing DPPC organic solvent solutions in excess at an air:buffer interface led to the formation of elongated structures which could be imaged by TEM. The structures appeared to be DPPC folds protruding into the sol. The structures were frequently ordered with respect to one another, suggesting that they arose during lateral compression due to excess DPPC and are characteristic of a type of monolayer collapse phase. The elongated folds are unstable and can resolve by forming vesicles. Fold formation occurred at defined lipid concentrations above which more vesicles were observed. SP-A did not influence fold or vesicle formation but bound to the edges of these structures preferentially. It suggested that DPPC monolayers can form bilayers spontaneously in the absence of surfactant apoproteins, other proteins or agents (Ridsdale et al. 2001).

The temperature dependence of 2,850 cm^{-1} phospholipid acyl chain CH2 symmetric stretching frequencies in infra red (IR) spectrum showed a broad, reversible, melting event

from about 15°C to 40°C in both the lipid extract and the native surfactant. The SP-A from bovine lung lavage was reconstituted into a binary lipid mixture of acyl chain perdeuterated DPPC-d62/DPPG (85:15 w/w) at a ratio approximating to that in surfactant. High levels of SP-A induced an ordering of the phospholipids, as shown by an increase in the transition temperature of DPPC-d62 compared to the lipid model. In contrast, a mixture of the other surfactant proteins induced a progressive disordering of the phospholipids and disruption of the cooperativity of the melting event (Reilly et al. 1989).

Structural Changes in SP-A Induced by Cations Reorient the Protein on Lipid Bilayers: Cations influence the interaction of bovine SP-A with phospholipid vesicles made of DPPC and unsaturated phosphatidylcholine (PC) (Gurel et al. 2001). The SP-A octadecamers exist in an "opened-bouquet" conformation in the absence of cations and interact with lipid membranes via one or two globular headgroups. Calcium-induced structural changes in SP-A resulted into the formation of a clearly identifiable stem in a "closed-bouquet" conformation. This change, in turn, results in all of SP-A's globular headgroups interacting with the lipid membrane surface and with the stem pointing away from the membrane surface. These results give direct evidence that the headgroups of SP-A (comprising CRDs), and not the stem (comprising the amino-terminus and collagen-like region), interact with lipid bilayers. Study supported models of tubular myelin in which headgroups, not the tails, interacted with lipid walls of the lattice (Palaniyar et al. 1998a).

24.2.4.3 Formation of Membrane Lattice Structures with SP-A

Tubular myelin (TM), the pulmonary surfactant membranous structure contains elongated tubes that form square lattices. Atomic force microscopy (AFM) imaging of the unstained sections containing TM indicated a highly heterogeneous surface topography with height variations ranging from 10 to 100 nm. In tapping-mode AFM, tubular myelin was seen as hemispherical protrusions of 30–70 nm in diameter, with vertical dimensions of 5–8 nm. This study suggests detection of 3-D nanotubes present in low abundance in a biological macromolecular complex (Nag et al. 1999).

Under TEM dipalmitoylphosphatidyl choline-egg phosphatidyl- choline (1:1 wt/wt) bilayers formed corrugations, folds, and predominantly 47-nm-square latticelike structures. SP-A specifically interacted with these lipid bilayers and folds. Other proteolipid structures could act as intermediates for reorganizing lipids and SP-As. Such a reorganization could result to the localization of SP-A in the lattice corners and could explain, in part, the formation of TM-like structures in vivo (Palaniyar et al. 1999).

Surfactant Lipid Peroxidation Damages SP-A and Inhibits Interactions with Phospholipid Vesicles: Lipid peroxidation (LPO) affects the function of SP-A. Exposure of SP-A to LPO is associated with an increase in the level of SP-A-associated carbonyl moieties and a marked reduction in SP-A-mediated aggregation of liposomes. LPO initiated by an azo-compound resulted in enhanced protein oxidation and inhibited SP-A-mediated liposome aggregation. Exposure of SP-A to LPO resulted in oxidative modification and functional inactivation of SP-A by phospholipid radicals (Kuzmenko et al. 2004). At the same time, pulmonary SP-A inhibits the lipid peroxidation stimulated by linoleic acid hydroperoxide of rat lung mitochondria and microsomes. Under similar conditions, BSA was unable to inhibit lipid peroxidation stimulated by LHP, indicating that the effect is specific to SP-A (Terrasa et al. 2005).

24.2.5 SP-A in Other Species

Canine surfactant SP-A sediments with a sedimentation coefficient of 14 S, giving a Mr of about 700 kDa. The hydrodynamic data can be approximated as a prolate ellipsoid having an axial ratio of about 20. SP-A aggregates into a complex of 18 monomers, which may form six triple-helices. The shape of the complex is considerably more globular than collagen and is not consistent with end-to-end binding of the helices to form fibrous structures. Canine SP-A contains a domain of 24 repeating triplets of Gly-X-Y, similar to that found in collagens. SP-A forms a collagen-like triple helix when in solution. The CD of protein demonstrated a relatively large negative ellipticity at 205 nm, with a negative shoulder ranging from 215 to 230 nm. There was no positive ellipticity, and the spectrum was not characteristic of collagen. Trypsin hydrolysis resulted in a fragment with peak negative ellipticity at about 200 nm, without the negative shoulder. Further hydrolysis of this fragment with pepsin resulted in a CD spectrum similar to that of collagen. The spectrum of the collagen-like fragment was reversibly sensitive to heating to 50°C, and was irreversibly lost after treatment with bacterial collagenase (King et al. 1989).

A full-length cDNA to guinea pig pulmonary SP-A consists of 1,839 bp and is highly conserved at both nucleotide and amino acid sequence levels with those from other species. As expected, guinea pig SP-A mRNA is abundantly expressed in adolescent lung tissue and is undetectable in nonpulmonary tissues. SP-A mRNA expression is confined to cells of the alveolar epithelium with no expression in the bronchiolar epithelial cells, whereas SP-B mRNA is expressed in both alveolar and bronchiolar epithelial cell populations. This distinct expression pattern suggests that

the guinea pig lung will be a useful model in which to study expression of transcription factors implicated in the regulation of SP genes (van Eijk et al. 2001; Yuan et al. 1997). The complete cDNAs for ovine SP-A consists of 1,901 bp and encodes a protein of 248 amino acids (Pietschmann and Pison 2000). SP-A is present in pulmonary tissues of horses. The equine SP-A predicted from a cDNA of 747 bp consists of 248 amino acids, showing highest homology with SP-A from sheep (85.01%). The genomic DNA of equine SP-A, as in other species, includes three introns. There was no indication for the existence of two different SP-A genes (Hospes et al. 2002).

24.3 Cell Surface Receptors for SP-A

Different receptors have been identified on cells that work as receptors for SP-A or SP-D. In human monocytes the antibacterial activity of SP-A is mediated by a receptor for SP-A. A receptor with a high degree of specificity has been suggested for SP-A on alveolar macrophages (AMΦs). Chen et al. (1996) showed that secretagogues stimulates surfactant uptake through recruitment of SP-A receptors to the type II cell surface. Alveolar type II cells express a high affinity receptor for pulmonary SP-A, and the interaction of SP-A with these cells leads to inhibition of surfactant lipid secretion. The binding of SP-A to type II cells required Ca^{2+} and exhibited high-affinity for AMΦs (Kuroki et al. 1988a). The cell membrane receptor for SP-A regulates secretion of alveolar type II cells by negative feedback. Because TGF-β also inhibits Ca^{2+} fluxes, SP-A and TGF-β could be representative of a group of physiologic regulators that act by modulating intracellular Ca signaling (Strayer et al. 1999).

C1qR for Phagocytosis (C1qRp) (or CD93): The 126-kDa polypeptide is a functional common receptor or component of the receptor that mediates the enhancement of phagocytosis. Expression of this receptor is limited to cells of myeloid origin, platelets and endothelial cells, consistent with a relatively selective function. The amino acid sequence for C1qRp indicates that this surface glycoprotein receptor is a type I membrane protein of 631 amino acid containing a region homologous to C-type lectin CRDs, 5 EGF-like domains, a single transmembrane domain and a 47 amino acid intracellular domain. C1qRp cross-linked directly by monoclonal anti-C1qRp or engaged as a result of cell surface ligation of SP-A, as well as C1q and MBL, enhances phagocytosis (Nepomuceno et al. 1999; Tenner 1998). Ligation of human monocytes with immobilized R3, a IgM mAb recognizing C1qRp, also triggers enhanced phagocytic capacity of these cells in absence of ligand, verifying the direct involvement of this polypeptide in the regulation of phagocytosis. A distinct C1q receptor that triggers superoxide in polymorphonuclear leukocytes has been functionally characterized and designated as C1qRO2^{-}. Thus, there are at least two C1q receptor/receptor complexes (C1qRp and C1qRO2^{-}), each triggering distinct cellular responses, that can be expressed on the same, as well as on different, cell types (Tenner 1998) (see Chap. 41).

The C1q receptor (C1qR), identified from U937 cells and human tonsil lymphocytes, interacts with the C1q, mannose-binding protein, conglutinin and lung SP-A (Malhotra et al. 1990). This C1qR is an acidic glycoprotein, which exists as a dimer of Mr 115,000 in soluble form under non-denaturing conditions. The interaction of SP-A with U937 cells was found to up-regulate the surface expression of C1qR and to trigger the expression of an intracellular pool of C1qR (Malhotra et al. 1992). The collagen triple helix in Clq, MBP and SP-A, a cluster of similar charged residues has been suggested to be associated with receptor binding. A similar region of charge density occurs close to the N-terminus of conglutinin (Malhotra et al. 1993). SP-A binds to both *S. aureus* and monocytes and mediates the phagocytosis of the bacteria by these cells. Results demonstrate that C1qR mediates the phagocytosis of SP-A-opsonized *S. aureus* by monocytes (Geertsma et al. 1994).

SP-A Receptor 210 (SP-R210): Both type II cells and macrophages express a 210-kDa SP-A-binding protein, which was purified from U937 macrophage membranes and rat lung membranes. The 210-kDa protein was functional cell-surface receptor on type II cells, and the macrophage SP-A receptor is involved in SP-A-mediated clearance of pathogens (Chroneos et al. 1996; Yang et al. 2005). However, bovine AMΦs expose binding sites that are specific to SP-A, and depend on Ca^{2+} and on mannose residues. Two receptor proteins bound SP-A in a Ca^{2+}-dependent manner: one comprised of a 40-kDa protein showing mannose dependency and second of a 210-kDa protein, showing no mannose sensitivity (Plaga et al. 1998).

Glycoprotein-340: A Member of Scavenger Receptor Superfamily: A glycoprotein (Mr 340 kDa, gp-340) was purified from human bronchioalveolar lung washings from a patient with alveolar proteinosis. The gp-340 was found to bind to lung SP-D and also SP-A. Its molecular mass was 340 kDa in reduced state and decreased to 290 kDa in unreduced state. Binding between gp-340 and SP-D and

SP-A required the presence of calcium, and was not inhibited by maltose. Binding between gp-340 and SP-D is a protein-protein interaction rather than a lectin-carbohydrate interaction and that the binding to gp-340 takes place via carbohydrate recognition domain CRD of SP-D (Holmskov et al. 1997). Glycoprotein-340 interacts directly with pathogenic microorganisms and induces their aggregation, suggesting its role in innate immunity. Differential regulation of gp-340 in epithelial cell lines by phorbol myristate acetate indicates that gp-340 s involvement in mucosal defense and growth of epithelial cells may vary at different body locations and during different stages of epithelial differentiation (Kang et al. 2002)

SP-A Specific Receptor (SPAR): A pulmonary protein of ~30 kDa binds SP-A. The cDNAs encoding this protein were identified in human (4.1-kb) and porcine (1.8-kb) lung expression libraries. Both cDNAs encode similar ~32-kDa proteins that bind SP-A. The human and porcine SP-A recognition (SP-A specific receptor; SPAR) proteins resemble each other, as well as other cell membrane receptors. Their projected structures are consistent with cell membrane receptors. SPAR transcripts are expressed primarily in lung. SPAR-producing cells resemble the alveolar cells expressing SP-B and SP-C transcripts in appearance, location, and distribution (Strayer et al. 1993)

SP-A increases intracellular $[Ca^{2+}]_i$. Increased SP-A concentrations lead to a higher percentage of responding cells. SP-A also leads to a dose-dependent and transient generation of IP3. Secretagogue-induced secretion is inhibited by SP-A, which binds to SPAR on the surface of type II cells. The mechanism of SP-A-activated SPAR signaling involves PI3K, since PI3K inhibitor LY294002 rescued surfactant secretion from inhibition by SP-A. Thus, SP-A signals to regulate surfactant secretion through SPAR, via pathways that involve tyrosine phosphorylation, include insulin receptor substrate-1 (IRS-1), an upstream activator of PI3K, and entail activation of PI3K. This activation leads to inhibition of secretagogue-induced secretion of pulmonary surfactant (White and Strayer 2000).

24.4 Interactions of SP-A

24.4.1 Protein-Protein Interactions

SP-A has been involved in the physiology of reproduction. Consistent with the activation of ERK-1/2 and COX-2-induced by SP-A in myometrial cells, there are two major proteins recognized by SP-A in these cells. One of these SP-A targets is intermediate filament (IF) desmin. SP-A recognizes especially its rod domain, which is known to play an important role during the assembly of desmin into filaments. SP-A is colocalized with desmin filaments in myometrial cells. Interestingly, vimentin, the IF characteristic of leukocytes, is one of the major proteins recognized by SP-A in protein extracts of U937 cells. The ability of SP-A to interact with desmin and vimentin, and to prevent polymerization of desmin monomers, shed light on unexpected and wider biological roles of this collectin (Garcia-Verdugo et al. 2008a; Tanfin 2008).

Schlosser et al. (2006) described the molecular interaction between the extracellular matrix protein microfibril-associated protein 4 (MFAP4) and SP-A. MFAP4 is a collagen-binding molecule containing a C-terminal fibrinogen-like domain and a N-terminal located integrin-binding motif and interacts with SP-A via the collagen region in vitro. MFAP4 and SP-A are colocated in different lung compartments indicating that the interaction may be operative in vivo (Schlosser et al. 2006). The SP-A binds to sTLR4 and MD-2 and the reaction is Ca^{2+}-dependent. The direct interaction between SP-A and TLR4/MD-2 suggests the importance of supratrimeric oligomerization in the immunomodulatory function of SP-A (Yamada et al. 2006). The α1-antitrypsin and SP-A are major lung defense proteins. SP-A can bind α1-antitrypsin. The CRD of SP-A appeared to be a major determinant of interaction, by recognizing α1-antitrypsin carbohydrate chains. However, binding of SP-A carbohydrate chains to the α1-antitrypsin amino acid backbone and interaction between carbohydrates of both proteins was also possible (Gorrini et al. 2005). At least three different proteins from bovine lung soluble fraction bind to SP-A in a Ca^{2+}-dependent manner. The main protein with molecular mass of 32 kDa was identified as annexin IV. The lung annexin IV augmented the $Ca2^{+}$-induced aggregation of lung lamellar bodies from rats. The SP-A binds annexin IV through protein-protein interaction, although, both proteins are phospholipid-binding proteins (Sohma et al. 1995). Among several polypeptides, the 200-kDa major polypeptide reacted with SP-A on ligand blots. This polypeptide corresponded completely with nonmuscle (cellular) myosin heavy chain. A smaller polypeptide of 135 kD also binds SP-A and appears to be a proteolytic fragment of the 200 kD peptide (Michelis et al. 1994).

Kresch and Christian (1998) isolated two SP-A specific binding proteins from type II cells with M_r of 86 and >200 kDa under nonreducing conditions, but dissociated into proteins with M_r of 65, 55, and 50 kDa. The 86-kDa protein is a glycoprotein with ~30% of its mass as carbohydrate. A protein band of 63 kDa under reduced conditions was identified as rat homolog of human type II transmembrane protein p63 (CKAP4/ERGIC-63/CLIMP-63) (Kresch and Christian 1998). The p63 closely interacts with SP-A and may play a role in trafficking or the biological function

of SP-A (Gupta et al. 2006). EM confirmed endoplasmic reticulum and plasma membrane localization of P63 in type II cells with prominent labeling of microvilli (Bates et al. 2008).

The SP-A and SP-D bind transmembrane inhibitory regulatory protein α (SIRPα) through their globular heads to initiate a signaling pathway that blocks proinflammatory mediator production. In contrast, their collagenous tails stimulate proinflammatory mediator production through binding to calreticulin/CD91. Together a model is implied in which SP-A and SP-D help maintain a non/anti-inflammatory lung environment by stimulating SIRPα on resident cells through their globular heads. However, interaction of these heads with PAMPs on foreign organisms or damaged cells and presentation of collagenous tails in an aggregated state to calreticulin/CD91 stimulates phagocytosis and proinflammatory responses (Gardai et al. 2003).

24.4.2 Protein-Carbohydrate Interactions

Haurum et al. (1993) studied the influence of carbohydrates on the binding of SP-A, MBP and conglutinin to mannan. The order of inhibiting potency on the binding of SP-A was: N-acetylmannosamine > L-fucose, maltose > glucose > mannose. These results were independent of the source or method of extraction of SP-A, and generally consistent with earlier studies that examined binding to various saccharide-substituted supports (Haagsman et al. 1987). However, they are in obvious disagreement with a study that characterized the binding of dog SP-A to various neoglycoproteins, glycolipids, and neoglycolipids (Childs et al. 1992). Notably, the latter studies demonstrated a preferential recognition of galactose and an inability to compete with free monosaccharides. Although residues corresponding to Glu^{185} and Asn^{187} are found in essentially all members of the mannose binding subgroup, SP-A has a substitution in the position corresponding to Asn^{187} (Arg in dog and rat and Ala in human). Drickamer has suggested that this correlates with the capacity of SP-A to bind to a variety of sugars with comparatively weak affinity (Drickamer 1992). In any case, mutagenesis of corresponding residues in the putative carbohydrate binding site of SP-A (Glu^{195} to Gln and Arg^{197} to Asp) reversed the preference from mannose to galactose in affinity chromatography assays using mannose- and galactose-substituted supports (McCormack 1998). Deletion of the consensus sequences for N-linked glycosylation showed no obvious effect on lectin activity (McCormack 1998). Human SP-A has been shown to bind to lactosylceramide (Childs et al. 1992), galactosylceramide, and gangliotriaosylceramide (Kuroki et al. 1992). The optimal binding conditions were drastically different for three receptors (lactosylceramide, galactosylceramide, and gangliotetraosylceramide). At pH 24.4 and at 5 mM Ca concentration the binding affinity of SP-A followed the order: galactosylceramide > lactosylceramide > gangliotetraosylceramide (Hynsjö et al. 1995).

Collectins bind DNA from a variety of origins, including bacteria, mice, and synthetic oligonucleo- tides. Pentoses, such as arabinose, ribose, and deoxyribose, inhibit the interaction between SP-D and mannan, one of the well-studied hexose ligands for SP-D and d-forms of the pentoses are better competitors than the l-forms (Palaniyar et al. 2004).

24.4.3 SP-A Binding with Lipids

SP-A exhibits high affinity binding interactions with lipid as well as carbohydrate ligands. SP-A binds specifically to DPPC, the most abundant phospholipid species in surfactant (Kuroki and Akino 1991), whereas SP-D binds to the minor surfactant component phosphatidylinositol (Ogasawara et al. 1992; Persson et al. 1992). Both bind to the lipopolysaccharide moieties that decorate bacterial surfaces; SP-A recognizes the lipid A component of lipopolysaccharide (Kalina et al. 1995), whereas SP-D binds to the core oligosaccharide and *O*-oligosaccharides (Sahly et al. 2002). Electron micrographs and other data indicate that the globular CRD and collagen-like domains in both surfactant proteins are oriented proximal and distal, respectively, to the membrane surface (Palaniyar et al. 1998, 2002).

SP-A Specifically Binds DPPC: Although a monolayer of DPPC, the major component of pulmonary surfactant, is thought to be responsible for the reduction of surface tension at the air-liquid interface of the alveolus, the participation of unsaturated and anionic phospholipids and the three surfactant-associated proteins is suggested in the generation and maintenance of this surface-active monolayer. Pulmonary SP-A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro (Cockshutt et al. 1990). SP-A interacts strongly with a mixture of surfactant-like phospholipids. Although SP-A binds phosphatidylcholine and Sphingomyelin, it showed very strong binding to DPPC, it did not bind to phosphatidylglycerol (PG), phosphatidyl- inositol, phosphatidylethanolamine, and phosphatidylserine. SP-A also exhibited strong binding to distearoylphosphatidyl-choline, but weak binding to dimyristoyl-, 1-palmitoyl-2-linoleoyl, and dilinoleoyl-phosphatidylcholine. Thus, it indicates that (1) SP-A specifically and strongly binds DPPC, (2) SP-A binds the nonpolar group of phospholipids, (3) the second positioned palmitate is involved in DPPC binding, and (4) the specificities of polar groups of dipalmitoylglycerophospholipids also appear to be important for SP-A binding, (5) the phospholipid binding activity of SP-A is dependent upon calcium ions and the integrity of the collagenous domain of SP-A, but not on the

oligosaccharide moiety of SP-A. SP-A may play an important role in the regulation of recycling and intra- and extracellular movement of DPPC (Kuroki and Akino 1991; Yu and Possmayer 1998). Yu et al. (1999) suggested the importance of CRD and N-terminal dependent oligomerization in SP-A-phospholipid associations.

Binding of SP-A to Glycolipids: The SP-A binds to galactosylceramide and asialo-GM2, and that both saccharide and ceramide moieties in the glycolipid molecule are important for the binding of SP-A to glycolipids (Kuroki et al. 1992). Though, ^{125}I-SP-A bound to galactosylceramide and asialo-GM2, but failed to exhibit significant binding to GM1, GM2, asialo-GM1, sulfatide, and Forssman antigen. The study of ^{125}I-SP-A binding to glycolipids coated onto microtiter wells also revealed that SP-A bound to galactosylceramide and asialo-GM2. SP-A bound to galactosylceramides with non-hydroxy or hydroxy fatty acids, but showed no binding to either glucosylceramide or galactosylsphingosine. Results provide evidence for binding specificity for proteins that have Ca^{2+}-dependent CRDs and raise the possibility that glycosphingolipids are endogenous ligands for SP-A (Childs et al. 1992).

Interaction with Phospholipid Liposomes: Ca^{2+} ions induce an active conformation in SP-A, which rapidly binds to liposomes and mediates their aggregation. SP-A/liposome interaction shows $K_D = 5$ μM for interaction between SP-A and DPPC liposomes. With POPC, the complex formation proceeds at half the rate, leading to a lower final equilibrium level of SP-A- DPPC interaction. Distearoylphosphatidylcholine (DSPC) shows a stronger interaction than DPPC. Among phospholipid headgroups, phosphatidyl inositol (PI) and sphingomyelin (SM) interact comparable to DPPC, while less interaction is seen with phosphatidylethanolamine (PE) or with phosphatidylglycerol (PG). Thus both headgroup and fatty acid composition determine SP-A phospholipid interaction. However, the protein does not exhibit high specificity for either the polar or the apolar moiety of phospholipids (Meyboom et al. 1999).

Whereas SP-A binds to DPPC and galactosylceramide (GalCer), MBP-A binds phosphatidylinositol (PI). SP-A also interacts with alveolar type II cells. Specific monoclonal antibodies inhibit the interactions between SP-A and lipids or alveolar type II cells. The amino acid residues 174–194 of SP-A and the corresponding region of MBP-A are critical for SP-A-type II cell interaction and Ca^{2+}-dependent lipid binding of collectins (Chiba et al. 1999; Tsunezawa et al. 1998).

Intermolecular Cross-Links Mediate Aggregation of Phospholipid Vesicles by SP-A: SP-A plays a central role in the organization of phospholipid bilayers in the alveolar air space. SP-A in lung lavage exists in oligomeric forms (N = 6, 12, 18 . . .) mediated by collagen-like triple helices and intermolecular disulfide bonds. These protein-protein interactions, involving the amino-terminal domain of SP-A, facilitate the alignment of surfactant lipid bilayers into unique tubular myelin structures. Accelerated aggregation of unilamellar vesicles required SP-A and 3 mM free calcium. The initial rate of aggregation was proportional to the concentration of canine SP-A over lipid:protein molar ratios ranging from 200:1 to 5,000:1. Data demonstrate the importance of the quaternary structure (triple helix and intermolecular disulfide bond) of SP-A for the aggregation of unilamellar phospholipid vesicles (Ross et al. 1991).

SP-A is thought to influence the surface properties of surfactant lipids and regulate the turnover of extracellular surfactant through interaction with a specific cell-surface receptor. SP-A induces a rapid Ca^{2+}-dependent aggregation of phospholipid vesicles and mediated by Ca^{2+}-induced interactions between carbohydrate-binding domains and oligosaccharide moieties of SP-A. This mechanism of membrane interactions may be relevant to the formation of the membrane lattice of tubular myelin, an extracellular form of surfactant (Haagsman et al. 1991)

24.5 Functions of SP-A

24.5.1 Surfactant Components in Surface Film Formation

Pulmonary surfactant is a mixture of phospholipids and proteins that lines the distal airways and stabilizes the gas-exchanging alveolar units of the lung. Surfactant membranes are decorated with SP-A, one of the four known surfactant proteins. The main function of surfactant is to reduce the surface tension at the air/liquid interface in the lung. This is achieved by forming a surface film that consists of a monolayer which is highly enriched in dipalmitoylphosphatidylcholine and bilayer lipid/protein structures closely attached to it. The molecular mechanisms of film formation and of film adaptation to surface changes during breathing in order to remain a low surface tension at the interface are unknown. The results of several model systems give indications for the role of the surfactant proteins and lipids in these processes. Veldhuizen and Haagsman (2000) described and compared the model systems that are used for this purpose and the progress that has been made. Despite some conflicting results using different techniques, workers concluded that surfactant protein B (SP-B) plays the major role in adsorption of new material into the interface during inspiration. SP-C's main functions are to exclude non-DPPC lipids from the interface during expiration and to attach the bilayer structures to the

lipid monolayer. The SP-A appears to promote most of SP-B's functions. Veldhuizen and Haagsman (2000) described a model proposing that SP-A and SP-B create DPPC enriched domains which can readily be adsorbed to create a DPPC-rich monolayer at the interface. Further enrichment in DPPC is achieved by selective desorption of non-DPPC lipids during repetitive breathing cycles. Reports that SP-A is required for the formation of tubular myelin (Korfhagen et al. 1996) and other large surfactant aggregates (Veldhuizen et al. 1996) and for the preservation of low surface tensions in presence of serum protein inhibitors (Cockshutt et al. 1990) suggest that the protein serves multiple roles in surfactant function.

24.5.2 Recognition and Clearance of Pathogens

SP-A interacts with viruses, bacteria and fungi. Furthermore, SP-A binds to various other inhaled glycoconjugates. SP-A receptors on phagocytic cells have been described that are important to ensure rapid pathogen clearance. This innate defence system of the lung is particularly important during infections in young children when the acquired immune system has not yet become fully established. Also in later life SP-A could be very important to prevent the lungs from infections by pathogens not previously encountered. Lung collectins, SP-A and SP-D have a relatively high affinity for oligosaccharides. This is an important determinant of self/non–self recognition, because most carbohydrates in animals are monosaccharides. Therefore, SP-A and SP-D enhance microbial phagocytosis by innate immune cells, such as macrophages and neutrophils, by opsonizing and aggregating bacteria and viruses, by acting as an activation ligand, and by upregulating the expression of immune cell surface receptors that recognize microbes. In addition, SP-A and SP-D also promote apoptotic cell uptake by innate immune cells and regulate cytokine and free radical production in a context-dependent manner. As an example, SP-A inhibits LPS-stimulated nitric oxide (NO) production by alveolar macrophages isolated from normal lungs, but promotes NO production in macrophages that have been activated by IFN-γ (Stamme et al. 2000). Both proteins possess direct bactericidal activity against bacteria and fungi through unknown mechanisms (Wu et al. 2003) (Table 24.1). In addition, SP-A may limit the inflammatory response in the lungs, thus preventing damage to the delicate lung epithelia. Evidences suggest that SP-A may modulate the allergic response to various glycosylated inhaled antigens. The presence of SP-A (and SP-D) in other organs indicates that these collectins may have a general role in mucosal immunity (Haagsman 1998; Tino and Wright 1998).

Table 24.1 Immune functions of SP-A and SP-D (Adapted by permission from Kishore et al. 2005 © Springer)

1. Endotoxin clearance and regulation of LPS-induced inflammation
2. Recognition, agglutination and phagocytosis of viral, bacterial and fungal pathogens[$]
3. Macrophage and neutrophil activation and chemotaxis
4. Microbial growth inhibition
5. Non-specific defense molecules in tears, saliva and body secretions against pathogens
6. Reduced viral infectivity
7. Protection against intrauterine infection and inflammatory reactions
8. Modulation of inflammation
9. Recognition and clearance of apoptotic and necrotic cells
10. Anti-proliferative effects on B and T lymphocytes
11. Pattern recognition of glycoprotein allergens
12. Inhibition of IgE-allergen cross-linking
13. Suppression of histamine release from basophils and mast cells
14. Modulation of Th cytokine profile
15. Modulation of maturation and antigen presentation by dendritic cells

Functions of SP-A and SP-D as collectins: SP-A and SP-D bind to and opsonize viruses, bacteria, allergens, and apoptotic cells. SP-A and SP-D enhance microbial phagocytosis by aggregating bacteria and viruses. SP-A and SP-D also possess direct bactericidal effects and potentially bind to a variety of receptors to modulate immune cell cytokine and inflammatory mediator expression (Wright 2005)

Although, the absence of SP-A changed the structure and in vitro properties of surfactant, the in vivo function of surfactant in SP-A$^{-/-}$ mice was not changed under the conditions of experiments. In the absence of SP-A, the structure of pulmonary surfactant large aggregates is altered, tubular myelin is absent, and SP-A is no longer available to contribute to the formation of the typical lattice-like structure. However, the surface tension–reducing function of surfactant appeared unaffected, suggesting that SP-A does not appear to play a primary role in surfactant homeostasis. Although the mice were able to survive with no apparent pathology in a sterile environment, and respond similarly to wild-type (WT) mice in exercised or hyperoxic conditions (Ikegami et al. 1998, 2000), their pulmonary immune responses are insufficient during immune challenge. *SP-D*–null mice have a more complex phenotype. Even in the absence of pathogens, the *SP-D*–null mice display advancing alveolar proteinosis and increased lipid pools, indicating that SP-D has a role in surfactant homeostasis. Metalloproteinases were also elevated in their lungs, which developed an emphysema-like phenotype. *SP-A*– and *SP-D*–null mice were more susceptible to bacterial and viral infections and LPS-mediated inflammation, confirming roles for SP-A and SP-D in modulating immune responses in the lung (Wright 2005) (Table 24.1).

SP-A facilitates phagocytosis by opsonizing bacteria, fungi and viruses, stimulates the oxidative burst by

phagocytes and modulates pro-inflammatory cytokine production by phagocytic cells. Pulmonary surfactant also exhibits immunomodulatory functions and plays a key role in host defense against infection. For example, surfactant lipids suppress a variety of immune cell functions, most notably lymphocyte proliferation. Both SP-A and SP-D improve phagocytosis of pathogens by polymorphonuclear neutrophils (Hartshorn et al. 1998). SP-A can also provide a link between innate and adaptive immune responses by promoting differentiation and chemotaxis of dendritic cells. The SP-A-deficient gene-targeted mouse is susceptible to lung infection with multiple organisms (LeVine et al. 1997). One view that reconciles these apparently divergent functions of SP-A is that its high affinity for surfactant membranes is a mechanism for concentrating the protein at the front lines of defense against inhaled pathogens (Hawgood and Poulain 2001; McCormack and Whitsett 2002). In addition, SP-A and SP-D each modifies the in vivo response to instilled endotoxin, leading to decreased lung injury and reduced inflammatory cell recruitment (Borron et al. 2000; Greene et al. 1999). The SP-A-mediated interaction of lipids with type II cell plasma membrane may contribute, in part, to the lipid uptake process by type II cells (Kuroki et al. 1996).

24.5.3 SP-A: As a Component of Complement System in Lung

There is a functional complement system present in the lung that helps in removal of pathogens. Complement proteins of alternative and classical pathways of complement have been observed in bronchoalveolar lavage fluids (BALF) from healthy volunteers (Watford et al. 2000). Because of structural and functional similarities between C1q and pulmonary SP-A, SP-A may interact with and regulate proteins of complement system. The SP-A binds directly to C1q, though only weakly to intact C1. The binding of SP-A to C1q prevents the association of C1q with C1r and C1s, and therefore the formation of active C1 complex required for classical pathway activation through a common binding site for C1r and C1s or Clq. Furthermore, SP-A blocked the ability of C1q to restore classical pathway activity in C1q-depleted serum. Thus, SP-A may down-regulate complement activity through its association with C1q and may serve a protective role in lung by preventing C1q-mediated complement activation and inflammation along the delicate alveolar epithelium (Tenner et al. 1989; Watford et al. 2001).

24.5.4 Modulation of Adaptive Immune Responses by SPs

Increasing evidence shows that SP-A and SP-D modulate the functions of adaptive immune cells, DCs and T cells. DCs form a tightly meshed network within the upper airways, parenchyma, and alveolar airspace, and are ideally positioned to sample inhaled antigens (Lipscomb and Masten 2002). DC density increases upon inflammatory stimuli, particularly in the lower airways as a result of recruited myeloid DC precursors (Jahnsen et al. 2001). Although SP-A and SP-D both bind to DCs in a calcium-dependent manner, they have differential effects on DC function. For example, SP-D enhances antigen uptake and presentation by bone marrow–derived DCs, but SP-A inhibits DC maturation and phagocytic and chemotactic function in vitro (Brinker et al. 2001, 2003). Also, SP-A and SP-D inhibit T-cell proliferation via two mechanisms: an IL-2–dependent mechanism observed with accessory cell–dependent T cell mitogens and specific Ag, as well as an IL-2–independent mechanism of suppression that potentially involves attenuation of calcium signaling (Borron et al. 1998a). Moreover, SP-A–mediated inhibition of T cell proliferation might partially result from TGF-ß present in the SP-A preparations (Kunzmann et al. 2006). In contrast to the results, obtained using bone marrow–derived DCs, SP-D was found to decrease antigen presentation by DCs isolated from the mouse lung during both resting and inflammatory conditions (Hansen et al. 2007). A role for SP-D in regulating T-cell responses in vivo is demonstrated in a study by Fisher et al. (2002) showing that *SP-D*–null mice have an accumulation of $CD4^+$ and $CD8^+$ T cells expressing activation markers CD69 and CD25 in the perivascular and peribronchial regions of the lung. Thus, studies suggest that surfactant may be a critical regulator of organ-specific immune regulation in the lung, and that the hyporesponsive immunologic environment of the lung is, in part, facilitated by the actions of SP-A and SP-D in an effort to thwart inflammatory cascades that could potentially damage the lung and impair gas exchange. SP-A regulates the differentiation of immature DCs into potent T cell stimulators. Studies demonstrate that SP-A participates in the adaptive immune response by modulating important immune functions of DCs (Brinker et al. 2003).

SP-A Inhibits Human Lymphocyte Proliferation and IL-2 Production: Studies suggest that purified SP-A suppress both PHA and anti-CD-3 activated proliferation of human peripheral blood and tonsillar mononuclear cells in a dose-

dependent manner at concentrations as low as 50 pM. In contrast, ConA-stimulated PBMC proliferation was slightly augmented by the addition of SP-A. The inhibition of PHA-stimulated proliferation by SP-A was accompanied by corresponding decline in IL-2 concentration. The in vitro inhibitory effect of SP-A was not blocked by C1q. The effect was not mediated by CRD of SP-A, but a 36-amino acid Arg-Gly-Asp (RGD) motif-containing span of collagen-like domain was responsible for the inhibition of T cell proliferation (Borron et al. 1998b).

In addition to SP-A, SP-D could also reduce the incorporation of ^{3}H-thymidine into PBMC in a dose-dependent manner. A recombinant peptide composed of neck and CRD of SP-D [SP-D(N/CRD)] was also suppressive for lymphocyte proliferation. Inhibitory effect of both SP-A and SP-D on histamine release in the early phase of allergen provocation and suppressing lymphocyte proliferation in the late phase of bronchial inflammation suggest that SP-A and SP-D may be protective against the pathogenesis of asthma (Wang et al. 1998). These experiments emphasize a potential role for SP-A in dampening lymphocyte responses to exogenous stimuli.

TGF-β1 and SP-A Influence Immune-Suppressive Properties of SP-A on CD4^{+} T Lymphocytes: SP-A and TGF-β1 have been shown to modulate the functions of different immune cells and specifically to inhibit T lymphocyte proliferation. Recombinant human SP-A1 suppressed T lymphocyte proliferation and IL-2 mRNA expression. The effect of rSP-A1m was mediated through TGF-βRII and could be antagonized by anti-TGF-β1 neutralizing antibodies and sTGF-βRII. The association between SP-A and latent TGF-β1 provides a possible mechanism that regulate TGF-β1-mediated inflammation and fibrosis reactions in lung but also leads to possible misinterpretation of immune-modulator functions of SP-A. Monitoring of SP-A preparations for possible TGF-β1 is essential (Kunzmann et al. 2006).

SP-A Differentially Regulates IFN-γ- and LPS-Induced Nitrite Production by Rat AMΦ: The role of SP-A and SP-D in regulating production of free radicals and cytokines is controversial. Haddad et al. (1994) suggest that peroxynitrite, but not .NO or superoxide and hydrogen peroxide, in concentrations likely to be encountered in vivo, caused nitrotyrosine formation and decreased the ability of SP-A to aggregate lipids.

The state and mechanism of activation of the immune cell influence its response to SP-A. SP-A inhibited production of NO and iNOS in rat AMΦs stimulated with smooth LPS, which did not significantly bind SP-A, or rough LPS, which avidly bound SP-A. In contrast, SP-A enhanced production of NO and iNOS in cells stimulated with IFN-γ or INF-γ plus LPS. Neither SP-A nor SP-D affected baseline NO production, and SP-D did not significantly affect production of NO in cells stimulated with either LPS or IFN-γ. These results suggest that SP-A contributes to the lung inflammatory response by exerting differential effects on the response of immune cells, depending on their state and mechanism of activation (Stamme et al. 2000).

SP-A$^{-/-}$ mice produced significantly more TNF-α and NO than SP-A$^{+/+}$ mice after intratracheal administration of LPS. Human SP-A to SP-A$^{-/-}$ mice restored regulation of TNF-α and NO production to that of SP-A$^{+/+}$ mice without affecting other markers of lung injury. Neither binding of LPS by SP-A, nor enhanced LPS clearance were the primary means of inhibition. It seems that SP-A acts directly on immune cells to suppress LPS-induced inflammation and that the endogenous or exogenous SP-A inhibits pulmonary LPS-induced cytokine and NO production in vivo (Borron et al. 2000). SP-A suppresses NO production by activated AMΦs by inhibiting TNF-α secretion and NF-kB activation (Hussain et al. 2003). SP-A also modulates NO production by AMΦ in vitro but results are contrasting. In AMΦ and type II cells, iNOS is known to generate NO, which is upregulated by SP-A of different origin in a concentration – dependent manner, whereas type II cells were unresponsive to SP-A. The increase in NO production was associated with elevation in expression of iNOS. Results indicated that SP-A is the agonist and not a contaminating LPS (Blau et al. 1997).

SP-A Modulates Production of Cytokines: SP-A enhances concanavalin A-induced proliferation and levels of TNF-α, IL-1 α, 1β, and IL-6 by human peripheral blood mononuclear cells. A similar enhancement of TNF-α release by SP-A was observed by rat peripheral blood cells, splenocytes, and alveolar macrophages. In combinations of SP-A with surfactant lipids, the enhanced levels of TNF-α resulting from SP-A treatment decreased as the lipids increased. At higher relative concentrations of SP-A, the lipids had little or no effect. SP-A also enhanced the production of IgA, IgG, and IgM by rat splenocytes. These data demonstrate that SP-A is capable of modulating immune cell function in lung by regulating cytokine production and Ig secretion (Kremlev et al. 1997; Huang et al. 2002).

SP-A Exhibits Inhibitory Effect on Eosinophils IL-8-Production: Eosinophils are one of the important sources of cytokines such as IL-8 at the site of allergic inflammation. Pulmonary SP-A plays a potential role in modifying inflammation and the immune function. SP-A could modify IL-8 production and release by eosinophils stimulated with ionomycin. The regulating effect of SP-A on eosinophil cytokine generation was verified. SP-A inhibits the secretion of IL-8 in a dose-dependent fashion. SP-A attenuated the production and release of IL-8 by eosinophils in a

concentration-dependent manner. Thus, SP-A may have the potential to modify allergic inflammation by inhibiting the release and production of IL-8 by eosinophils (Cheng et al. 1998, 2000).

SP-A Inhibits Lavage-Induced Surfactant Secretion in Newborn Rabbits: Various agents stimulate the secretion of lung surfactant from alveolar type II cells by increasing intracellular Ca^{2+}, c-AMP, or diacylglycerol. In vivo and in vitro experiments with granular pneumocytes suggest that SP-A inhibits secretion of pulmonary surfactant (Corbet et al. 1992). Stilbene disulfonic acids are potent but reversible inhibitors of lung surfactant secretion (Chander and Sen 1993). While searching the role of C-terminal domain of SP-A in binding to type II cells and regulation of phospholipid secretion, Murata et al. (1993) demonstrated that the non-collagenous, C-terminal, domain of SP-A is responsible for the protein's inhibitory effect on lipid secretion and its binding to type II cells.

24.5.5 SP-A Stimulates Chemotaxis of AMΦ and Neutrophils

Besides accelerating pathogen clearance by pulmonary phagocytes, SP-A also stimulates alveolar macrophage chemotaxis and directed actin polymerization. Moreover, SP-A also stimulates neutrophil chemotaxis and supports a role in regulating neutrophil migration in pulmonary tissue (Schagat et al. 2003). The stimulatory effect of surfactant on macrophage migration was mediated and stimulated by SP-A. Heat treatment or reduction and alkylation of SP-A reduced its stimulatory effect. The SP-A stimulates migration primarily by enhancing chemotaxis (directed movement) rather than chemokinesis (random movement). The interaction of SP-A with macrophages may be mediated partly by the collagen-like domain of SP-A (Wright and Youmans 1993).

24.5.6 SP-A Inhibits sPLA2 and Regulates Surfactant Phospholipid Break-Down

Secretory type IIA phospholipase A2 (sPLA2-IIA) is one of the key enzymes that may potentially play a role in the pathogenesis of inflammatory diseases because its presence is observed in sera of patients with bacterial infection and in the airspaces of animals with endotoxin-induced acute lung injury (Arbibe et al. 1997). Lyso-phospholipids are generated from surfactant mediated by type-II PLA2. Lyso-phospholipids exert a major injurious effect on lung cell membranes during ARDS. This hydrolysis was inhibited by SP-A through a direct and selective protein-protein interaction between SP-A and sPLA2-II (Arbibe et al. 1998). The absence of SP-A exacerbates the susceptibility of surfactant to degradation by $sPLA_2$-IIA. SP-A inhibits Ca^{2+}-independent acidic PLA2 of rat lung homogenate or isolated lamellar bodies but does not affect Ca^{2+}-dependent alkaline enzyme. Thus, inhibition of sPLA2 activity by SP-A may be a protective mechanism by maintaining surfactant integrity during lung injury (Arbibe et al. 1998; Chabot et al. 2003a; Fisher et al. 1994). SP-A and DOPG/PG play a role in the surfactant-mediated inhibition of sPLA2-IIA expression in AMΦs and that this inhibition occurs via a downregulation of NF-kB activation (Wu et al. 2003). Peroxiredoxin 6 (Prdx6), a protein with both GSH peroxidase and PLA_2 activities, degrades internalized DPPC of lung surfactant. The PLA_2 activity is inhibited by SP-A. A direct interaction between SP-A and Prdx6 may be a mechanism for regulation of the PLA_2 activity of Prdx6 by SP-A (Wu et al. 2006).

24.5.7 SP-A Helps in Increased Clearance of Alveolar DPPC

SP-A is necessary for lungs to respond to hyperventilation or secretagogues with increased ^{3}H-DPPC uptake. SP-A improves the surfactant activity of lipid extracts by enhancing the rate of adsorption and/or spreading of phospholipid at the air/liquid interface resulting in the formation of a stable lipid monolayer at lower bulk concentrations of either phospholipid or calcium (Chung et al. 1989). The surfactant phospholipids are mainly phosphatidylcholine and phosphatidylglycerol species, but analysis has shown that dipalmitoylphosphatidyl choline (DPPC) and dioleylphosphatidylglycerol (DOPG), in particular, play a major role in maintaining the biophysical properties of the surfactant film (Hawgood and Poulain 2001). SP-A enhances the uptake of liposomes containing DPPC, 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC), or 1,2-dihexadecyl-sn-glycero-3-phosphocholine (DPPC-ether) by alveolar type II cells (Tsuzuki et al. 1993). SP-A also modulates the PLA_2-mediated degradation of internalized DPPC (Jain et al. 2003). Pulmonary SP-A binds the lipids DPPC and galactosylceramide and induces aggregation of phospholipid vesicles.

The SP-A also inhibits lipid secretion and enhances the uptake of phospholipid by alveolar type II cells. Kuroki et al. (1994) suggested that the CRD is essential for the SP-A functions of lipid binding, liposome aggregation, the inhibitory effect on lipid secretion, and the augmentation of lipid uptake by type II cells, and these activities are largely attributable to amino acid residues within the steric inhibitory footprint of antibody, 1D6, bound to the small disulfide loop region. The neck domain of SP-A may also be involved

in the process of SP-A-mediated uptake of phospholipids by alveolar type II cells.

In vitro studies have revealed that the surface activity of oxidized surfactant was impaired and that this effect could be overcome by SP-A. Mechanically ventilated, surfactant-deficient rats were administered either bovine lipid extract surfactant (BLES) or in vitro oxidized BLES. Instillation of an in vitro oxidized surfactant caused an inferior physiological response in a surfactant-deficient rat (Bailey et al. 2004). Both C-reactive protein (CRP) and SP-A affect the surface activity of bovine lipid extract surfactant (BLES), a clinically applied modified natural surfactant. Although SP-A and CRP both bind PC, there is a difference in the manner in which they interact with surface films (Nag et al. 2004).

Isolated perfused rat lung showed that both clathrin- and actin-mediated pathways are responsible for endocytosis of dipalmitoylphosphatidylcholine (DPPC)-labeled liposomes by granular pneumocytes in the intact lung. Further studies may strengthen for a major role of receptor-mediated endocytosis of DPPC by granular pneumocytes, a process critically dependent on SP-A (Lang et al. 2008).

24.5.8 Protection of Type II Pneumocytes from Apoptosis

Activation of SPAR by SP-A binding initiates a signal through pathways that involve tyrosine phosphorylation, include IRS-1, and entail activation of PI3K. In other cell types, cytokines that activate the PI3K signaling pathway promote cell survival. SP-A affects apoptosis as measured by DNA laddering, and other techniques. The protective effects of SP-A were abrogated by inhibition of either tyrosine-specific protein kinase activity or PI3K (White et al. 2001). Analysis of anti-apoptotic signaling species downstream of IRS-1 showed activation of PKB/Akt but not of MAPK. Phosphorylation of IkB was minimally affected by SP-A. However, FKHR was rapidly phosphorylated in response to SP-A and its DNA-binding activity was significantly reduced. Since FKHR is pro-apoptotic, this may play an important role in signaling the anti-apoptotic effects of SP-A. Therefore, survival-enhancing signaling activated by SP-A include SPAR through IRS-1, PI3K, PKB/Akt, and FKHR (White and Strayer 2002).

SP-A and SP-D are potent modulators of macrophage function and may suppress clearance of apoptotic cells through activation of the transmembrane receptor signal inhibitory regulatory protein alpha (SIRPα). Gardai et al. (2003) provided evidence that SP-A and SP-D act in a dual manner to enhance or suppress inflammatory mediator production depending on binding orientation. In this concept, a model is implied in which SP-A and SP-D help maintain a non/anti-inflammatory lung environment by stimulating SIRPα on resident cells through their globular heads (Janssen et al. 2008).

24.5.9 Anti-inflammatory Role of SP-A

SP-A Modulates the Cellular Response to Smooth and Rough LPSs by Interaction with CD14: Lung SP-A modulates cellular inflammatory responses by their direct interactions with pattern-recognition receptors (PRRs) and has been implicated in the regulation of pulmonary host defense. Pathogen derived components such as LPS, peptidoglycan (PGN) or zymosan are potent stimulators of inflammation. SP-A binds to rough forms but not to smooth forms of LPS. SP-A interacts with CD14 on AMΦ and inhibits the binding of smooth LPS to CD14 and reduces TNF-α expression induced by smooth LPS (Sano et al. 1999). In this interaction, SP-A associates with LPS via lipid A moiety of rough LPS and involved in the anti-bacterial defences of the lung. The direct interaction of SP-A with CD14 constitutes a likely mechanism by which SP-A modulates LPS-elicited cellular responses (Sano et al. 1999; Stamme et al. 2002). Studies show that the direct interaction of SP-A with TLR2 alters PGN-induced cell signaling and that SP-A modulates inflammatory responses against the bacterial components by interactions with PRRs (Murakami et al. 2002; Crowther et al. 2004). SP-A also down-regulates TLR2-mediated signaling and TNF-α secretion stimulated by zymosan. This supports an important role of SP-A in controlling pulmonary inflammation caused by microbial pathogens (Sato et al. 2003). NF-kB activation and TNF-α expression induced by PGN or zymosan were significantly inhibited in presence of SP-A. AMΦs are prototypical alternatively activated macrophages, with limited production of reactive oxygen intermediates (ROI) in response to stimuli (Weissbach et al. 1994). Crowther et al. (2004) support an anti-inflammatory role for SP-A in pulmonary homeostasis by inhibiting MΦ production of ROI through a reduction in NADPH oxidase activity. SP-A has been shown to stimulate the phagocytosis of apoptotic neutrophils (PMNs) and anti-inflammatory cytokine release by inflammatory AMΦs. Furthermore, SP-A enhances TGF-β 1 release from both AMΦ populations (Reidy and Wright 2003).

However, Alcorn and Wright (2004) suggested that SP-A inhibits inflammatory cytokine production in a CD14-independent manner and also by mechanisms independent of LPS signaling pathway (Alcorn and Wright 2004). SP-A inhibited LPS-induced mRNA levels for TNF-α, IL-1α, and IL-1β as well as NF-kB DNA binding activity. Significantly, SP-A also diminished ultra pure LPS-stimulated TNF-α produced by wild-type and CD14-null mouse alveolar macrophages. Additionally, SP-A inhibited TNF-α stimulated by PMA in both wild-type and TLR4-mutant

macrophages. Salez et al. (2001) highlighted the inhibitory role of SP-A in the anti-inflammatory activity of macrophages through inhibition of IL-10 production.

SP-A Regulates Surfactant Phospholipid Clearance After LPS-Induced Injury In Vivo: SP-A plays a role in surfactant homeostasis after acute lung injury. In bacterial LPS- induced injury into the lungs surfactant phospholipid levels were increased 1.6-fold in injured SP-$A^{-/-}$ animals, although injury did not alter ^{3}H-choline or ^{14}C-palmitate incorporation into dipalmitoylphosphatidylcholine (DPPC), suggesting no change in surfactant synthesis/secretion after injury. SP-A may play a role in regulating clearance of surfactant phospholipids after acute lung injury (Quintero et al. 2002). As SP-A inhibits LPS-induced in vitro IL-10 formation by bone marrow-derived macrophages, Chabot et al. (2003b) demonstrated an in vivo inhibitory role of SP-A on anti-inflammatory activity of mononuclear phagocytes, through inhibition of IL-10 production.

SP-A Regulates TNF-α Production: SP-A inhibited the LPS-induced TNF-α response of both interstitial (iMΦ) and alveolar human MΦ (AMΦ), as well as IL-1 response in iMΦ. Studies lend a credit to a physiological function of SP-A in regulating alveolar host defense and inflammation by suggesting a fundamental role of this protein in limiting excessive proinflammatory cytokine release in AMΦ during ARDS (Arias-Diaz et al. 2000; McIntosh et al. 1996). Surfactant lipids inhibit cytokine production by immune cells, and SP-A stimulates it. The increases in TNF-α mRNA and protein induced by SP-A were inhibited by surfactant lipids. SP-A exerts its action, at least in part, via activation of NF-kB. Moreover, the NF-kB inhibitors blocked SP-A-dependent increases in TNF-α mRNA levels. Observations suggest a mechanism by which SP-A plays a role in induced inflammation in the lung. The SP-A-induced increase in TNF-α levels differ among SP-A variants and appear to be affected by SP-A genotype and if SP-A is derived from one or both genes (Wang et al. 2000). Further more, different serotypes of LPS respond to SP-A differently on regulation of inflammatory cytokines in vitro (Song and Phelps 2000a, b).

SP-A Up-Regulates Activity of ManR and TLRs Expression by MΦs: SP-A selectively enhances ManR expression on human monocyte-derived macrophages. SP-A up-regulation of ManR activity provides a mechanism for enhanced phagocytosis of microbes by AMΦs, thereby enhancing lung host defense against extracellular pathogens (Beharka et al. 2002). Not only SP-A, but other lung collectins also enhance the uptake of *S. pneumoniae* or *M. avium* by AMΦs. The direct interaction of lung collectins with MΦs resulted in increased cell surface expression of scavenger receptor A or mannose receptor, which are responsible for phagocytosis. A more complete understanding of the molecular mechanism is required (Sano et al. 2006).

Henning et al. (2008) reported the SP-A-induced transcriptional and posttranslational regulation of TLR2 and TLR4 expression during the differentiation of primary human monocytes into MΦs. Despite SP-A's ability to up-regulate TLR2 expression on human MΦs, it dampens TLR2 and TLR4 signaling in these cells. SP-A decreases the phosphorylation of IkBα, a key regulator of NF-kB activity, and nuclear translocation of p65 which result in diminished TNF-α secretion in response to TLR ligands. SP-A also reduces the phosphorylation of TLR signaling proteins upstream of NF-kB, including members of the MAPK family. Finally, SP-A decreases the phosphorylation of Akt, a major cell regulator of NF-kB and potentially MAPKs. These data identify a critical role for SP-A in modulating the lung inflammatory response by regulating MΦ TLR activity (Henning et al. 2008). Molecular mechanisms related to the immunostimulatory activity of SP-A using MΦs from C3H/HeJ mice, which carry an inactivating mutation in the TLR-4 gene, and TLR-4-transfected CHO cells, suggest that SP-A-induced activation of NF-kB signaling pathway and up-regulation of cytokine synthesis such as TNF-α and IL-10 are critically dependent on TLR-4 functional complex. These findings support the concept that TLR-4 signals in response to both foreign pathogens and endogenous host mediators (Guillot et al. 2002).

SP-A Increases MMP-9 Production by THP-1 Cells: MMP-9 from alveolar MΦs is a major source of elastolytic activity in lung. SP-A may regulate MMP-9 expression. SP-A induced the expression of MMP-9 in THP-1 cells and peripheral blood mononuclear cells. It is believed that SP-A action is mediated through TLR-2. These observations suggest the presence of a locally controlled mechanism by which MMP-9 levels may be regulated in alveolar AΦs. Perhaps, SP-A influences the protease/antiprotease balance in lungs of patients with changes in surfactant constituents favoring an abnormal breakdown of extracellular matrix components (Vazquez et al. 2003). However, $Mmp9^{-/-}/Spd^{-/-}$ and $Mmp12^{-/-}/Spd^{-/-}$ mice developed air space enlargement similar to $Spd^{-/-}$ mice, supporting the concept that increased expression of each metalloproteinase seen in $Spd^{-/-}$ lungs is not major cause of emphysema (Zhang et al. 2006).

SP-A Provides Immunoprotection in Neonatal Mice: SP-A acts as an autocrine cytokine: it binds its receptor and specifically regulates transcription of surfactant proteins and other genes. SP-A null pups, reared on corn dust bedding had significant mortality The exogenous SP-A delivered by mouth to newborn SP-A null pups with SP-A

null mothers improved newborn survival in the corn dust environment. The lack of SP-D did not affect newborn survival, while SP-A produced by either the mother or the pup or oral exogenous SP-A significantly reduced newborn mortality associated with environmentally induced infection in newborns (George et al. 2008). The beneficial role of surfactant in improving oxygenation has already been established in clinical trials, whereas the immunomodulating effects are promising but remain to be elucidated (Kneyber et al. 2005).

24.6 Reactive Oxygen and Nitrogen-Induced Lung Injury

A pathway for the generation of potential oxidants with the reactivity of hydroxyl radical without the need for metal catalysis has been described. In response to various inflammatory stimuli, lung endothelial, alveolar, and airway epithelial cells, as well as activated AMΦ, produce both .NO and superoxide anion radicals (O_2^-). .NO regulates pulmonary vascular and airway tone and plays an important role in lung host defense against various bacteria. However, .NO may be cytotoxic by inhibiting critical enzymes such as mitochondrial aconitase and ribonucleotide reductase, by S-nitrosolation of thiol groups, or by binding to their iron-sulfur centers. In addition, .NO reacts with O_2^- at a near diffusion-limited rate to form the strong oxidant peroxynitrite ($ONOO^-$), which can nitrate and oxidize key amino acids in various lung proteins such as SP-A, and inhibit their functions. The presence of $ONOO^-$ in the lungs of patients with ARDS has been demonstrated. Various studies have shown that inhalation or intratracheal instillation of various respirable mineral dusts or asbestos fibers increased levels of inducible NOS mRNA. Zhu et al. (1998) reviewed the evidence for the upregulation of .NO in the lungs of animals exposed to mineral particulates and assess the contribution of reactive nitrogen species in the pathogenesis of the resultant lung injury (Zhu et al. 1998).

24.6.1 Decreased Ability of Nitrated SP-A to Aggregate Surfactant Lipids

The .NO can modify oxidant stress by limiting superoxide (O_2^-)-mediated injury. However, the product of .NO reaction with O_2^-, peroxynitrite ($ONOO^-$), is a potent oxidizing and nitrating agent. At high concentrations, .NO inhibit O_2-induced lipid peroxidation. However, ONOO., formed by reaction of .NO and O_2^-, nitrates SP-A leading to decreased ability to aggregate lipids and bind mannose (Zhu et al. 1998). Depending on the pH, tetranitromethane (TNM) acts either as a nitrating (pH $>$ or $=$ 24.4) or an oxidizing agent (pH $<$ or $=$ 6). SP-A, treated with TNM at pH 6, 24.4, 8, or 10, was tested for its ability to aggregate lipids. Treatment of SP-A with 0.5 mM TNM decreased its ability to aggregate lipids by 30% at pH 24.4, and 90% at pH 8, but had no effect on disulfide-dependent oligomeric state of SP-A. In contrast, SP-A exposed to 1 mM TNM at pH 6 had background levels of nitrotyrosine and exhibited normal lipid aggregation properties. It appeared that tyrosine nitration selectively inhibits the SP-A-mediated lipid aggregation without affecting its ability to bind lipids (Haddad et al. 1996). Sequencing of nitrated peptides demonstrated that the nitration was equally distributed on Tyr164 and Tyr1624. Nitrated SP-A exhibited decreased ability to aggregate surfactant lipids in the presence of Ca^{2+} (Greis et al. 1996). Peroxynitrite, produced by activated AMΦs, can nitrate SP-A and that CO_2 increased nitration by enhancing enzymatic nitric oxide production (Zhu et al. 2000).

Nitrated SP-A Does Not Enhance Adherence of *P. carinii* to Alveolar MΦs: Nitration of SP-A alters its ability to bind to mannose-containing saccharides on *P. carinii*. Human SP-A nitrated by ONOO– or tetranitromethane decreases its binding to *P.* carinii by increasing its dissociation constant from 24.8×10^{-9} to 1.6×10^{-8} or 2.4×10^{-8} M, respectively, without significantly affecting the number of binding sites. Furthermore, ONOO – nitrated SP-A failed to mediate the adherence and phagocytosis of *P. carinii* to rat AM as compared with normal SP-A. Binding of SP-A to rat AMΦs was not altered by nitration. These results indicate that nitration of SP-A interferes with its ability to serve as a ligand for *P. carinii* adherence to AMΦs at the site of the SP-A molecule (Zhu et al. 1998). Nitration of SP-A impairs its host- defense properties. Pulmonary edema fluid from patients with ALI has significantly higher levels of nitrite (NO_2^-) + nitrate (NO_3^-) compared with pulmonary edema fluid from patients with hydrostatic pulmonary edema. Nitrated pulmonary SP-A was also detected in the edema fluid of patients with ALI. This study indicated that reactive oxygen-nitrogen species may play a role in the pathogenesis of human ALI (Zhu et al. 2001).

24.7 Non-Pulmonary SP-A

24.7.1 SP-A in Epithelial Cells of Small and Large Intestine

Although, lung is the major source of surfactant proteins, SP-A expression has been reported in extra-pulmonary tissues. Several lines of evidence indicate that pulmonary and gastrointestinal epithelium produce closely related surface-active materials. Small intestine and colon express SP-A

constitutively (Rubio et al. 1995). Both SP-A genes, SP-A1 and SP-A2 genes, are expressed in human small and large intestine. The size of intestinal SP-A mRNA is the same as that in human lung (Lin et al. 2001). SP-A mRNA transcripts are readily amplified from the trachea, prostate, pancreas, intestine and thymus. SP-A sequences derived from lung and thymus mRNA revealed the presence of both SP-A1 and SP-A2, whereas only SP-A2 expression was found in the trachea and prostate (Madsen et al. 2003a). The middle ear protein has the same epitope as human lung SP-A. SP-A is also expressed in pig Eustachian tube. The secreted antimicrobial molecules of the tubotympanum include lysozyme, lactoferrin, beta defensins, and the SP-A, SP-D. Defects in the expression or regulation of these molecules may also be the major risk factor for otitis media (Lim et al. 2000). Human vaginal mucosa and vaginal lavage fluid show presence of SP-A protein and its transcripts. Transcripts of SP-A were identified in vaginal wall, derived from SP-A genes, SP-A1 and SP-A2.

24.7.2 SP-A in Female Genital Tract and During Pregnancy

SP-A1 is found in female genital tract. Evidence suggests that SP-A is produced in a squamous epithelium, namely the vaginal mucosa, and has a localization that would allow it to contribute to both the innate and adaptive immune response. In vagina, as in lung, SP-A is an essential component of the host-defence system (MacNeill et al. 2004). The bronchiolar 16 kD Clara cell secretory protein (CC16) and the alveolar SP-A are secreted in the amniotic fluid, where they reflect the growth and the maturity of the fetal lung (Cho et al. 1999). There is compelling evidence to suggest that the fetus signals the initiation of labor by secretion into amniotic fluid of major lung SP-A. SP-A protein has been demonstrated in human chorioamniotic membranes. Parturition at term, gestational age and chorioamnionitis in preterm delivery are associated with changes in the expression of SP-A in the chorioamniotic membranes (Han et al. 2007). The maternal smoking during pregnancy does not alter secretory functions of the epithelium of the distal airways and the alveoli at term (Hermans et al. 2001, 2003).

Exogenous administration of SP-A into mouse amniotic fluid at 15 dpc caused preterm labor. SP-A activated amniotic fluid macrophages in vitro to produce NF-kB and IL-1beta. These macrophages, which are of fetal origin, migrate to the pregnant uterus causing an inflammatory response and increase uterine NF-kB activity. It is suggested that the increase in NF-kB within the maternal uterus both directly increases expression of genes that promote uterine contractility and negatively impacts the capacity of the PR to maintain uterine quiescence, contributing to the onset of labor. Findings, therefore, indicate that SP-A secreted into amniotic fluid/the maturing fetal lung serves as a hormone of parturition (Condon et al. 2004; Mendelson and Condon 2005). It was proposed that IL-1 from extrapulmonary sources induces the SPs in premature lung and is responsible for decreased risk of RDS in intra-amniotic infection (Väyrynen et al. 2002). However, Sun et al. (2006) suggested that SP-A can be synthesized locally in human fetal membranes, which can be induced by glucocorticoids. SP-A appeared to induce PGE2 synthesis in chorionic trophoblasts via induced expression of cyclooxygenase type 2. Levels of SP-A and SP-B, in Amniotic fluid, change during human parturition. Whereas SP-A was detected in all amniotic fluid samples SP-B was detected in 24.1% of mid-trimester samples and in samples at term. The median amniotic fluid concentrations of SP-A and SP-B were significantly higher in women at term than in women in the mid-trimester, and decreases during spontaneous human parturition at term (Garcia-Verdugo et al. 2008a).

Human myometrial cells express functional SP-A1 binding sites and respond to SP-A1 to initiate activation of signaling related to human parturition. SP-A1 is not produced in rat uterus, but detected transiently in rat myometrium at the end (Days 19 and 21) of gestation, but not postpartum. SP-A1 binds myometrium through its collagenlike domain and rapidly activated MAPK1/3 in myometrial cells. Bacterial LPS, known to trigger uterine contractions and preterm birth, also activated MAPK1/3. Results provide the evidence for inhibitory cross talk between SP-A1 and LPS signals, and new insight into the mechanisms of normal and preterm parturition (Garcia-Verdugo et al. 2007, 2008b). Various microorganisms colonize this area and may cause intrauterine infection or trigger preterm labor.

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Surfactant Protein-D

25

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The collectins can be classed into two distinct group, with MBP and SP-A being hexamers and SP-D, conglutinin and collectin-43 (CL-43) being tetramers, with proteins in the latter group also having significantly larger dimensions with respect to the length of their collagen-like 'stalks' (Lu et al. 1993). MBP, lung SP-D, conglutinin, CL-43 and CL-46 are important components of innate immune defence system. They all bind complex glycoconjugates on microorganisms thereby inhibiting infection, enhancing the clearance by phagocytes and modulating the immune response. In addition, SP-D inhibits the generation of radical oxygen species or the propagation of lipid peroxidation. Knock-out mice deficient in SP-D have a disturbed homeostasis of pulmonary surfactant and suffer from oxidative stress leading to pulmonary inflammation upon microbial challenge. Moreover, both SP-D and SP-A (Chap. 24) have been shown to enhance oxygen radical production by alveolar macrophages. The structural and functional relationships of this group of collectins have been reviewd (Hansen and Holmskov 2002; Jinhua et al. 2003).

25.1 Pulmonary Surfactant Protein-D (SP-D)

Surfactant proteins, SP-A and SP-D contribute significantly to surfactant homeostasis and pulmonary immunity. Their basic structures include a triple-helical collagen region and a C-terminal homotrimeric lectin or CRD. The trimeric CRDs can recognize carbohydrate or charge patterns on microbes, allergens and dying cells, while the collagen region can interact with receptor molecules present on a variety of immune cells in order to initiate clearance mechanisms. Gene knock-out mice models of lung hypersensitivity and infection, and functional characterization of cell surface receptors have revealed the diverse roles of SP-A and SP-D in control of lung inflammation. A model based on studies with the calreticulin-CD91 complex as a receptor for SP-A and SP-D has suggested an anti-inflammatory role for SP-A and SP-D in naïve lungs which would help minimise the potential damage that continual low level exposure to pathogens, allergens and apoptosis can cause. However, when lungs are overwhelmed with exogenous insults, SP-A and SP-D can assume pro-inflammatory roles in order to complement pulmonary innate and adaptive immunity. The structural and functional aspects of SP-A and SP-D emphasize on their roles in controlling pulmonary infection, allergy and inflammation (Kishore et al. 2006)

Lung SP-D can directly interact with carbohydrate residues on pulmonary pathogens and allergens, stimulate immune cells, and manipulate cytokine and chemokine profiles during the immune response in lungs. Therapeutic administration of rfhSP-D, a recombinant homotrimeric fragment of human SP-D comprising the alpha-helical coiled-coil neck plus three CRDs, protects mice against lung allergy and infection caused by the fungal pathogen *Aspergillus fumigatus*. Curcumin inhibits inflammatory response reducing significantly all histopathological parameters in different pulmonary aspiration models. Curcumin is a potential therapeutic agent in acute lung injury (Guzel et al. 2009).

The lung is the main site of synthesis for SP-D, but transcripts were readily amplified from trachea, brain, testis, salivary gland, heart, prostate gland, kidney, and pancreas. Minor sites of synthesis were uterus, small intestine, placenta, mammary gland, and stomach. The sequence of SP-D derived from parotid gland mRNA was identical with that of pulmonary SP-D. SP-D immunoreactivity was found in alveolar type II cells, Clara cells, on and within alveolar macrophages, in epithelial cells of large and small ducts of the parotid gland, sweat glands, and lachrymal glands, in epithelial cells of the gall bladder and intrahepatic bile ducts, and in exocrine pancreatic ducts. SP-D was also present in epithelial cells of the skin, esophagus, small intestine, and urinary tract, as well as in the collecting ducts of the kidney. SP-D is present in mucosal lined surfaces throughout the human body, including the male reproductive tract and not

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restricted to a subset of cells in the lung. The localization and functions of SP-D indicated that this collectin is the counterpart in the innate immune system of IgA in the adaptive immune system (Madsen et al. 2000). The extra-pulmonary expression of SP-A and SP-D indicates the systemic roles of these proteins. SP-D is localized in the cytoplasm of a subpopulation of bronchiolar epithelial cells as well as type II cells. Anti-SP-D selectively decorated secretory compartments of nonciliated bronchiolar cells (Clara cells) with strong and specific labeling of apical electron-dense secretory granules. Studies provide evidence that SP-D is a secretory product of nonciliated bronchiolar cells. It was suggested that Clara cell-derived SP-D is a component of bronchiolar lining material, consistent with the hypothesis that SP-D contributes to surfactant metabolism and/or host defense within small airways (Crouch et al. 1992).

Alveolar Type II cells bind and recycle SP-D in vitro to lamellar bodies: Alveolar type II cells secrete, internalize, and recycle pulmonary surfactant complex that increases alveolar compliance and participates in pulmonary host defense. SP-D has been described as a modulator of surfactant homeostasis. Mice lacking SP-D accumulate surfactant in their alveoli and type II cell lamellar bodies, organelles adapted for recycling and secretion of surfactant. However, SP-D binding did not alter type II cell surfactant lipid uptake. Thus, type II cells bind and recycle SP-D in vitro to lamellar bodies, but SP-D may not directly modulate surfactant uptake by type II cells (Herbein and Wright 2001).

25.1.1 Human Pulmonary SP-D

25.1.1.1 Human Pulmonary SP-D Protein

Human pulmonary SP-D was identified in lung lavage by its similarity to rat SP-D in both its molecular mass and its Ca^{2+}-dependent-binding affinity for maltose. On SDS/PAGE human SP-D behaved as a single band of 150 kDa in non-reducing and 43 kDa band in reducing conditions. The presence of a high concentration of glycine (22%), hydroxyproline and hydroxylysine in the amino acid composition of human SP-D pointed towards its collagen-like structure. Human SP-D has a similar carbohydrate-binding specificity to rat SP-D, but differed from that of other lectins, such as conglutinin. Amino acid sequence analysis showed the presence of collagen-like Gly-Xaa-Yaa triplets in human SP-D. The derived amino acid sequence indicated that mature SP-D polypeptide chain is 355 residues long, having a short non-collagen-like N-terminal section of 25 residues, followed by a collagen-like region of 177 residues and a C-terminal C-type lectin domain of 153 residues. The human SP-D and bovine serum conglutinin showed 66% identity despite their marked differences in carbohydrate specificity (Lu et al. 1993). SP-D differs in its quaternary structure from SP-A and other members of the family, such as C1q, in that it forms large multimers held together by N-terminal domain, rather than aligning the triple helix domains in traditional "bunch of flowers" arrangement. SP-D is an oligomeric complex of four set of homotrimers. Purified trimeric and multimeric SP-D represent separate and only partly interconvertible molecular populations with distinct biochemical properties (Soerense et al. 2009).

25.1.1.2 Genomic Organization of Human SP-D

SP-D gene sequences spann > 11 kb on long arm of chromosome 25. Genomic sequencing revealed that signal peptide/amino-terminal domain, the CRD, and the linking sequence between the collagen domain, and CRD are each encoded by a single exon, as seen for SP-A and MBP-C. However, sequencing also demonstrated a unique intron-exon structure for collagen domain which is encoded on five exons, including four tandem exons of 117 bp. The latter exons show marked conservation in the predicted distribution of hydrophilic amino acids, consistent with tandem replication of this collagen gene sequence during evolution (Crouch et al. 1993). Human SP-D gene (*Sftp4 or Sftpd*) was assigned to chromosome 25. A regional mapping panel for five loci was mapped to 10q. S*ftp4*, the SP-A gene (*Sftp1*/*Sftpa*), and the microsatellite D10S109 were placed in the interval 10q22–q23. In situ hybridization of metaphase chromosomes using genomic probes gave selective labeling of 10q22.2–23.1 (Crouch et al. 1993; Kölble et al. 1993).

Rust et al. (1996) described the characterization of a human genomic fragment (H5E7) that encodes entirety of first translated exon (Exon 2), Intron 1, a short transcribed untranslated sequence (Exon 1; 39 bp), and approximately 4 kb of sequence upstream from transcription initiation site. The start site comprises of a putative TATA box (CATAAATA) of ~30 bp upstream of the start site. Complete sequencing of a HS-1674 fragment encoding approximately 1.7 kb of sequence 5′ to TATA demonstrated multiple potential cis-regulatory elements including half-site glucocorticoid response elements (GRE), a canonical AP-1 consensus, several AP-1 like sequences, E-box sequences, NF-IL-6 and PEA3 motifs, and putative interferon response elements. Studies support the hypothesis that the effects of glucocorticoids on SP-D production in vivo are regulated at level of transcription.

While SP-D is the product of a single gene whereas SP-A is the product of two highly homologous genes SP-A1 and SP-A2. The relative location and orientation of each of the SP-A and SP-D genomic sequences have been characterized. Characterization of two overlapping genomic clones revealed that SP-A pseudogene lies in a reverse orientation

15 kb away from the 5′ side of SP-A1. Both SP-A2 and SP-D are on the 5′ side of SP-A1 at approximate distances of 40 and 120 kb, respectively. The SP-A and SP-D loci were also oriented relative to centromere, with overall order being: centromere-SP-D-SP-A2-pseudogene-SP-A1- telomere (Hoover and Floros 1998). Several single nucleotide polymorphisms (SNP) have been identified in the SP-A1, SP-A2 and SP-D genes (Pantelidis et al. 2003).

25.1.1.3 Transcriptional Regulation of SP-D Promotor

Proximal Promoter of SP-D Gene: Because AP-1 proteins regulate cellular responses to diverse environmental stimuli, He et al. (2000) hypothesized that conserved AP-1 motif (at -109) and flanking sequences in human SP-D promoter contribute to the regulation of SP-D expression. Davé et al. (2004) identified the role of nuclear factor of activated T cells (NFATs) in regulation of murine SP-D gene (*Sftpd*) transcription. Components of calcineurin/NFAT pathway were identified in respiratory epithelial cells of the lung that potentially augment rapid assembly of a multiprotein transcription complex on *Sftpd* promoter inducing SP-D expression via direct interaction with TTF-1 in lung epithelial cells

CCAAT/Enhancer-Binding Proteins (C/EBP): CCAAT/enhancer-binding proteins (C/EBP) constitute a family of transcription factors that are involved in regulation of proliferation and differentiation in several cell types. In epithelial lung cells the C/EBPα isoform seems to play a role in the regulation of SPs and Clara cell specific protein (CCSP). In vitro results suggest that C/EBPδ alone is not related to the maintenance of proteins involved in differentiation (Låg et al. 2000). It was hypothesized that conserved C/EBP motifs in the near-distal and proximal promoters contribute to the regulation of SP-D expression by C/EBPs. Five SP-D motifs (-432, -340, -319, -140, and -90) homologous to C/EBP consensus sequence specifically bound to C/EBPs in gel shift assays, and four of the five sites (-432, -340, -319, and -90) efficiently competed for binding of C/EBPα, C/EBPβ, or C/EBPδ to consensus oligomers. The conserved AP-1 element at -109 was required for maximal promoter activity, but not for transactivation of SS698 by C/EBPs. Thus, interactions among C/EBP elements in near-distal promoter can modulate promoter activity of SP-D (He and Crouch 2002).

Retinoblastoma Protein Activates C/EBP-Mediated Transcription of SP-D: The retinoblastoma protein (RB) through histone deacetylase (HDAC) plays a critical role in cell cycle regulation. RB is also involved in activation of expression of a number of tissue specific- and differentiation-related genes and stimulates the expression of a differentiation-related gene, SP-D in lungs. RB specifically stimulated the activity of human SP-D gene promoter. Activation by RB was mediated through a NF-IL6 (C/EBPβ) binding motif in the human SP-D promoter, and this sequence specifically bound to C/EBPα, C/EBPβ, and C/EBPδ. Furthermore, the complexes containing RB and C/EBP proteins directly interacted with C-EBP binding site on DNA. Thus, RB plays a direct role in C/EBP-dependent transcriptional regulation of human SP-D expression (Charles et al. 2001).

25.1.2 Rat Lung SP-D

Ontogeny of Surfactant Apoprotein D, SP-D in Rat Lung: In rat lungs SP-A increased during late gestation and reached its maximum on day 1 of neonate, and then gradually declined until at least day 5. SP-D content during early gestation was less than 10 ng/mg protein until day 18, but on day 19 there was a fourfold increase in SP-D (compared to that on day 18). It increased twice between day 21 and the day of birth, when it reached the adult level of 250 ng/mg protein, which is about one fourth that of the adult level of SP-A. Unlike SP-A there seemed to be no decrease in SP-D content after birth. SP-D is regulated developmentally as with other components of surfactant. In contrast to humans, rat lungs are immature at birth. Alveolarization starts on postnatal day 4. Studies support the concept that postnatal alveolarization in rat lungs is associated with significant increases in the SP-B content in lb and volume fraction of lb in type II pneumocytes. The postnatal compartment-specific distribution of SP-A, precursors of SP-C and SP-D does not change (Schmiedl et al. 2005).

Rat SP-D Protein: Rat SP-D was SP-D, purified from the supernatant of rat bronchoalveolar lavage fluids, and delipidated form does not exhibit interaction with lipids in the same fashion as SP-A. SP-D consists of three regions: an NH2-terminal segment of 25 amino acids, a collagen-like domain consisting of 59 Gly-X-Y repeats, and a COOH-terminal carbohydrate recognition domain of 153 amino acids. There are six cysteine residues present in rat SP-D: two in NH2-terminal noncollagenous segment and four in COOH-terminal carbohydrate-binding domain. The collagenous domain contains one possible N-glycosylation site. The protein is preceded by a cleaved, NH2-terminal signal peptide. Rat SP-D is encoded by a 1.3-kb mRNA which is abundant in lung and highly enriched in alveolar type II cells. Extensive homology exists between rat SP-D and bovine conglutinin (Shimizu et al. 1992; Lim et al. 1993; Ogasawara et al. 1991).

In electron microscopy, rat SP-D is made of four identical rod-like arms (46 nm in length), each with an 8–9-nm

diameter globular terminal expansion. The arms, which are similar in diameter to the type I collagen helix (~4 nm), emanate from central "hub" in two pairs that closely parallel each other for their first 10 nm. This structure is consistent with hydrodynamic studies that predict a highly asymmetric and extended molecule (f/f0 = 3.26) with a large Stokes radius of 18 nm. Pepsin digestion gave glycosylated, trimeric collagenous fragments. Trimeric subunits containing intact triple helical domains were also liberated from SP-D dodecamers by sulfhydryl reduction under non-denaturing conditions. Digestion of rSP-D with bacterial collagenase generated a COOH-terminal carbohydrate binding fragment and a smaller peptide (~12 kDa, unreduced) that contains interchain disulfide bonds. Higher orders of multimerization with as many as eight molecules associated at the hub were also identified. Rat SP-D molecules are assembled as tetramers of trimeric subunits (12 mers). Dodecamers can participate in higher orders of molecular assembly involving interactions of NH_2-terminal peptide domains. Interactions between amino-terminal domains of trimers are stabilized by interchain disulfide bonds. SP-D molecules can associate to form complex multimolecular assemblies (Crouch et al. 1994b).

Studies demonstrated that a single genetically distinct chain type can account for various and complex molecular assemblies of SP-D (Crouch et al. 1994a). The SP-D forms large multimers held together by N-terminal domain, rather than aligning the triple helix domains in traditional "bunch of flowers" arrangement. The two cysteine residues within hydrophobic NH_2-terminus are critical for multimer assembly and have been proposed to be involved in stabilizing disulfide bonds. These cysteins exist within reduced state in dodecameric SP-D and form a specific target for S-nitrosylation both in vitro and by endogenous, pulmonary derived NO within a rodent acute lung injury model (Crouch et al. 1994a; Guo et al. 2008).

25.1.3 Mouse SP-D

The Protein: A cDNA deduced sequence predicts a 19-amino acid signal sequence, a 25-amino acid long NH_2-terminus with two cysteines, followed by an uninterrupted collagen domain with 59 Gly-X-Y repeats. Next, a short "neck" domain of 28 amino acids, with a potential to form trimeric alpha-helical coiled coil is found ending in a COOH-terminal 125-amino acid CRD. The mature mouse SP-D protein of 355 amino acids shows strong homology to rat (92% identity), human (76%), and bovine (72%) SP-D amino acid sequences. The mouse SP-D gene is expressed predominantly in lung and also in heart, stomach, and kidney but not in brain. In contrast, mouse SP-A mRNA expression was found to be restricted to lung. Histochemically, SP-A and SP-D mRNA were detected in a significant number of non-pulmonary tissues but proteins have a more limited distribution. SP-D protein was detected in lung, uterus, ovary, and lacrimal gland, whereas SP-A protein was detected only in lung (Akiyama et al. 2002). Mouse SP-D gene (*Sftp4*) has been localized to chromosome 14 (to a region syntenic to human chromosome 10), closely linked to genes for *Mbl1*, and SP-A (*Sftp1*) (Motwani et al. 1995).

Genomic Organization of Mouse Lung SP-D Gene: The mouse SP-D gene (*Sftpd*), which spans eight exons over 14 kb of sequence and shows an overall organization similar to other collectin genes. The complete 5′ untranslated region of mRNA is encoded by a single exon. Analysis of 3.5 kb of 5′ flanking nucleotide sequence for *Sftpd* reveals positional conservation of a number of transcription factor binding sites when compared with human SP-D gene and bovine conglutinin gene (Lim et al. 2003). The single copy SP-D-like gene is present in mammals, birds, and amphibians but is absent in fish. An atypical, rodent-specific, long terminal repeat of retroviral origin containing a minisatellite that has become inserted in *Sftpd* is described. Three new polymorphic microsatellites are also described, one of which is just 160 bp upstream of *Sftpd*. This microsatellite was used to map the gene to central region of chromosome 14. Fine-scale mapping indicates that it lies in a 5.64-centimorgan area between D14Mit45 and D14Mit60 (Lawson et al. 1999).

The Collectin Gene Locus: Three of four known mouse collectin genes have been mapped to chromosome 14. To characterize spatial relationship of these genes, one large clone hybridized to both SP-A and SP-D cDNAs was found to contain sequences from one of the mouse *Mbl1*. The SP-A, *Mbl1*, and SP-D genes reside contiguously within a 55-kb region. The SP-A and *Mbl1* genes are in same 5′–3′ orientation and 16 kb apart. The SP-D gene is in opposite orientation to two other collectin genes, 13 kb away from 3′ end of the *Mbl1* gene. The size (13 kb) and organization of mouse gene was similar to that of human SP-D. Exon I is untranslated. The second exon is a hybrid exon that contains signal for initiation of translation, signal peptide, N-terminal domain, and first seven collagen triplets of collagen-like domain of the protein. Four short exons (III to VI) encode collagen-like domain of protein, and exons VII and VIII, the linking and the CRDs, respectively (Akiyama et al. 1999).

Developmental Expression: SP-D mRNA and protein were readily detected in alveolar type II and nonciliated bronchiolar epithelial cells of lung, as well as in cells of the tracheal epithelium and tracheal submucosal glands of the adult mouse. However, SP-A mRNA or protein was not detected in murine trachea. Expression of murine SP-D mRNA was first detected on Ed 16 of gestation in pregnant mice, and this

increased dramatically before birth and during the immediate postnatal period. The developmental expression of murine SP-A mRNA paralleled that of SP-D except that there was a small decrease in mRNA on postnatal Ed 5. Thus, SP-D is synthesized not only in the lung but also in submucosal glands of trachea (Wong et al. 1996).

25.1.4 Bovine SP-D

Gjerstorff et al. (2004) characterized the gene encoding bovine SP-D and its proximal promoter. Cloning and sequencing of the bSP-D gene, including the complete 5′-untranslated sequence, reveal that the gene comprises nine exons spanning ~ 25.5 kb and resembling bovine conglutinin gene. The gene localizes to the same locus as conglutinin gene on Bos taurus chromosome 28 at position q1.8, which also includes the genes for CL-43 and CL-46. Several potential cis-regulatory elements, similar to elements known to regulate transcription of human SP-D, were identified in the 5′-upstream sequence. Bovine SP-D is heavily expressed in lung and the trachea, but also in segments of gastrointestinal tract, mammary glands and salivary glands. By genotyping two potential polymorphisms leading to variations in amino acid composition of CRD (242 Glu/Val and 268 Ala/Gly) have been assigned.

Human and bovine SP-D from late amniotic fluid and BAL gave a Mr of a trimeric subunit in the range 115–125 kDa for human SP-D and 110–123 kDa for bovine SP-D. A single polypeptide chain was determined at 37–41 and 36–40 kDa for human and bovine species respectively. Primary structures, determined by MS and Edman degradation, showed heterogeneity in SP-D, caused mainly by high number of post-translational modifications in the collagen-like region. Proline and lysine residues were partly hydroxylated and lysine residues were further O-glycosylated with disaccharide galactose-glucose. A partly occupied N-linked glycosylation site was characterized in human SP-D. The carbohydrate was determined as a complex type bi-antennary structure, with a small content of mono-antennary and tri-antennary structures. No sialic acid residues were present on the glycan, but some had an attached fucose and/or an N-acetylglucosamine residue linked to the core. Bovine SP-D was having a similar structure (Leth-Larsen et al. 1999).

25.1.5 Porcine Lung SP-D

The complete cDNA sequence of porcine SP-D, including the 5′ and 3′ untranslated regions, was determined from two overlapping clones. Three unique features were revealed from porcine sequence in comparison to SP-D from other species, making porcine SP-D an intriguing species addition to the collectin family. The collagen region contains an extra cysteine residue, which may have important structural consequences. The other two differences, a potential glycosylation site and an insertion of three amino acids lie in the loop regions of CRD, close to carbohydrate binding region and thus may have functional implications. The genes for SP-D (*Sftpd*) and SP-A (*Sftpa*) also co-localized to a region of porcine chromosome 14 band 14q25—>q26 that is syntenic with human and murine collectin loci (van Eijk et al. 2000, 2001).

The porcine SP-D appeared as a band of ~ 53 kDa in reduced state and ~138 kDa in unreduced state (Soerensen et al. 2005a). The monomeric porcine SP-D (50–53 kDa) is larger than that of SP-D from humans (43 kDa). Carbohydrate moiety is a highly heterogeneous, complex type oligosaccharide which is sialylated. The heterogeneity of oligosaccharide sialylation results in existence of many differently charged porcine SP-D isoforms. The removal of carbohydrate moiety reduces the inhibitory effect of porcine SP-D on IAV haemagglutination. Ultrastructural analysis showed the presence of both dodecameric and higher order oligomeric complexes of SP-D (van Eijk et al. 2000, 2002). Porcine tissues showed SP-D predominantly present in Clara cells and serous cells of bronchial submucosal glands, and to a lesser extent in alveolar type II cells, epithelial cells of intestinal glands (crypts of Lieberkuhn) in duodenum, jejunum and ileum and serous cells of dorsolateral lacrimal gland (Soerensen et al. 2005b). SP-A and D are expressed in the porcine Eustachian tube (ET) originating from upper airways and were present in epithelial cells of ET (Paananen et al. 2001). Porcine SP-D is an important reagent for use in existing porcine animal models for human lung infections.

25.2 Interactions of SP-D

The SP-A and SP-D bind carbohydrates, lipids, and nucleic acids with a broad-spectrum specificity and initiate phagocytosis of inhaled pathogens as well as apoptotic cells. Investigations on gene-deficient and conditional overexpressed mice indicated that lung SP-A and SP-D directly modulate innate immune cell function and T-cell-dependent inflammatory events and have a unique, dual-function capacity to induce pathogen elimination and control proinflammatory mechanisms (Botas et al. 1998; Haczku 2008).

25.2.1 Interactions with Carbohydrates

SP-D showed specific calcium-dependent binding to alpha-D-glucosidophenyl isothiocyanate-BSA and maltosyl-BSA, but negligible binding to beta-D-glucosidophenyl

isothiocyanate-BSA or unconjugated BSA. The most efficient inhibitors of SP-D binding were alpha-glucosyl-containing saccharides (e.g. isomaltose, maltose, malotriose). Studies demonstrate that SP-D is a calcium dependent lectin-like protein and that the association of SP-D with surfactant is mediated by carbohydrate-dependent interactions with specificity for alpha-glucosyl residues. Crystallographic studies of trimeric human SP-D neck + CRD domains have shown that maltose, a preferred saccharide ligand, binds to calcium via the vicinal 3- and 4-OH groups of the non-reducing glucose, previously designated calcium ion 1 and glucose 1 (Glc1), respectively (Shrive et al. 2003).

Oligo and Polysaccharide Recognition by SP-D: SP-D binding to *Aspergillus fumigatus* is strongly inhibited by a soluble β-(1—>6)-linked but not by a soluble β-(1—>3)-linked glucosyl homopolysaccharide (pustulan and laminarin, respectively), suggesting that SP-D recognizes only certain polysaccharide configurations. Docking studies predict that α/β-(1—>2)-, α-(1—>4)-, and α/β-(1—>6)-linked but not α/β-(1—>3)-linked glucosyl trisaccharides can be bound by their internal glucosyl residues and that binding also occurs through interactions of the protein with the 2- and 3-equatorial OH groups on the glucosyl ring. Given the sequence and structural similarity between SP-D and other C-type lectins, many of the predicted interactions should be applicable to this protein family (Allen et al. 2001).

SP-D binds to various synthetic fucosylated oligosaccharides. Fucα1-3GalNAc and Fuc α1-3GlcNAc elements show strong binding to SP-D. Fucosylated glycoconjugates are present at the surface of *Schistosoma mansoni*, a parasitic worm that transiently resides in lung during development. In line with this observation, SP-D was found to bind to larval stages of *S. manson* and may interact with multicellular lung pathogens (van de Wetering et al. 2004). The SP-A and SP-D bind gram-positive bacteria through lipoteichoic acid (LTA) and peptidoglycan, components of cell wall of gram-positive bacteria. The CRD is responsible for this binding (van de Wetering et al. 2001).

Interaction with LPS: Both SP-A and SP-D bind bacterial lipopolysaccharide (LPS). Wang et al. (2008a) demonstrated specific binding between CRD and heptoses in the core region of LPS. The geometry suggested that all three CRDs are simultaneously bound to LPS under conditions that support Ca^{2+}-mediated interaction. Mutant trimeric recombinant neck + CRDs (NCRDs) of SP-D is known to bind with rough LPS (R-LPS). Crystallographic analysis of hNCRD demonstrated a novel binding orientation for LD-heptose, involving hydroxyl groups of side chain. Similar binding was observed for a synthetic α1—>3-linked heptose disaccharide corresponding to heptoses I and II of inner core region in many LPS. 7-O-Carbamoyl-l,D-heptose and D-glycero-α-D-manno-heptose were bound via ring hydroxyl groups. Interactions with side chain of inner core heptoses provide a potential mechanism for recognition of diverse types of LPS by SP-D (Wang et al. 2008b). SPD binds to human α2-macroglobulin, that protects the collectin from proteolytic degradation by elastase (Craig-Barnes et al. 2010).

SP-D Binds MD-2 (lymphocyte antigen 96) and TLRs Through CRD: SP-A interacts with MD-2 and alters LPS signaling. SP-D modulates LPS-elicited inflammatory cell responses. Ligand blot analysis revealed that SP-D bound to N-glycopeptidase F-treated sMD-2. In addition, the biotinylated SP-D pulled down the mutant sMD-2 with Asn (26) —>Ala and Asn (114) —>Ala substitutions, which lacks the consensus for N-glycosylation. Results demonstrated that SP-D directly interacts with MD-2 through CRD (Nie et al. 2008).

Human SP-D exhibits specific binding, through its CRD, to extracellular domains of rTLR-2 and TLR-4 by a mechanism different from its binding to PI and LPS (Ohya et al. 2006). SP-D bound to a complex of soluble forms of TLR4 and MD-2 with high affinity and down-regulates TNF-α secretion and NF-kB activation elicited by rough and smooth LPS, in alveolar macrophages and TLR4/MD-2-transfected HEK293 cells. Study demonstrates that SP-D down-regulates LPS-elicited inflammatory responses by altering LPS binding to its receptors and reveals the importance of correct oligomeric structure of the protein in this process (Yamazoe et al. 2008).

Binding with Glycoprotein-340: A glycoprotein (Mr 340 kDa, gp-340) from human bronchioalveolar lung washings from a patient with alveolar proteinosis was found to bind to lung SP-D. Like gp-340, CRP-ductin binds human SP-D in a calcium-dependent manner. CRP-ductin also showed calcium-dependent binding to both gram-positive and -negative bacteria (Holmskov et al. 1997).

Interactions with Decorin and Biglycan and Other Proteoglycans: Decorin and biglycan are closely related abundant extracellular matrix proteoglycans that bind to C1q and MBL. The human decorin, a SP-D-binding protein, from amniotic fluid co-purifies with SP-D. SP-D and decorin interact with each other (K_D = 4 nM) by two mechanisms: (1) the CRD of SP-D binds, in a calcium dependent-manner, to sulfated N-acetyl galactosamine moiety of the glycosaminoglycan chain and (2) C1q, a complement protein that is known to interact with decorin core protein via its collagen-like region, partially blocks the interaction between decorin and native SP-D. It is suggested that decorin core protein binds the collagen-like region of SP-D and CRDs of SP-D interact with dermatan sulfate moiety of decorin via lectin activity (Nadesalingam et al. 2003).

Other members of collectin family, including collectin-43 and conglutinin also bind to decorin and biglycan. Thus, decorin and biglycan act as inhibitors of activation of complement cascade and pro-inflammatory cytokine production mediated by C1q. These proteoglycans are likely to down-regulate proinflammatory effects mediated by C1q (Groeneveld et al. 2005).

25.2.2 Binding with Nucleic Acids

Collectins can bind nucleic acid, which is a pentameric sugar-based anionic polymer. SP-D and MBL bind to both DNA and RNA effectively. SP-D also enhances the uptake of DNA by human monocytic cells. Therefore, nucleic acids can act as ligands for collectins (Palaniyar et al. 2003b). Pentoses, such as arabinose, ribose, and deoxyribose, inhibit the interaction between SP-D and mannan, one of the well-studied hexose ligands for SP-D; biologically relevant D-forms of the pentoses are better competitors than the L-forms. SP-D binds apoptotic cells through DNA present on these cells. SP-D binds and aggregates mouse alveolar macrophage DNA effectively. Alveolar macrophages of SP-D$^{-/-}$ mice contained more nicked DNA than those of SP-A$^{-/-}$ and wild type mice. Results also suggested that CRDs of SP-D may recognize DNA present on apoptotic cells. Therefore, cell-surface DNA could be a ligand for recognition of apoptotic cells by collectins and may have important biological implications, such as the alleviation of DNA-mediated tissue inflammation (Palaniyar et al. 2003a)

SP-D enhances the uptake of Cy3-labeled fragments of DNA and DNA-coated beads by U937 human monocytic cells, in vitro. Analysis of DNA uptake by alveolar macrophages shows that SP-D, but not SP-A deficiency results in reduced clearance of DNA, ex vivo. Additionally, both SP-A- and SP-D-deficient mice accumulate anti-DNA Abs in sera in an age-dependent manner. Thus, SP-A and SP-D may reduce the generation of anti-DNA autoantibody, which may be explained in part by defective clearance of DNA from lungs in absence of these proteins. These findings establish two new roles for these innate immune proteins and that SP-D enhances efficient pinocytosis and phagocytosis of DNA by macrophages and minimizes anti-DNA Ab generation (Palaniyar et al. 2005).

25.2.3 Interactions with Lipids

Surfactant proteins interact with phospholipids and are believed to play important roles in alveolar spaces. Pulmonary SP-D binds glycosylated lipids such as phosphatidylinositol (PI) and glucosylceramide (GlcCer). The major known surfactant-associated ligand for lung SP-D is PI. SP-D binds to PI in various phospholipids or a fraction containing phospholipids. Interaction of SP-D with PI is dependent on calcium and inhibited by competing saccharides. SP-D binds with similar efficiency to liposomes (Ogasawara et al. 1992; Persson et al. 1992). On TLC plates, SP-D bound exclusively to GlcCer, whereas it failed to bind to GalCer, GM1, GM2, asialo-GM1, asialo-GM2, sulfatide, Forssman antigen, ceramide dihexoside, ceramide trihexoside, globoside, paragloboside or ceramide. SP-D bound to ceramide monohexoside in glycolipids isolated from rat lung and bronchoalveolar lavage fluids of rats (Kuroki et al. 1992). PI interaction, at least in part, involves the carbohydrate moiety. Results suggest that: (1) carbohydrate binding specificity of SP-D (Glu-321—>Gln and Asn-323—>Asp) was changed from a mannose-glucose type to a galactose type; (2) the GlcCer binding property of SP-D is closely related to its sugar binding activity; and (3) the PI binding activity is not completely dependent on its carbohydrate binding specificity (Ogasawara and Voelker 1995a). SP-D counteracts the inhibitory effect of SP-A on phospholipid secretion by alveolar type II cells. Native SP-D alters SP-A activity in type II cells through interaction with it via SP-D-associated lipids (Kuroki et al. 1991).

SP-D binds specifically to saturated, unsaturated, and hydroxylated fatty acids (FA). Maximal binding to FA was dependent on calcium, and was localized to neck and CRD region in recombinant trimeric neck + CRDs. Saccharide ligands showed complex, dose-dependent effects on FA binding, and FAs showed dose- and physical state-dependent effects on the binding of SP-D to mannan (DeSilva et al. 2003).

Trimeric neck-carbohydrate recognition domains (NCRDs) of rat and human SP-D exhibited dose-dependent, calcium-dependent, and inositol-sensitive binding to solid-phase PI and to multilamellar PI liposomes. Studies directly implicate the CRD in PI binding and reveal unexpected species differences in PI recognition that can be largely attributed to the side chain of residue 343. In addition, oligomerization of trimeric subunits is an important determinant of recognition of PI by human SP-D (Crouch et al. 2007, 2009).

25.3 Structure: Function Relations of Lung SP-D

25.3.1 Role of NH_2 Domain and Collagenous Region

SP-A and SP-D possess similar structures in mammalian C-type lectin superfamily. Both proteins are composed of four characteristic domains which are: (1) an NH_2-terminal domain involved in interchain disulfide formation (denoted

A1 domain for SP-A or D1 for SP-D); (2) a collagenous domain (denoted A2 or D2); (3) a neck domain (denoted A3 or D3); and (4) a CRD (denoted A4 or D4). A collagen domain deletion mutant (CDM) of SP-D and a second variant lacking both the amino-terminal region and collagen-like domain were generated and collagenase-resistant fragment (CRF) was purified. Studies on CDM and CRF demonstrated that collagen-like domain is required for dodecamer but not covalent trimer formation of SP-D and plays an important, but not essential, role in the interaction of SP-D with phosphatidylinositol (PI) and glucosylceramide (GlcCer). Removal of amino-terminal domain of SP-D along with collagen-like domain diminished PI binding and effectively eliminated GlcCer binding (Ogasawara and Voelker 1995b). Amino-terminal domains of SP-A and SP-D are critical for surfactant phospholipid interactions and surfactant homeostasis, respectively.

To further assess the importance of N-terminal domains of SP-D/SP-A in surfactant structure and function, a chimeric SP-D/SP-A gene was constructed by substituting nucleotides encoding amino acids Asn-1-Ala7 of rat SP-A with the corresponding N-terminal sequences from rat SP-D, Ala1-Asn25. Studies indicated that N terminus of SP-D: (1) can functionally replace the N terminus of SP-A for lipid aggregation and tubular myelin formation, but not for surface tension lowering properties of SP-A, and (2) is not sufficient to reverse the structural and metabolic pulmonary defects in SP-D$^{-/-}$ mouse (Palaniyar et al. 2002).

25.3.2 Role of NH2-Terminal Cysteines in Collagen Helix Formation

The NH2-terminal sequence of each monomer in SP-D contains two conserved cysteine residues. The SP-D is preferentially secreted as dodecamers consisting of four collagenous trimers cross-linked by disulfide bonds. Although mutants with serine substituted for Cys15 and Cys20 (RrSP-Dser15/20) are secreted as trimeric subunits, proteins with single cysteine substitutions were retained in the cell. Studies suggest that the most important and rate-limiting step for the secretion of SP-D involves the association of cross-linked trimeric subunits to form dodecamers stabilized by specific inter-subunit disulfide cross-links. Interference with collagen helix formation prevents secretion by interfering with efficient disulfide cross-linking of the NH2-terminal domain (Brown-Augsburger et al. 1996a). The two conserved cysteine residues participate in interchain disulfide bonds formation. Substitution of serine for Cys15 and Cys 20 in recombinant rat SP-D (RrSP-Dser15/20) bound to the hemagglutinin of influenza A virus. However, it failed to aggregate virus and did not enhance the binding of influenza A to neutrophils (PMN), augment PMN respiratory burst, or protect PMNs from deactivation. It indicates that amino-terminal disulfides are required to stabilize dodecamers, and support the hypothesis that the oligomerization of trimeric subunits contributes to the anti-microbial properties of SP-D (Brown-Augsburger et al. 1996b; Crouch et al. 2005).

25.3.3 D4 (CRD) Domain in Phospholipid Interaction

As discussed earlier that SP-A specifically binds to DPPC, the major lipid component of surfactant, and can regulate the secretion and recycling of this lipid by alveolar type II cells. SP-D binds to PI and glucosylceramide (GlcCer). The D3 (neck) plus D4 (CRD) domains of SP-D play a role in lipid binding and that CRD domain is essential for PI binding. Furthermore, the A3 domain of SP-A cannot account for all the lipid binding activity of this protein. The results implicate the A4 domain of SP-A as an important structural domain in lipid aggregation phenomena (Ogasawara et al. 1994). Saitoh et al. (2000) showed that (1) SP-A region Leu-219-Phe-228 is required for liposome aggregation and interaction with alveolar type II cells, (2) SP-A region Cys-204-Cys-218 is required for DPPC binding, (3) the SP-D region Cys-331-Phe-355 is essential for minimal PI binding, and (4) the epitope for mAb 1D6 is located at the region contiguous to SP-A region Leu-219-Phe-228. Analysis of chemotactic properties of trimeric CRD demonstrated that CRD was chemotactic for neutrophils (polymorphonuclear leukocytes). The chemotactic activity was abolished by maltose, which did not suppress the chemotactic response to fMLP (Cai et al. 1999).

The recombinant structure, containing three CRDs, was comparable to native SP-D in terms of carbohydrate binding specificity, binding to LPSs of Gram-negative bacteria, and interaction with phospholipids. The CRD of SP-D, without the neck region peptide that was also expressed, showed a very weak affinity for LPSs and phospholipids. The α-helical neck region on its own showed affinity for phospholipids and thus might contribute to the binding of SP-D to these structures. These results show the importance of the neck region as a trimerizing agent in bringing together three CRDs and suggest that multivalency is important in the strong binding of SP-D to carbohydrate targets (Kishore et al. 1996).

Zhang et al. (2001a) provided evidence that amino-terminal heptad repeats of the neck domain are necessary for intracellular, trimeric association of SP-D monomers and for the assembly and secretion of functional dodecamers (Zhang et al. 2001b). A recombinant homotrimer, composed of the α-helical neck region of human surfactant protein D and C1q B chain globular domain, was an inhibitor of classical complement pathway (Kishore et al. 2001).

25.3.4 A Three Stranded α-Helical Bundle at Nucleation Site of Collagen Triple-Helix Formation

A short stretch of 35 amino acids comprising a structural motif is responsible for tight parallel association and trimerization of three identical polypeptide chains of lung SP-D, which contains both collagen regions and C-type lectin domains. This 'neck-region' is located at nucleation site at which collagenous sequences fold into a staggered triple-helix to consist of a triple-stranded parallel α-helical bundle in a non-staggered, and extremely strong, non-covalent association. This type of association between three polypeptide chains may represent a common structural feature immediately following C-terminal end of triple-helical region of collagenous proteins (Hoppe et al. 1994).

The SP-D is assembled predominantly as dodecamers consisting of four homotrimeric subunits each. Trimerization of SP-D monomers is required for high affinity saccharide binding, and the oligomerization of trimers is required for many of its functions. A peptide containing α-helical neck region can spontaneously trimerize in vitro. Håkansson et al. (1999) determined the crystal structure of a trimeric fragment of hSP-D at 2.3 A resolution. The structure comprises an α-helical coiled-coil and three carbohydrate-recognition domains (CRDs). A deviation from symmetry was found in the projection of a single tyrosine side chain into the centre of coiled-coil; the asymmetry of this residue influences orientation of one of the adjacent CRDs. The cleft between the three CRDs presents a large positively charged surface. The fold of CRD of hSP-D is similar to that of MBP, but its orientation relative to α-helical coiled-coil region differs somewhat to that seen in MBP structure. The novel central packing of tyrosine side chain within the coiled-coil and the resulting asymmetric orientation of CRDs has unexpected functional implications. The positively charged surface might facilitate binding to negatively charged structures, such as LPS (Håkansson et al. 1999).

Association of trimeric subunits is stabilized by interchain disulfide bonds involving two conserved amino-terminal cysteine residues (Cys-15 and Cys-20). Mutant recombinant rat SP-D lacking these residues (RrSP-Dser15/20) is secreted in cell culture as trimeric subunits rather than as dodecamers. Activity of SP-D in lungs is dependent on oligomeric structure. In transgenic mice that express mutant RrSP-Dser15/20 showed that the activity of lung SP-D in vivo is dependent on oligomeric structure. Disulfide cross-linked SP-D oligomers are required for regulation of surfactant phospholipid homeostasis and prevention of emphysema and foamy macrophages in vivo (Zhang et al. 2001a; McAlinden et al. 2002).

The solution structure of a 64-residue peptide encompassing the coiled-coil domain of human SP-D confirmed that the domain forms a triple-helical parallel coiled coil. Symmetry-ADR (ambiguous distance restraint) structure calculations demonstrated that leucine zipper region of SP-D is an autonomously folded domain and agreed with X-ray crystal structure, differing mainly at a single residue, Tyr248. This residue is completely symmetric in solution structure, and markedly asymmetric in crystalline phase. This difference may be functionally important, as it affects orientation of antigenic surface presented by SP-D (Kovacs et al. 2002).

25.3.5 Ligand Binding Amino Acids

Arg343 in SP-D Discriminates Between Glucose and N-acetylglucosamine Ligands: SP-D binds glucose (Glc) stronger than N-acetylglucosamine (GlcNAc). Structural superimposition of hSP-D with MBP-C complexed with GlcNAc revealed steric clashes between the ligand and the side chain of Arg343 in hSP-D. Computational model of Arg343—>Val (R343V) mutant hSP-D demonstrated that Arg343 is critical for hSP-D recognition specificity and plays a key role in defining ligand specificity differences between MBP and SP-D. Additionally, the number of binding orientations contributes to monosaccharide binding affinity (Allen et al. 2004). Asp325, in addition to Arg343, is other important determinant of ligand selectivity, recognition, and binding; and that differences in crystal contact interfaces exert, through Asp325, significant influence on preferred binding modes (Shrive et al. 2009).

Contributions of Phenylalanine-335 to Ligand Recognition: A trimeric fusion protein encoding the human neck + CRD bound to aromatic glycoside p-nitrophenyl-α-D-maltoside with a higher affinity than maltose. Maltotriose, which has the same linkage pattern as the maltoside, bound with intermediate affinity. Substitution of leucine Phe-335 decreased affinities for maltoside and maltotriose without altering affinity for maltose or glucose, and substitution of tyrosine or tryptophan for leucine restored binding to maltotriose and maltoside. Crouch et al. (2006a) indicated that Phe-335, which is evolutionarily conserved in all known SP-Ds, plays important role in SP-D function. The ligand binding of homologous human, rat, and mouse trimeric neck + CRD fusion proteins, each with identical N-terminal tags remote from ligand-binding surface was compared. The ligand recognition by human SP-D involves a complex interplay between saccharide presentation, valency of trimeric subunits, and species-specific residues that flank primary carbohydrate binding site (Crouch et al. 2006b).

25.3.6 Ligand Binding and Immune Cell-Recognition

Crystallographic studies of trimeric human SP-D neck +CRD domains have shown that maltose, a preferred saccharide

ligand, binds to calcium via the vicinal 3- and 4-OH groups of the non-reducing glucose, previously designated calcium ion 1 and glucose 1 (Glc1), respectively (Shrive et al. 2003). These interactions are further stabilized by hydrogen bonding of Glc1 to amino acid side chains that also coordinate with calcium ion 1. The crystal structures of maltose-bound rfhSP-D (recombinant homotrimeric fragment of human SP-D comprising α-helical coiled-coil neck plus three CRDs) at 1.4A, and of rfhSP-D at 1.6A provided insights on the mode of carbohydrate recognition and fine details of SP-D binding to allergens/antigens or whole pathogens, and details on engagement of effector cells and molecules of humoral immunity. A calcium ion, located on trimeric axis in a pore at the bottom of funnel formed by the three CRDs and close to neck-CRD interface, is coordinated by a triad of glutamate residues which are, to some extent, neutralised by their interactions with a triad of exposed lysine residues in the funnel. The spatial relationship between neck and CRDs is maintained internally by these lysine residues, and externally by a glutamine, which forms a pair of hydrogen-bonds within an external cleft at each neck-CRD interface. Structural links between central pore and cleft suggested a possible effector mechanism for immune cell surface receptor binding in presence of bound, extended LPS and phospholipid ligands. The structural requirements for such an effector mechanism, involving both the trimeric framework for multivalent ligand binding and recognition sites formed from more than one subunit, are present in both native hSP-D and rfhSP-D, providing a possible explanation for significant biological activity of rfhSP-D (Shrive et al. 2003).

25.4 Regulation of Sp-D by Various Factors

25.4.1 Glucocorticoids

Surfactant proteins are known to be regulated by glucocorticoids. Lung explants from fetuses depend on hydrocortisone for their growth. The addition of hydrocortisone resulted in increase in SP-D mRNA expression (Gonzales et al. 2001; Shannon et al. 2001). The accelerated lung maturation accompanying glucocorticoid exposure in utero is associated with a precocious increase in SP-D gene transcription and protein production by pulmonary epithelial cells (Mariencheck and Crouch 1994). Glucocorticoid treatment in vivo of fetal, neonatal and adult rats for short durations exhibits stimulatory effect on contents of lung SP-D. Human lung is under developmental and glucocorticoid regulation occurring at a pre-translational level. SP-D is not influenced by inflammatory mediators that regulate SP-A, suggesting that these two proteins are not coordinately regulated in response to lung infection (Dulkerian et al. 1996). Early postnatal use of Dex in infants with respiratory distress syndrome (RDS) was shown to improve pulmonary status and to allow early weaning off mechanical ventilation. Early use of Dex can improve pulmonary status and also increase SP-A and SP-D levels in tracheal fluid in premature infants with RDS (Wang et al. 1996).

25.4.2 1α,25-Dihydroxyvitamin D3

The active form of 1α,25-dihydroxyvitamin D_3 [1,25 $(OH)_2D_3$], has been shown to stimulate lung maturity, alveolar type II cell differentiation and fetal lung development. In fetal rat lung, 1,25$(OH)_2D_3$ increases the synthesis and secretion of surfactant lipids and accelerates the appearance of the morphological features of alveolar type II cells (Marin et al. 1993). It was stated that a natural metabolite of 1,25 $(OH)_2D_3$ increases surfactant phospholipid and SP-B mRNA and protein synthesis in human NCI-H441 cells (Rehan et al. 2002; Phokela et al. 2005). Phokela et al. (2005) suggest that regulation of surfactant protein gene expression in human lung and type II cells by 1,25$(OH)_2D_3$ is not coordinated where as 1,25$(OH)_2D_3$ decreases SP-A mRNA and protein levels in both fetal lung tissue and type II cells.

25.4.3 Growth Factors

Keratinocyte growth factor (KGF, FGF-7) is a potent mitogen of pulmonary bronchial and alveolar epithelial cells. Airway epithelial cells (AEC) proliferate in response to KGF. KGF instillation resulted in epithelial cell hyperplasia in rats and the mRNA levels for SP-A, SP-B, and SP-D were increased in whole lung tissue on days 1 and 2 after KGF treatment (Yano et al. 2000). In presence of KGF, rat serum mRNAs for surfactant proteins were maintained at high levels. Secretion of SP-A and SP-D was found to be independent of phospholipid secretion (Mason et al. 2002). Clara cells also responded to human KGF in vivo by proliferation as well as by changes in protein expression, whereas no significant response was observed in pulmonary neuroendocrine cells (Fehrenbach et al. 2002; Shannon et al. 2001). Growth hormone significantly increases MBL and SP-D levels in Turner Syndrome. A link between endocrine and immune system with clinical consequences needs to be studied (Gravholt et al. 2004). VEGF is an autocrine proliferation and maturation factor for developing alveolar type II cells. But VEGF does not change the transcriptional level of SP-A, SP-C, and SP-D though it increases SP-B. The effects of VEGF isoform on type II

cells are likely to be exerted indirectly through reciprocal paracrine interactions involving other lung cell types (Raoul et al. 2004). Pretreatment therapy by recombinant human VEGF (rhVEGF) decreased RSV disease in perinatal lamb respiratory syncytial virus (RSV) model (Meyerholz et al. 2007). Increased expression of SP-D mRNA at 6 and 24 h and decreased expression of SP-A mRNA at 12 h were observed after treatment with all-trans retinoic acid (RA) (Grubor et al. 2006).

25.5 SP-D in Human Fetal and Newborns Lungs

The SP-D was not detected in fetuses at 8–19 weeks gestation. At 21 weeks gestation, SP-D was weakly localized, in some cases (5/9), in the epithelial lining of both bronchioles and terminal airways. In contrast at 21 weeks gestation, SP-A was more markedly detected in the epithelial lining of both bronchioles and terminal airways in all cases but not detected in bronchioles and terminal airways at 8–19 weeks gestation. The production of SP-D in fetal human lungs begins in bronchiolar and terminal epithelium from about 21 weeks of gestation (Mori et al. 2002). Stahlman et al. (2002) detected SP-D on airway surfaces by 10 weeks' gestation and indicated that SP-D is secreted onto luminal surfaces by epithelial cells lining ducts of many organs, where it likely plays a role in innate host defense. The SP-D concentrations in umbilical cord blood and capillary blood are highly variable and depend on several perinatal conditions. In preterm infants, significant changes occur in collectin umbilical cord blood concentrations and pulmonary SP-D levels. Further studies are needed to elucidate the effect of respiratory distress and infection on SP-D concentrations (Dahl et al. 2006; Hilgendorff et al. 2005).

Serum SP-D and MBL levels are significantly low in preterms between 28 and 32 week gestational age (GA) compared to term infants and positively correlated with history of antenatal corticosteroids and chorioamnionitis. The SP-D levels in tracheal aspirates (TA) were increased in preterm infants between 28 and 32 week GA with respiratory distress syndrome compared to control subjects in contrast to extremely immature infants <28 week GA suffering from respiratory distress syndrome (RDS). SP-D concentrations in umbilical cord blood and capillary blood in premature infants are twice as high as in mature infants and depend on several perinatal conditions. High SP-D levels in umbilical cord blood and capillary blood on day 1 were found to be related to increased risk of RDS and infections (Dahl et al. 2006).

25.6 Non-Pulmonary SP-D

25.6.1 Human Skin and Nasal Mucosa

The SP-D has been detected in basal layers of normal human skin. It is more abundant in the stratum spinosum of lesional psoriatic and atopic skin due to more cells producing molecule rather than up-regulation of production in single cells of diseased skin (Hohwy et al. 2006). The surface tension of SP-deficient artificial sebum is (a) lowered by skin-extracted SP-B and (b) further reduced to a level comparable to normal sebum by additional presence of skin-extracted SP-A and SP-D, consistent with their surface tension-lowering capabilities in lung (Mo et al. 2007). SP-A2, SP-B, and SP-D mRNAs were expressed normal human nasal epithelial (NHNE) cells and human nasal mucosa and localized in ciliated cells of the surface epithelium and serous acini of submucosal glands (Kim et al. 2007; Woodworth et al. 2006, 2007).

25.6.2 Digestive Tract, Mesentery, and Other Organs

The fact that surfactant-like materials composed of phospholipids are secreted by a number of other organs prompted several groups to search for SP expression in these organs also. The SP-D occurs in gastric mucosa at the luminal surface and within gastric pits of mucus-secreting cells. The hydrophilic proteins SP-A and SP-D and their transcripts have been found in a number of tissues, including gastric and intestinal mucosae, mesothelial tissues (mesentery, peritoneum, and pleura), synovial cells, Eustachian tube and sinus, and possibly in salivary glands, pancreas, and urinary tract. SP-D is expressed in mucus-secreting cells of gastric mucosa. SP-D protein and mRNA were not detected in the duodenum and remainder of the gastrointestinal tract (Herias et al. 2007). It is possible that SP-D may participate in the regulation of secretion or assembly of gastric acid barrier. Alternatively, SP-D may participate in gastric mucosal host defense (Fisher and Mason 1995). By contrast, the hydrophobic proteins SP-B and SP-C actually appear to be expressed in lung epithelium only. The expression of SP-A and SP-D appears as a general feature of organs exposed to pathogens because they present an interface with the external milieu. Although this function has been investigated in lung only through the gene-targeting approach, increased expression of SP-A in the infected middle ear and of SP-D in the *Helicobacter*-infected

antrum argues for such a function also in other organs. In organs that are not exposed to external pathogens, their role is likely to exert anti-inflammatory and immunomodulatory functions, as suggested by increased SP-A immunoreactivity in rheumatoid disease. SP-A and SP-B have been found in association with phospholipids in the lung of all air-breathing vertebrates, including the most primitive forms represented by lungfish, which implies that the surfactant system had a single evolutionary origin. Immunochemical proximity of the proteins among vertebrates indicates considerable conservation during evolution. Infection with the gastroduodenal pathogen *Helicobacter pylori* up-regulates expression of SP-D in human patients with gastritis, and its influence on colonization has been demonstrated in a *Helicobacter* SP-D-deficient (SP-$D^{-/-}$) mouse model. SP-D binds and agglutinates *H. pylori* cells in a lectin-specific manner, and has been shown to bind *H. pylori* lipopolysaccharide. Furthermore, evidence indicates that *H. pylori* varies LPS O-chain structure to evade SP-D binding which is speculated aids persistence of this chronic infection (Bourbon and Chailley-Heu 2001; Moran et al. 2005). To explore the similarity between lung and peritoneal surfactants, Chailley-Heu et al. (1997) suggested that mesothelial cells also produce SP-A and SP-D, although they are of embryonic origin (mesodermal) and are different from those of lung and digestive tract (endodermal) that secrete these surfactants. Porcine SP-D bound to solid-phase mannan in a Ca^{2+}-dependent manner with a saccharide specificity similar to rat and human SP-D. Whereas SP-D and SP-A are abundant in the peripheral lung, their presence in sputum derived from the larger airways is variable and their carbohydrate binding capacity is lost. Sputum sol fraction and LPS inhibited the binding of the collectins to carbohydrate in the presence of calcium (Griese et al. 2001).

Bands for SP-A were detected in human Eustachian tube (ET) (SP-A, 34 kDa) and in kidney extracts, and for SP-D (43 kDa) in Eustachian tube and in kidney extracts (SP-D, 86 kDa), and for SP-B (8 kDa) in human ET and organ of Corti extracts. Dysfunction of local mucosal immunity in ET may predispose infants to recurrent otitis media. The protein was found in the granules of microvillar epithelial cells. SP-A and SP-D may be important for antibody-independent protection of the middle ear against infections (Kankavi 2003; Paananen et al. 2001).

25.6.3 Male Reproductive Tract

SP-A and SP-D have been identified in testis and epididymis. The presence of SP-A, SP-D and components of molecular weight 34 and 43 kDa have been detected in human spermatozoa. SP-A is localized in mid-piece, the tail, and sometimes at equatorial region of spermatozoa (Kankavi et al. 2008). SP-D mRNA and protein are present throughout the mouse male reproductive tract, including the prostate. Castration increases prostate SP-D mRNA levels. SP-D protects the human prostate from infection by pathogens. The SP-D inhibits infection of prostate epithelial cells by *Chlamydia trachomatis* in an in vitro infection assay. The SP-D binds to C. *trachomatis* via its carboxy-terminal lectin domains. SP-D protein levels are increased at sites of inflammation in prostate, suggesting SP-D may also contribute more generally to inflammatory regulation in prostate (Oberley et al. 2005). Results suggest that infection and androgens regulate SP-D in the prostate (Oberley et al. 2007b).

Surfactant proteins in the stallion reproductive tract contribute to immune surveillance and to active barrier defense mechanism (Kankavi et al. 2007). SP-A and SP-D are present in the mare genital tract, vulva, vagina, ovarium, uterus and tuba uterina. The SPs are present not only in just lamellar bodies associated with lung, but also in genital system of mare (Kankavi et al. 2007). SP-D protein was localized in the apical portion of the reproductive epithelial cells. One of the functions of the SP-D protein may be to protect cervical epithelial cells from infection by C. *trachomatis*. *Chlamydia muridarum* infection caused an increase in the SP-D protein content of reproductive tract epithelial cells. Data were suggestive that SP-D may play a role in innate immunity in the female reproductive tract in vivo (Oberley et al. 2004, 2007a).

25.6.4 Female Genital Tract

The SP-D is present in the female genital tract, the placenta and in amniotic fluid. In the placenta, SP-D was seen in all villous and extravillous trophoblast subpopulations. Endometrial presence of SP-D in non-pregnant women varied according to stage of the menstrual cycle and was up-regulated towards the secretory phase. Endometrial SP-D may prevent intrauterine infection at the time of implantation and during pregnancy (Leth-Larsen et al. 2004). Findings suggest that SP-A and SP-D interact with chlamydial pathogens and enhance their phagocytosis into macrophages. *Chlamydia muridarum* infection increases SP-D protein content of reproductive tract epithelial cells, suggestiing that SP-D plays a role in innate immunity in the female reproductive tract in vivo (Oberley et al. 2004, 2007).

Amniotic Fluid: MBP, SP-A and SP-D are present in amniotic fluid and localize on the surface of amniotic epithelium. MBP levels in amniotic fluid were found to increase sharply from about 32 weeks of gestation. Both SP-A and SP-D were detected in amniotic fluid as early as 26 weeks gestation and, SP-A levels rose sharply from 32 weeks towards term. By

contrast, SP-D levels rose only moderately. Collecins appear to play a role in antibody-independent recognition and clearance of pathogens in amniotic cavity, towards term (Malhotra et al. 1994; Miyamura et al. 1994b). The production of SP-D is increased shortly prior to birth, and the increases in total lung SP-D and SP-D mRNA are temporally correlated with SP-D secretion and the appearance of SP-D in amniotic fluid.

Intra-Amniotic Endotoxin Accelerates Lung Maturation: Intra-amniotic LPS and cytokines may decrease RDS and increase chronic lung disease in the newborn. Intrauterine exposure to LPS increases surfactant protein expression and improves lung stability and aeration in preterm animals (Bry and Lappalainen 2001). Intra-amniotic endotoxin from *E. coli* 055:B5 induces lung maturation within 6 days in fetal sheep of 125 days gestational age. SP-A, SP-B, and SP-C mRNAs were maximally induced at 2 days. SP-D mRNA was increased fourfold at 1 day and remained at peak levels for up to 7 days. The alveolar pool of SP-B was significantly increased between 4 and 7 days in conjunction with conversion to the fully processed active airway peptide. All SPs were significantly elevated in the BAL fluid by 7 days (Bachurski et al. 2001). IL-1 and glucocorticoid affect the expression of SP-A, -B, and –C in lung explants from rabbit fetuses. The study revealed beneficial additive effects of glucocorticoid and cytokine on lung surfactant (Vayrynen et al. 2004).

25.7 Functions of Lung SP-D

25.7.1 Innate Immunity

Surfactant is a complex of lipids and proteins that reduces surface tension at the air/liquid interface of the lung and regulates immune cell function. Surfactant immune function is primarily attributed to two proteins: SP-A and SP-D. It has been known for several years that surfactant lipids suppress a variety of immune cell functions, most notably lymphocyte proliferation, which, conversely, is augmented by SP-A. Thus surfactant lipids and proteins may be counterregulatory, and changes in lipid-to-protein ratios may be important in regulating the immune status of the lung. That these ratios change in disease states is clear, but it is not known whether the alterations are a cause or an effect. Studies with mice in which the SP-A and SP-D genes have been ablated are helping to clarify the role of surfactant in immune function (Kingma and Whitsett 2006)

The SP-A and SP-D bind carbohydrates, lipids, and nucleic acids with a broad-spectrum specificity and initiate phagocytosis of inhaled pathogens as well as apoptotic cells. Investigations on gene-deficient and conditional overexpressed mice indicated that lung SP-A and SP-D directly modulate innate immune cell function and T-cell-dependent inflammatory events and have a unique, dual-function capacity to induce pathogen elimination and control proinflammatory mechanisms. Surfactant protein-D participates in innate response to inhaled microorganisms and organic antigens, and contributes to immune and inflammatory regulation within lung. Porcine SP-D promotes uptake of pathogenic bacteria by epithelial cells. This reflects a scavenger function for SP-D in intestine, which enables the host to generate a more rapid response to infectious bacteria. SP-D binds to surface glycoconjugates expressed by a wide variety of microorganisms, and to oligosaccharides associated with the surface of various complex organic antigens. After binding of SP-D to microbial surface effector mechanisms such as agglutination, neutralizing or opsonization of microorganisms for phagocytosis are initiated. SP-D also specifically interacts with glycoconjugates and other molecules expressed on the surface of macrophages, neutrophils, and lymphocytes. Presence and activity of SP-D in porcine coronary endothelial cells depend on PI3K/Akt, Erk and nitric oxide and decrease after multiple passaging. They suggest a protective role of SP-D in these cells (Lee et al. 2009). In addition, SP-D binds to specific surfactant-associated lipids and can influence the organization of lipid mixtures containing phosphatidylinositol in vitro. SP-D-deficient transgenic mice show abnormal accumulations of surfactant lipids, and respond abnormally to challenge with respiratory viruses and bacterial lipopolysaccharides. The phenotype of macrophages isolated from lungs of SP-D-deficient mice is altered. SP-D stimulates chemotaxis of phagocytes and once bound to the phagocytes, the production of oxygen radicals can be induced. Circumstantial evidence indicates that abnormal oxidant metabolism and/or increased metalloproteinase expression contributes to the development of emphysema. The expression of SP-D is increased in response to many forms of lung injury, and deficient accumulation of appropriately oligomerized SP-D might contribute to the pathogenesis of a variety of human lung diseases (Crouch 2000; Hogenkamp et al. 2007; Reid 1998; Wright 2004; Awasthi et al. 2001) (Figs. 25.1, 25.2, 25.3).

25.7.2 Effects on Alveolar Macrophages

SP-D opsonizes pathogens and enhances their phagocytosis by alveolar macrophages and neutrophils. SP-D was found to bind alveolar Type II cells, Clara cells, and alveolar macrophages. In Type II cells abundant binding was

observed in the endoplasmic reticulum, whereas Golgi complex and multivesicular bodies were labeled to a limited extent. Anti-surfactant SP-A and SP-D showed both SP-A and SP-D in same granules (Kuan et al. 1994). Both SP-D and SP-A have been shown to enhance oxygen radical production by alveolar macrophages. SP-D binds specifically to alveolar macrophages and the receptor involved is different from that of C1q (Miyamura et al. 2004a). SP-D can bind to specific surfactant phospholipids and to glycoconjugates associated with the surface of various microorganisms, consistent with possible roles in surfactant metabolism and pulmonary host defense. Administration of a truncated 60-kDa fragment of human rSP-D reduces the number of apoptotic and necrotic alveolar macrophages and partially corrects the lipid accumulation in SP-D-deficient mice. The same SP-D fragment binds preferentially to apoptotic and necrotic alveolar macrophages in vitro, that suggests that SP-D contributes to immune homeostasis in lung by recognizing and promoting removal of necrotic and apoptotic cells (Clark et al. 2002, 2003) (Fig. 25.3). Findings indicate that collagen domain of SP-D is not required for assembly of disulfide-stabilized oligomers or the innate immune response to viral pathogens. However, collagen domain of SP-D is required for regulation of pulmonary macrophage activation, airspace remodeling, and surfactant lipid homeostasis (Kingma et al. 2006). Surfactant protein D regulates the cell surface expression of alveolar macrophage β2-integrins (Senft et al. 2007).

Opsonization of Gram-Negative Bacteria Independent of Macrophages: SP-A and SP-D bind LPS, opsonize microorganisms, and enhance the clearance of lung pathogens. The pulmonary clearance of *E. coli K12* was reduced in SP-A-null mice and was increased in SP-D-overexpressing mice. Purified SP-A and SP-D inhibited bacterial synthetic functions of several, but not all, strains of *E. coli, Klebsiella pneumoniae,* and *E. aerogenes*. In general, rough *E. coli* strains were more susceptible than smooth strains, and collectin-mediated growth inhibition was partially blocked by coincubation with rough LPS vesicles. Data indicate that SP-A and SP-D are antimicrobial proteins that directly inhibit proliferation of Gram-negative bacteria in a macrophage- and aggregation-independent manner by increasing the permeability of the microbial cell membrane (Wu et al. 2003).

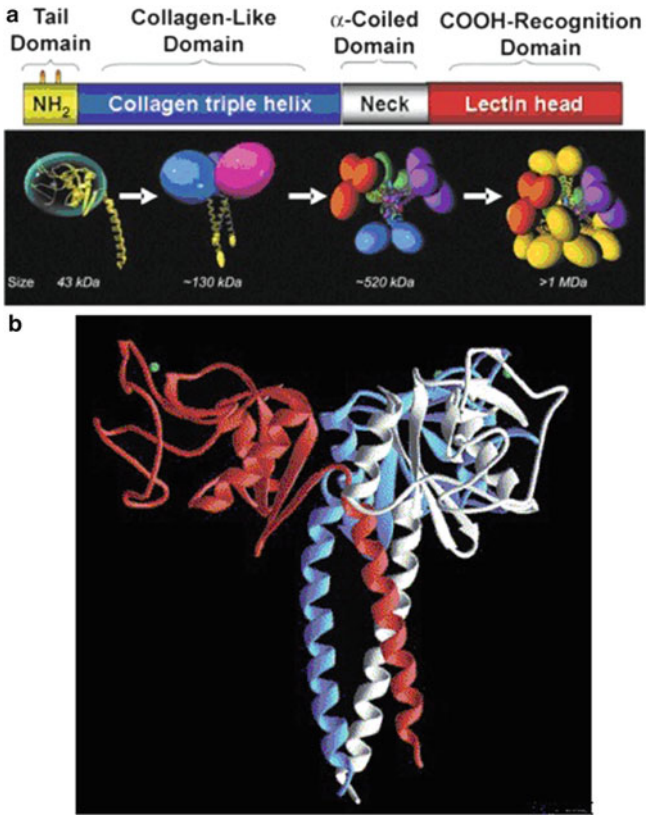

Fig. 25.1 SP-D structure: (*Upper Panel*) **A model of SP-D structure**. The SP-D monomer (43 kDa) consists of a carbohydrate recognition domain which forms the globular head structure. This domain is connected to the collagen-like helical tail domain by a short, 30–amino acid, neck domain. At the end of the tail domain is the amino terminus in which cysteines 15 and 20 are positioned (shown as *yellow* projections). (*Central panel*) Stylized representation of SP-D multimer assembly (note tail domains are shown *shortened* for ease of visualization). The head and neck domains drive the aggregation of the SP-D monomer to form a trimer of ~130 kDa. These trimers associate to form a dodecamer (~520 kDa). The forces holding this dodecamer together are unclear, although there is a dependency upon the amino-terminal cysteines as mutant lacking these cysteines do not form dodecamers. These dodecamers can assemble to a multimer of greater than 1 MDa. It is unclear whether the dodecamer is an essential intermediate in multimer formation. It should be noted that neither the trimer nor the dodecamer are globular proteins, due to the presence of the long collagen tail and thus under native conditions will behave as molecules with greater molecular radius (Adapted from Guo et al. PLoS Biol. 2008; 6: e266). (*Lower Panel*) *Ribbon diagram* shows the overall main chain structure of human lung surfactant protein SP-D. Each monomer is shown in a different colour and calcium ions are depicted as *green spheres* (Adapted with permission from Håkansson et al. 1999 © Elsevier)

25.7.3 Functions of Neutrophils

Calcium-Dependent Neutrophil Uptake of Bacteria: Because neutrophils (PMN) and monocytes are recruited into the airspaces in association with many types of infection or lung injury, Crouch et al. examined the interactions of these cells with SP-D. Natural or recombinant rat SP-D showed dose-dependent effects on human PMN and monocyte migration. Studies established that SP-D can bind to specific sites on neutrophils and monocytes involving saccharide binding domains of SP-D (Crouch et al. 1995). The SP-A and SP-D increased calcium-dependent neutrophil uptake of *E. coli, S. pneumoniae,* and *S. aureus*. Collectins

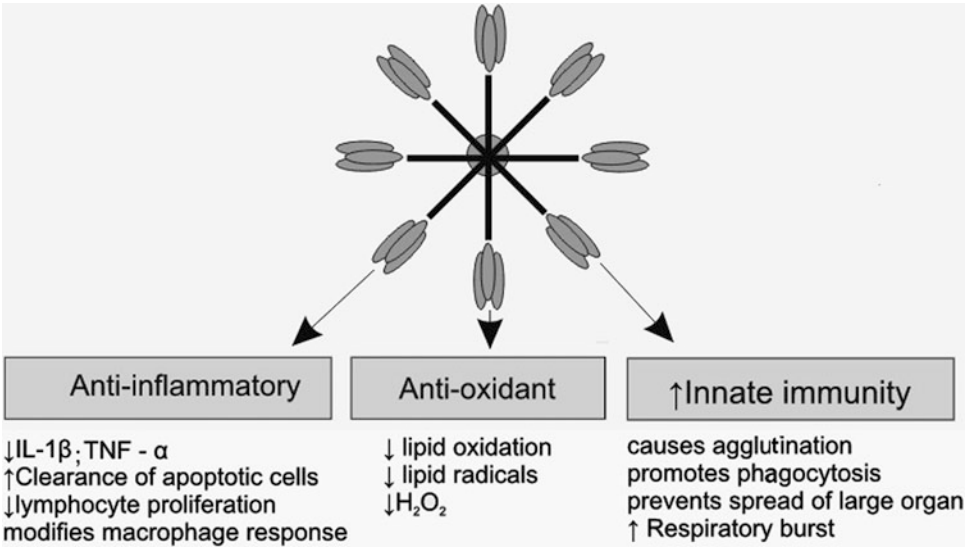

Fig. 25.2 The potential actions of surfactant protein D that may be beneficial in protecting lungs from COPD. SP-D is illustrated as a dodecamer (containing 12 monomeric chains of the carbohydrate recognition domains). SP-D has anti-inflammatory properties by attenuating the expression of pro-inflammatory cytokines such as interleukin (*IL*)-1β, 6, 8 and tumor necrosis factor-α and reducing lymphocyte accumulation in the perivascular tissues in the lungs. SP-D reduces lipid peroxidation and generation of hydrogen peroxide in vivo. SP-D also plays an important role in the innate immunity by promoting phagocytosis and agglutination of pathogens and recruiting neutrophils into lung tissues during active infections (see text for details)

enhanced bacterial uptake occurred through a mechanism that involved both bacterial aggregation and direct actions on neutrophils. The degree of multimerization of SP-D preparations was a critical determinant of both aggregating activity and potency in enhancing bacterial uptake. Results provided evidence that surfactant collectins may promote neutrophil-mediated clearance of bacteria in the lung independently of opsonizing antibody (Hartshorn et al. 1998, 2002).

Neutrophil Serine Proteinases Inactivate SP-D: Because SP-D specifically interacts with neutrophils that infiltrate the lung in response to acute inflammation and infection, the neutrophil-derived serine proteinases (NSPs): neutrophil elastase, proteinase-3, and cathepsin G degrade SP-D. All three human NSPs specifically cleaved recombinant rat and natural human SP-D dodecamers in a time- and dose-dependent manner at the sites of inflammation with potential deleterious effects on its biological functions (Hirche et al. 2004; Griese et al. 2003). However, excess NSPs in lungs play a central role in pathology of inflammatory pulmonary disease. The serpinb1, an efficient inhibitor of the three NSPs, preserves cell and molecular components responsible for host defense against *P. aeruginosa*. The regulation of pulmonary innate immunity by serpinb1 is nonredundant and is required to protect two key components, the neutrophil and SP-D, from NSP damage during host response to infection (Benarafa et al. 2007).

25.7.4 Protective Role in Allergy and Infection

SP-A and SP-D modulate allergic reactions, and resolution of inflammation. SP-A and SP-D can interact with receptor molecules present on immune cells leading to enhanced microbial clearance and modulation of inflammation. SP-A and SP-D also modulate the functions of cells of the adaptive immune system including DCs and T cells. SP-D has multiple functions in innate immunity in lung. The generation of SP-D knock-out mice has revealed a central role for this protein in the control of lung inflammation. Accumulating evidence in mouse models of infection and inflammation indicates that truncated recombinant forms of SP-D are biologically active in vivo. Clark and Reid (2002) addressed structural requirements for recognised activities of SP-D in vitro and in vivo, with emphasis on evidence arising from studies with transgenic mice and mouse models of inflammatory lung disease. The potential of truncated recombinant forms of surfactant protein D as novel therapy for infectious and inflammatory disease is discussed. Constitutive absence of SP-D in mice is associated with lung inflammation, alteration in surfactant lipid homeostasis, and increased oxidative-nitrative stress. The extracellular pool size of SP-D fluctuates significantly during acute inflammation. Clearance of SP-D into lung tissue is increased during inflammation and that tissue-associated neutrophils significantly contribute to this process. Studies using murine models of allergy and infection have raised the

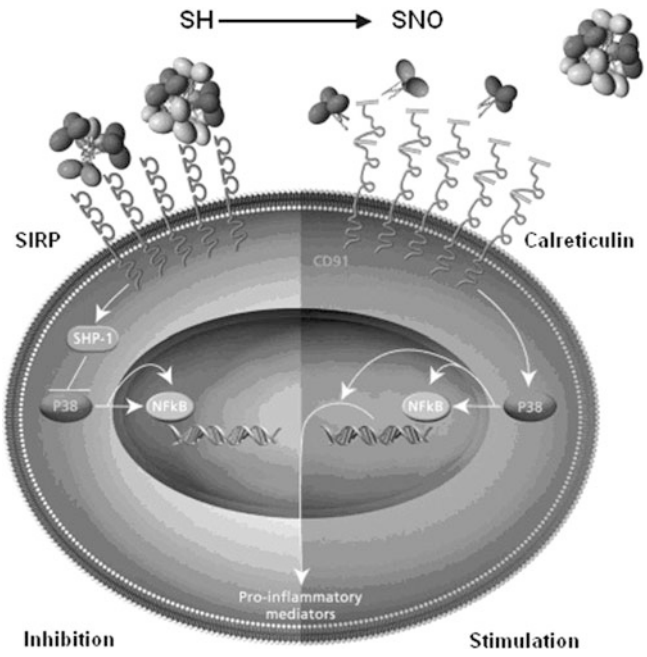

Fig. 25.3 A model of the pro- and anti-inflammatory functions of SP-D: Under non-inflammatory conditions, SP-D remains in large multimeric or dodecameric forms in which the tail domains remain buried. The head domains bind to SIRP-1α and activate the kinase SHP-1. SHP-1 activation inhibits p38 activations, potentially resulting in the blockaged of NF-κB action and the inhibition of infammatory function. Under inflammatory conditions the production of NO leads to the formation of SNO-SP-D and the disruption of the multimeric structure. The tail domains now become exposed and bind to calreticulin. This results in p38 phosphorylation via CD91, potentially leading to NF-κB activation and the production of pro-inflammatory mediators. Presumably other actions which result in disruption of SP-D multimeric structure may also be pro-inflammatory (Adapted from Guo et al. PLoS Biol. 2008; 6: e266)

possibility that recombinant forms of SP-A and SP-D may have therapeutic potential in controlling pulmonary infection, inflammation, and allergies in humans (Haczku 2008).

Using an opportunistic fungal pathogen *Aspergillus fumigatus* (Afu), Kishor et al. (2002) studied the role of SP-A and SP-D in the host immunity. Afu causes a systemic infection via lungs, called invasive aspergillosis (IPA) in immunocompromised subjects. In immunocompetent subjects, it can cause an allergic disorder, called allergic bronchopulmonary aspergillosis (ABPA). Therapeutic administration of these proteins in a murine model of IPA can rescue mice from death. Treating mice, having ABPA, can suppress IgE levels, eosinophilia, pulmonary cellular infiltration and cause a marked shift from a pathogenic Th2 to a protective Th1 cytokine profile. These results highlight the potential of SP-A, SP-D and their recombinant forms, as novel therapeutics for lung allergy and infection (Brandt et al. 2008; Kishor et al. 2002).

Susceptibility of SP-A or SP-D Genetically Deficient Mice: Madan et al. (2005) examined the susceptibility of SP-A (AKO) or SP-D gene-deficient (DKO) mice to Afu allergen challenge. Both AKO and DKO mice showed intrinsic hypereosinophilia and several-fold increase in levels of IL-5 and IL-13, and lowering of IFN-γ to IL-4 ratio in the lungs, suggesting a Th2 bias of immune response. The AKO and DKO mice showed distinct immune responses to Afu sensitization. Intranasal treatment with SP-D or rhSP-D (a recombinant fragment of SP-D) was effective in rescuing Afu-sensitized DKO mice, while SP-A-treated Afu-sensitized AKO mice showed several-fold elevated levels of IL-13 and IL-5, resulting in increased pulmonary eosinophilia and damaged lung tissue. These studies suggest a role for SP-A and SP-D in offering resistance to pulmonary allergenic challenge. Fungi have been implicated in pathogenesis of chronic rhinosinusitis (CRS) with eosinophilic mucus (EMCRS). SP-D is expressed in nasal mucosa and is up-regulated in vitro in response to fungal allergens. SP-D offers a potential therapy for treatment of CRS (Ooi et al. 2007).

Regulation of Chemotaxis and Degranulation of Human Eosinophils: Alveolar macrophages express receptors specific for SP-A and SP-D and provide insight into potential roles of collectins in recruitment and maturation of mononuclear phagocytes in lung (Tino and Wright 1999). SP-D markedly inhibited chemotaxis of eosinophils triggered by eotaxin, a major tissue-derived CC-chemokine. In addition, degranulation of ECP in response to Ca^{2+} ionophore, immobilized IgG and serum from allergic patients was inhibited by SP-D. Data support the concept of an anti-inflammatory function of SP-D in the lung of patients with allergic diseases (von Bredow et al. 2006).

Surfactant Proteins and Lipids as Modulators of Inflammation: SP-A binds with *Mycoplasma pneumoniae* with high affinity and enhances inflammatory response of human and rat macrophages. The interaction of SP-A with bacteria involved phospholipids as major ligand. The SP-A reactive lipid consisted of several disaturated molecular species of phosphatidylglycerol (PtdGro). The disaturated PtdGro failed to alter the anti-inflammatory action of SP-A, but could modify the host response to LPS. These findings reveal that both the lipids and proteins of pulmonary surfactant play a role in regulating the host response to invading microorganisms (Chiba et al. 2006) (Fig. 25.3).

Exogenous surfactants and surfactant phospholipids without SP-A and SP-D inhibit secretion of pro-inflammatory cytokines and NO in NR8383 AMφ. The inhibitory effects of surfactant on oxygen radical and LPS-induced NO formation may result from mechanisms of low cell signaling. The anti-inflammatory activity of surfactant products used in the treatment of neonatal

respiratory distress syndrome (RDS) may depend upon the specific preparation used (Kerecman et al. 2008). SP-D would protect against acute lung injury from hyperoxia in vivo. Local expression of SP-D protects against hyperoxic lung injury through modulation of proinflammatory cytokines and antioxidant enzymatic scavenger systems (Jain et al. 2008). Murine models of pulmonary hypersensitivity suggest that SP-D may be a potent anti-allergic protein.

Anti-inflammatory Protein in Human Coronary Artery SMCs: Immunoreactive SP-D protein is present in smooth muscle cells (SMCs) and endothelial cells. SP-D was also detected in human coronary artery SMCs (HCASMCs). Treatment of HCASMCs with endotoxin (LPS) stimulated the release of IL-8, a proinflammatory cytokine. This release was inhibited >70% by recombinant SP-D. It is stated that SP-D in human coronary arteries functions as an anti-inflammatory protein in HCASMCs (Snyder et al. 2008).

25.7.5 Adaptive Immune Responses

SP-A and SP-D are part of innate immune system and regulate the functions of other innate immune cells, such as macrophages. They also modulate the adaptive immune response by interacting with antigen-presenting cells and T cells, thereby linking innate and adaptive immunity. Emerging studies suggest that SP-A and SP-D function to modulate the immunologic environment of the lung so as to protect the host and, at the same time, modulate an overzealous inflammatory response that could potentially damage the lung and impair gas exchange. Numerous polymorphisms of SPs have been identified that may potentially possess differential functional abilities and may act via different receptors to ultimately alter the susceptibility to or severity of lung diseases (Pastva et al. 2007; Haley et al. 2002).

Nadesalingam et al. (2005) showed that SP-D binds various classes of Igs, including IgG, IgM, IgE and secretory IgA, but not serum IgA. SP-D recognizes IgG, aggregates IgG-coated beads and enhances their phagocytosis by murine macrophage RAW 264.7 cells. Therefore, SP-D effectively interlinks innate and adaptive immune systems (Nadesalingam et al. 2005). IL-4 selectively up-regulates SP-D expression in type II alveolar rat epithelial cell cultures. Since SP-D has a potent anti-inflammatory function, this mechanism may be part of a negative feedback loop providing a regulatory link between adaptive and innate immunity during allergic inflammation (Cao et al. 2004).

SP-A and D Suppress $CD3^+/CD4^+$ Cell Function: SP-A and SP-D inhibit lymphocyte proliferation in presence of accessory cells. SP-A and SP-D directly suppress Th cell function. Both proteins inhibited $CD3^+/CD4^+$ lymphocyte proliferation induced by PMA and ionomycin in an IL-2-independent manner. Both proteins decreased the number of cells entering the S and mitotic phases of the cell cycle. Inhibition of T cell proliferation by SP-A and SP-D occurs via two mechanisms, an IL-2-dependent mechanism observed with accessory cell-dependent T cell mitogens and specific Ag, as well as an IL-2-independent mechanism of suppression that potentially involves attenuation of $[Ca^2]_I$ (Borron et al. 2002).

SP-D Enhances Bacterial Antigen Presentation by DCs SP-D interacts with DCs to enhance uptake and presentation of bacterial antigens. It binds to immature DCs in a dose-, carbohydrate-, and calcium-dependent manner, whereas its binding to mature DCs is reduced. SP-D also binds to *E. coli HB101* and enhances its association with DCs. In addition, SP-D enhances antigen presentation of an ovalbumin fusion protein to ovalbumin-specific MHC class II T cell hybridomas. Studies demonstrate that SP-D augments antigen presentation by DCs and suggest that innate immune molecules such as SP-D may help initiate an adaptive immune response for the purpose of resolving an infection (Brinker et al. 2001, 2002).

As DC function varies depending on the tissue of origin, studies were extended to APCs isolated from mouse lung. Though the SP-D binds specifically to lung CD11c-positive cells, results show that SP-D increases the opsonization of pathogens, but decreases the antigen presentation by lung DCs, and thereby, potentially dampens the activation of T cells and an adaptive immune response against bacterial antigens—during both steady-state conditions and inflammation (Hansen et al. 2007)

The SP-D shares target cells with the proinflammatory cytokine TNF-α, an important autocrine stimulator of DCs and macrophages in the airways. TNF-α can contribute to enhanced SP-D production in the lung indirectly through inducing IL-13. The SP-D, on the other hand, can antagonize the proinflammatory effects of TNF-α on macrophages and DCs, at least partly, by inhibiting production of this cytokine (Hortobágyi et al. 2008). Soerensen et al. (2005b) showed that porcine SP-D (pSP-D) expression in lung surfactant is induced by bacterial infection by an aerogenous route rather than by a haematogenous route and that the protein interacts specifically with alveolar macrophages and with DCs in microbial-induced in bronchus-associated lymphoid tissue (BALT). The purpose of the interaction between pSP-D and DCs in BALT remains unclear, but pSP-D could represent a link between the innate and adaptive immune system, facilitating the bacterial antigen presentation by DCs in BALT.

Lymphocyte Activation in Lungs of SP-D Null Mice: In vitro evidence suggests that SP-D may suppress local T cell responses. In vivo, the SP-D deficient mice ($SP\text{-}D^{-/-}$) showed

marked T cell activation in the lungs, as reflected by an increased percentage of both $CD4^+$ and $CD8^+$ T cells expressing CD69 and CD25. CD4 lymphocytes and the fraction expressing CD69 was also increased in BAL. Increases in CD69-positive CD8 lymphocytes was not significant. Among proinflammatory cytokines, the expression of IL-12 and IL-6 was increased in the lungs of $SP\text{-}D^{-/-}$ mice. The lack of local pulmonary production of SP-D may lead to a state of persistent T cell activation, possibly in response to exogenous antigens (Fisher et al. 2002).

25.7.6 Apoptosis

Emerging results indicate that collectins are able to bind self-derived ligands in the form of apoptotic cells and regulate inflammatory responses. Stuart et al. (2006) discussed the understanding of the process of collectin recognition of dying and damaged cells and its implications for autoimmune and inflammatory diseases. The SP-A, SP-D, and C1q all enhance apoptotic cell ingestion by resident murine and human alveolar macrophages in vitro. However, only SP-D altered apoptotic cell clearance from the naive murine lung, suggesting that SP-D plays a particularly important role in vivo. Similar to C1q and MBL, SP-A and SP-D bind apoptotic cells in a localized, patchy pattern and drive apoptotic cell ingestion by phagocytes through a mechanism dependent on calreticulin and CD91 (Vandivier et al. 2002). SP-A binds to and enhances macrophage uptake of other nonself particles, specifically apoptotic polymorphonuclear neutrophils (PMNs). PMNs are recruited into the lungs during inflammation, but as inflammation is resolved, PMNs undergo apoptosis and are phagocytosed by *MΦ*s. SP-A enhances phagocytosis via an opsonization-dependent mechanism and binds apoptotic cells. Data suggest that SP-A and SP-D facilitate the resolution of inflammation by accelerating apoptotic PMN clearance (Schagat et al. 2001) and that SP-D influences innate host defense by regulating sCD14 in a process mediated by MMP-9 and MMP-12 (Senft et al. 2005).

25.7.7 Other Effects of SP-D

Enhanced Pulmonary Lipoidosis: Targeted disruption of SP-D gene caused a marked pulmonary lipoidosis characterized by increased alveolar lung phospholipids, demonstrating a unexpected role for SP-D in surfactant homeostasis. Pulmonary-specific expression of SP-D corrects pulmonary lipid accumulation in SP-D gene-targeted mice (Fisher et al. 2000). SP-D deficient mice have three to four times more surfactant lipids in air spaces and lung tissue than control mice. Relative to saturated phosphatidylcholine (Sat PC), SP-A and SP-C were decreased in alveolar surfactant and the large-aggregate surfactant fraction in SP-D deficienct mice. SP-D deficiency results in multiple abnormalities in surfactant forms and metabolism that cannot be attributed to a single mechanism (Ikegami et al. 2000). Development of dementia, including Alzheimer's disease (AD), is associated with lipid dysregulation and inflammation. As SP-D has multiple effects in lipid homeostasis and inflammation, the correlation between SP-D concentrations and development of dementia as well as to augmented mortality has been indicated (Nybo et al. 2007).

Body Mass Index: Reports have demonstrated that body mass index (BMI; kg/m^2) is influenced by genes in common with SP-D. In Danish twins, serum SP-D was significantly and inversely associated with weight and waist circumference in men and to BMI in both genders. The $SP\text{-}D^{-/-}$ mice and wild-type mice gained significantly increased weight, with 90 mg/week on normal chow and significantly increased fat in $SP\text{-}D^{-/-}$ male mice. It suggests that there is an association between low levels or absent SP-D and obesity (Sorensen et al. 2006b). Racial differences in SP-D expression exist since median plasma SP-D in Chinese population was approximately two times lower than the median serum SP-D previously measured in a Danish population using same immuno-assay. The inverse association between serum SP-D and BMI found in Chinese population is related to obesity in similar ways in Chinese and Danes (Zhao et al. 2007).

25.8 Oxidative Stress and Hyperoxia

Contributor to the Protection of Lung from Oxidative Stresses: The surfactant lining is exposed to highest ambient oxygen tension of any internal interface and encounters a variety of oxidizing toxicants including ozone and trace metals contained within the 10 kl of air that is respired daily. The pathophysiological consequences of surfactant oxidation in humans and animals include airspace collapse, reduced lung compliance, and impaired gas exchange. In many instances, normal surfactant inhibits many immune cell functions including proliferation resulting from various stimuli and production of reactive oxidative species, inflammatory mediators, and some cell surface markers. The predominant surfactant lipids appear to be responsible for these suppressive effects. Conversely, surfactant proteins SP-A and SP-D stimulate many aspects of immune cell behavior. The SP-A and SP-D directly protect surfactant phospholipids and macrophages from oxidative damage. Both proteins block accumulation of thiobarbituric acid-reactive substances and conjugated dienes during copper-

induced oxidation of surfactant lipids or LDL particles by a mechanism that does not involve metal chelation or oxidative modification of proteins. Low density lipoprotein oxidation is instantaneously arrested upon SP-A or SP-D addition, suggesting direct interference with free radical formation or propagation. The antioxidant activity of SP-A is located to the carboxyl-terminal domain of the protein, which, like SP-D, contains a C-type lectin CRD. Thus, the SP-A and SP-D, which are ubiquitous among air breathing organisms, contribute to the protection of lung from oxidative stresses due to atmospheric or supplemental oxygen, air pollutants, and lung inflammation (Bridges et al. 2000; Phelps 2001).

To evaluate the effects of O3 exposure in mouse strains with genetically different expression levels of SP-D Kierstein et al. (2006) exposed Balb/c, C57BL/6 and SP-D knockout mice to O3 or air. Ozone-exposed Balb/c mice demonstrated significantly enhanced acute inflammatory changes and higher levels of SP-D and released more IL-10 and IL-6 in BAL. IL-6 contributes to the up-regulation of SP-D after acute O3 exposure; elevation of SP-D in the lung is associated with resolution of inflammation. Absence or low levels of SP-D predispose to enhanced inflammatory changes following acute oxidative stress.

Permeabilization of Bacteria by SPs: Studies suggest that SP-A and SP-D have a direct effect on growth and viability of Gram-negative bacteria, *Mycoplasma pneumoniae and Histoplasma capsulatum*. Permeability assays indicate membrane permeabilization by BAL fluid of mice that were sufficient or deficient in surfactant proteins, of rough and smooth LPS-containing membranes, and of genetically altered bacteria. The permeabilizing activity of concentrated BAL material from SP-$A^{+/+}$ mice was substantially greater than that from SP-$A^{-/-}$ animals, and was sensitive to hyperoxic exposure. Reports suggest that pulmonary collectins directly permeabilize bacteria in an LPS-dependent and rough LPS-specific manner. Oxidative damage blocks the permeabilizing activity of alveolar lining fluid and purified proteins (McCormack 2006).

Effect of Hyperoxia: The mRNA level of SP has been reported to be increased in lungs of animals exposed to hyperoxia. Relative amounts of SP-A, SP-B, and SP-D mRNA expression in congenital diaphragmatic hernia (CDH) lung were significantly decreased compared to controls at birth. The inability of O_2 to increase SP mRNA expression in hypoplastic CDH lung suggests that the hypoplastic lung is not responsive to increased oxygenation for the synthesis of SP (Shima et al. 2000). The hypoplastic lung in CDH has both a quantitative and qualitative reduction in surfactant. Aderibigbe et al. (1999) evaluated early effects of hyperoxia (95% O_2) on expression of SP-D in adult male rat lung. Hyperoxia had differential effects on SP-D abundance in alveolar epithelial and bronchiolar epithelial cells, and therefore may influence the availability of SP-D to bind microbial pathogens in airways depending on cell type and location. Alternatively, absence of SP-D aggravates hyperoxia-induced injury. This was tested in SP-D-deficient (SP-$D^{-/-}$) and wild-type (SP-$D^{+/+}$) mice which were exposed to 80% or 21% oxygen. Paradoxically, SP-$D^{-/-}$ mice had 100% survival during 14 days of hyperoxia, vs. 30% in SP-$D^{+/+}$. Perhaps, resistance of SP-D-deficient mice to hyperoxia reflects homeostatic changes in SP-$D^{-/-}$ phenotype involving both phospholipid and SP-B-mediated induced resistance of surfactant to inactivation as well as changes in immunomodulatory BAL cytokine profile (Jain et al. 2007).

Environmental Influences on SP-D Genes: Genetic risk for respiratory distress in infancy has been recognized with increasing frequency in neonatal intensive care units. Examples of genetic variations known to be associated with or cause respiratory distress in infancy have been reviewed (Cole et al. 2001). Serum SP-D levels in children are genetically determined. A SNP located in NH_2-terminal region ($Met^{11}Thr$) of mature protein is significantly associated with the serum SP-D levels. In a classic twin study, the serum SP-D levels increased with male sex, age, and smoking status. The intraclass correlation was higher for monozygotic (MZ) twin pairs than for dizygotic (DZ) twin pairs. Multivariate analysis of MZ and DZ covariance matrixes showed significant genetic correlation among serum SP-D and metabolic variables (Sørensen et al. 2006a). Like MBL**,** structural as wells as promoter variants linked to disease states are known for SP-D. In children MZ and DZ like-sexed twin pairs aged 6–9 years, intraclass correlations were significantly higher in MZ than in DZ twins, indicating substantial genetic influence on both MBL and SP-D levels (Husby et al. 2002).

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Part IX

C-Type Lectins: Selectins

L-Selectin (CD62L) and Its Ligands

26

G.S. Gupta

26.1 Cell Adhesion Molecules

Cell adhesion molecules are glycoproteins expressed on the cell surface and play an important role in inflammatory as well as neoplastic diseases. There are four main groups: the integrin family, the immunoglobulin superfamily, selectins, and cadherins. The integrin family has eight subfamilies, designated as β1 through β8. The most widely studied subfamilies are β1 (CD29, very late activation [VLA] members), β2 (leukocyte integrins such as CD11a/CD18, CD11b/CD18, CD11c/CD18, and αd β2), β3 (CD61, cytoadhesions), and β7 (α4 β7 and αE β7). The immunoglobulin superfamily includes leukocyte function antigen-2 (LFA-2 or CD2), leukocyte function antigen-3 (LFA-3 or CD58), intercellular adhesion molecules (ICAMs), vascular adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PE-CAM-1), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). The selectin family includes L-selectin (CD62L), P-selectin (CD62P), and E-selectin (CD62E). Cadherins are major cell-cell adhesion molecules and include epithelial (E), placental (P), and neural (N) subclasses. The binding sites (ligands/receptors) are different for each of these cell adhesion molecules (e.g., ICAM binds to CD11/CD18; VCAM-1 binds to VLA-4). The specific cell adhesion molecules and their ligands that may be involved in pathologic conditions and potential therapeutic strategies by modulating the expression of these molecules have been discussed (Elangbam et al. 1997). The main classes of adhesion molecule involved in lymphocyte interactions are the selectins, the integrins, members of the Ig superfamily, and some mucinlike molecules. Most adhesion molecules play fairly broad roles in the generation of immune responses. The three selectins act in concert with other cell adhesion molecules (e.g., intracellular adhesion molecule (ICAM-1), vascular cell adhesion molecule- 1 (VCAM-1), and leukocyte integrins (Springer 1990; Shimizu et al. 1992) to effect adhesive interactions of leukocytes, platelets, and endothelial cells. Hence selectins which belong to C-type lectins family are reviewed in Chaps. 26–28

26.1.1 Selectins

The selectin family of lectins consists of three closely related cell-surface molecules with differential expression by leukocytes (L-selectin), platelets (P-selectin), and vascular endothelium (E- and P-selectin). Structural identity of a selectins resides in its unique domain composition (Fig. 26.1). E-, P-, and L-selectin are >60% identical in their NH2 terminus of 120 amino acids, which represent the lectin domain (Siegelman et al. 1989; Lasky et al. 1989; Johnston et al. 1989; Bevilacqua et al. 1989; Camerini et al. 1989; Tedder et al. 1989). The ligands (counter structures) of selectins are sialylated and fucosylated carbohydrate molecules which, in most cases, decorate mucin-like glycoprotein membrane receptors. Their common structure consists of an N-terminal Ca^{2+}-dependent lectin-type domain, an epidermal growth factor (EGF)-like domain, multiple short consensus repeat (SCR) domains similar to those found in complement regulatory proteins, a transmembrane region, and a short cytoplasmic C-terminal domain. Together this arrangement results in an elongated structure which projects from the cell surface, ideal for initiating interactions with circulating leucocytes**.** The lectin domain forms the main ligand binding site, interacting with a carbohydrate determinant typified by fucosylated, sialylated, and usually sulphated glycans such as sialyl Lewis X (s-Le^X). The EGF domain may also play a role in ligand recognition. The multiple SCR domains (two for L-selectin, six for

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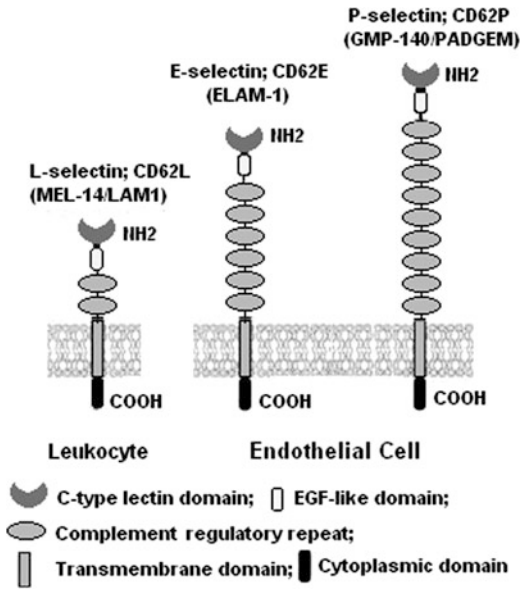

Fig. 26.1 Domain composition of the three known human selectins. The extracellular portion of each selectin contains an amino terminal domain homologous to C-type lectins and an adjacent epidermal growth factor-like domain. The single EGF element that is found in each selectin is followed by a varying number of repetitive elements, each ~ 60 amino acids long, which resemble protein motives found in complement regulatory proteins and a transmembrane sequence. A short cytoplasmic sequence is at the carboxyl terminus of each selectin. The number of amino acids present in the mature proteins as deduced from the cDNA sequences are: L-selectin, 385; P-selectin, 789; and E-selectin, 589

E-selectin, and nine for P-selectin) probably act as spacer elements, ensuring optimum positioning of the lectin and EGF domains for ligand interaction. The EGF repeats have comparable sequence similarity. Each complement regulatory-like module is 60 amino acids in length and contains six cysteinyl residues capable of disulfide bond formation. This feature distinguishes the selectin modules from those found in complement binding proteins, such as complement receptors 1and 2, which contain four cysteines (Hourcade et al. 1989).

The selectins cell-surface receptors play a key role in the initial adhesive interaction between leukocytes and endothelial cells at sites of inflammation (Fig. 26.2). Activation of endothelial cells (EC) with different stimuli induces the expression of E- and P-selectins, and other adhesion molecules (ICAM-1, VCAM-1), involved in their interaction with circulating cells. Lymphocytes home to peripheral lymph nodes (PLNs) via high endothelial venules (HEVs) in the subcortex and incrementally larger collecting venules in the medulla. HEVs express ligands for L-selectin, which mediates lymphocyte rolling.

26.2 Leukocyte-Endothelial Cell Adhesion Molecule 1 (LECAM-1) (L-Selectin/CD62L or LAM-1)

26.2.1 Leukocyte-Endothelial Cell Adhesion Molecule 1 in Humans

Lymphocyte trafficking is a fundamental aspect of the immune system that allows B and T lymphocytes with diverse antigen recognition specificities to be exposed to various antigenic stimuli in spatially distinct regions of an organism. A lymphocyte adhesion molecule that is involved with this trafficking phenomenon has been termed the homing receptor. The Leukocyte-endothelial cell adhesion molecule 1 (LECAM-1) (L-selectin/MEL-14) or leukocyte adhesion molecule-1 (LAM-1, TQ1, Leu-8), a cell surface component, expressed by human lymphocytes, neutrophils, monocytes, and their precursors, is the member of the selectin family of cellular adhesion/homing receptors which play important roles in leukocyte-endothelial cell interactions. LECAM-1 mediates leukocyte rolling and leukocyte adhesion to endothelium at sites of inflammation. The migration of naive T cells into lymphoid tissues is mediated by the chemokine SLC (secondary lymphoid tissue chemokine), which is expressed by the high vascular endothelium, stromal cells, and dendritic cells in lymphoid tissue, and binds to the CCR7 chemokine receptor on naive T cells. In addition, L-selectin mediates the binding of lymphocytes to HEV of PLN, which is an essential process in lymphocyte recirculation and hence also known as PLN homing receptor. Metabolic studies revealed that thymocytes synthesize markedly less L-selectin than do thoracic duct lymphocytes (TDL) or LN lymphocytes. However, Northern blot studies indicated that thymocytes possess more L-selectin RNA than do TDL. These results provide evidence that post-transcriptional events contribute to regulation of L-selectin expression in thymocytes.

26.2.1.1 Lymphocyte-Associated Molecule (LAM-1) and L-Selectin Are Homologous

A cDNA encoding a human lymphocyte cell surface molecule named lymphocyte-associated molecule (LAM-1) composed of multiple distinct domains, one domain homologous with animal lectins was identified by Tedder et al. (1989). This cDNA clone hybridized with RNAs found in B cell lines and T lymphocytes, but not with RNA from other cell types. The amino acid sequence of LAM-1 is 77% homologous with the sequence of the mouse lymphocyte homing receptor, suggesting that LAM-1 may function in human lymphocyte adhesion. The LAM-1 gene is located on chromosome 1q23–25, as is another member of this adhesion family, suggesting that this new family

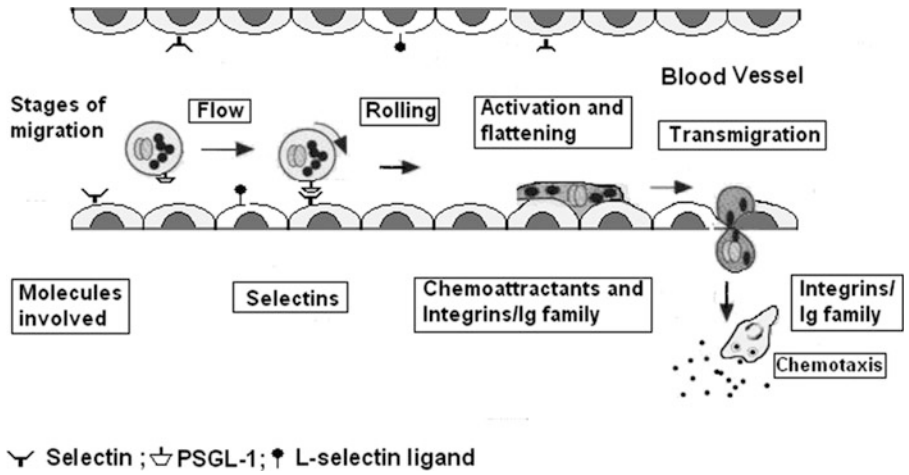

Fig. 26.2 Schematic outline of the stages involved in leucocyte migration through post-capillary venular endothelium. Leucocytes flowing in the vein become tethered to the vessel wall, a step mediated largely by the selectin family of adhesion receptors. The leucocytes then roll along the vessel wall until they come into contact with a chemoattractant and become activated. Activation leads to engagement of members of the integrin family of receptors binding to immunoglobulin (Ig) family adhesion receptors on the endothelium. This causes them to become more firmly attached, stop rolling, flatten on the surface of the endothelial cell, and transmigrate

of proteins may be encoded by a cluster of "adhesion protein" loci. The human homologue of murine peripheral lymph node-specific receptor is highly homologous to the murine receptor in overall sequence that may be involved with human lymphocyte homing. The extracellular region of the human receptor contained an NH2-terminally located carbohydrate binding domain followed by an EGF-like domain and a domain containing two repeats of a complement binding protein/motif (CRP). Interestingly, the human receptor showed a high degree of sequence homology to endothelial cell adhesion molecule ELAM (Bowen et al. 1989; Camerini et al. 1989). Genomic analysis of human DNA suggests a low-copy gene under high-stringent conditions. The nucleotide sequence predicts a mature protein of 334 amino acids, identical in length to mouse lymph node homing receptor core protein (mLHR) (Siegelman and Weissman 1989). The cDNA encoding baboon L-selectin predicts a protein of 372-aa, which is 95% identical to that of human L-selectin (Tsurushita et al. 1996). The carbohydrate binding domain is apparently involved in the adhesive interaction between murine lymphocytes and PLN endothelium. The lectin and EGF-like regions are the most homologous, while CRP domains are less conserved between species. The two CRP units in hLHRc are distinct from those in mLHRc in that they are homologous to one another rather than identical, suggesting strong pressure for maintenance of two repeats in this molecule (Siegelman and Weissman 1989).

26.2.2 Gene Structure of L-Selectin

The leukocyte adhesion molecule-1 (LAM-1) or L-selectin gene expressed by human lymphocytes, neutrophils, monocytes, and their precursors spans greater than 30 kbp of DNA and is composed of at least 10 exons. The 5′ end of LAM-1 mRNA revealed a single initiation region for transcription. Exons II through X contain translated sequences; exon II encodes the translation initiation codon; exon III, the leader peptide; IV, the lectin-like domain; V, the epidermal growth factor-like domain; VI and VII, the short consensus repeat units; exon VIII, the transmembrane region; exon IX encodes seven amino acids containing a potential phosphorylation site; and exon X encodes the five remaining amino acids of the cytoplasmic tail and the long 3′ untranslated region. Sequences of *Lam-1* cDNA clones and protein expressed by neutrophils are identical to cDNA sequences and the protein expressed by lymphocytes. Therefore, the usage of exons II through X results in the generation of a single major LAM-1 protein product expressed by lymphocytes and neutrophils (Ord et al. 1990; Watson et al. 1990).

26.2.3 Murine PLN Homing Receptor/mLHR3

Characterization of the cDNAs encoding the murine lymphocyte homing receptor (mLHR) has revealed mosaic structure containing three well-known protein motifs: a

C-type lectin domain, an epidermal growth factor-like domain, and two exact copies of a short consensus repeat sequence homologous to those found in a family of complement regulatory proteins (CRP), in addition to a signal sequence, a transmembrane anchor, and a short cytoplasmic tail. The receptor molecule is potentially highly glycosylated, and contains an apparent transmembrane region. Analysis of mRNA transcripts reveals a predominantly lymphoid distribution in direct relation to the cell surface expression of the MEL-14 determinant, and the cDNA clone is shown to confer the MEL-14 epitope in heterologous cells. These features, including ubiquitination, embodied in this single receptor molecule form the basis for numerous approaches to the study of cell-cell interactions (Lasky et al. 1989; Siegelman et al. 1989). Characterization of genomic clones encoding mLHR gene revealed a high degree of correlation between various structure/function motifs and exons that specify them. Comparison of the exons encoding the two identical copies of the complement regulatory motif revealed that short intronic regions 5′ and 3′ of these exactly repeated exons are also identical. The mLHR core peptide locus is localized to chromosome 1, the portion syntenic with chromosome 1 in man. The gene on chromosome 1 is very near to a site that contains the genes for the family of complement regulatory proteins which encode short consensus repeats similar to those found in the homing receptor (Dowbenko et al. 1991; Siegelman et al. 1990).

The partial sequencing of a rat cDNA revealed a putative peptide of 372 aa, including a signal peptide of 38 aa. The protein has three tandem domains: a lectin domain, an EGF-like domain and two repeats of complement regulatory proteins (CRP domain). The lectin binding domain has 93.2% and 81.4% and the EGF-like domain has 85.3% and 76.5% aa identity with those of mouse and human, respectively. In the CRP repeat domain, the amino acid identity was 72.6% between human and rat and 71.8% between mouse and rat. The main transcript is of about 3 kb in peripheral blood mononuclear cells (PBMC), spleen and thymus. Sackstein et al. (1995) identified the complete coding sequence of 105-bp 5′-untranslated region and a 359-bp 3′-untranslated region. Expression studies gave evidence that cDNA represented rat L-selectin. The cDNA encoding rabbit L-Selectin encodes a peptide of 377 aa, including a signal peptide of 38 amino acids. Sequence analysis demonstrated extensive homology with L-Selectins from other species (Qian et al. 2001). The bovine L-selectin is predicted amino acid sequence with an overall high identity to that of human and murine L-selectin. However, the cytoplasmic tail of bovine L-selectin showed little similarity to that of human and murine L-selectin (Bosworth et al. 1993).

26.3 Functions of L-Selectin

The selectin family of adhesion molecules (E-, P- and L-selectins) is involved in leukocyte recruitment to the sites of inflammation and tissue damage. In contrast to most other adhesion molecules, selectin function is restricted to leukocyte interactions with vascular endothelium. Multiple studies indicate that the selectins mediate neutrophil, monocyte, and lymphocyte rolling along the venular wall. The generation of selectin-deficient mice has confirmed these findings and provided further insight into how the overlapping functions of these receptors regulate inflammatory processes. These proteins also play a critical part in the subsequent interactions of lymphocytes with antigen-presenting cells and later with their target cells (da Costa Martins et al. 2004). L-selectin dimerization enhances tether formation to properly spaced ligand. Ezrin-radixin-moesin proteins are required for microvillar positioning of L-selectin and that this is important both for leukocyte tethering and L-selectin shedding (Ivetic and Ridley 2004; Ivetic et al. 2004). L-selectin is involved not only in leukocyte tethering and rolling, but also in leukocyte activation.

26.3.1 Lymphocyte Homing and Leukocyte Rolling and Migration

Selectins participate in leukocyte homing to particular tissues, and can be expressed either on leukocytes (L-selectin, CD62L) or on vascular endothelium (Fig. 26.2). L-Selectin is expressed on naive T cells and guides their exit from blood into peripheral lymphoid tissues. The interaction between L-selectin and the vascular addressins is responsible for the specific homing of naive T cells to lymphoid organs but does not, on its own, enable the cell to cross the endothelial barrier into the lymphoid tissue. Studies have shown that inhibition of selectin function can ameliorate a range of inflammatory processes, offering the possibility that antagonists of selectin function may be useful in the treatment of inflammatory lung diseases such as asthma (Symon and Wardlaw 1996). Experiments on L-sel$^{-/-}$ mice showed that L-sel$^{-/-}$ leukocytes were severely impaired in their ability to respond to a directional cue. These findings indicate that L-selectin is important in

enabling leukocytes to respond effectively to chemotactic stimuli in inflamed tissues (Hickey et al. 2000). L- selectin, ICAM-1, and both β1- and β-integrins seem to function synergistically to mediate optimal leukocyte rolling and entry into tissues, which is essential for the generation of effective inflammatory responses in vivo (Steeber et al. 1999; Diacovo et al. 2005). Lymphocyte homing and leukocyte rolling and migration are impaired in L-Sel$^{-/-}$ mice. Whether L-selectin-mediated rolling can promote leukocyte adhesion in vivo independent of P- and E-selectin, Jung et al. (1998) concluded that L-selectin can mediate rolling that results in sufficient leukocyte recruitment to account for the robust inflammatory response seen in E^{-}/P^{-} mice at later times.

There are numerous in vitro studies that demonstrate that engagement of L-selectin leads to the activation of several signaling pathways potentially contributing to subsequent adhesion, emigration, or even migration through the interstitium. Ligation of L-selectin through conserved regions within the lectin domain activates signal transduction pathways and integrin function in human, mouse, and rat leukocytes (Steeber et al. 1997). L-selectin signal transduction requires protein kinase Cα, ι, and θ binding (Kilian et al. 2004). This actually induces cellular events in vivo.

L-Selectin-Mediated Lymphocyte Rolling Is Regulated by Endothelial Chemokines: CXCR2- and E-selectin-induced neutrophils arrest during inflammation in vivo (Smith et al. 2004). L-selectin mobilized intracellular CXCR4 significantly increases surface CXCR4 stimulation and inhibited SDF-1 induced CXCR4 internalization (Ding et al. 2003). Engagement of both CD4 and CXCR4 is required for HIV-induced shedding of L-selectin on primary resting CD4^{+} T cells (Wang et al. 2004).

Intracellular Mechanisms of L-Selectin Induced Capping: Capping of surface receptors is an ubiquitous mechanism but least understood. Junge et al. (1999) demonstrated that L-selectin triggering results in receptor capping of the L-selectin molecules in lymphocytes. This process involves intracellular signaling molecules. L-Selectin capping seems to be independent on activation of p56lck-kinase, but requires the neutral sphingomyelinase, small G proteins and the cytoskeleton. Capping of L-selectin upon stimulation might play an important role in the very early phase of lymphocyte trafficking.

26.3.2 Immune Responses

The mouse strains, deficient in selectin leukocyte adhesion receptors, have been very informative in finding the roles of cell adhesion molecules in leukocyte-endothelium interaction and produced some surprises.

26.3.2.1 Impaired Primary T Cell Responses in L-Sel$^{-/-}$ mice

L-selectin-deficient mice show impaired leukocyte recruitment into inflammatory sites and impaired primary T cell responses. L-selectin-deficient mice are defective in cutaneous delayed-type hypersensitivity (DTH) responses when tested after conventional intervals of immunization (4d). L-selectin plays an important role in the generation of primary T cell responses but may not be essential for humoral and memory T cell responses (Tedder et al. 1995). Lymphocyte migration in L-selectin-deficient mice is due to altered subset migration and aging of the immune system (Steeber et al. 1996). Circulating lymphocyte numbers or subpopulations were not altered in young L-Sel$^{-/-}$ mice, but circulating monocyte numbers were increased nearly threefold. In contrast, older L-Sel$^{-/-}$ mice had disproportionate increases of both naive and memory CD4^{+} T cells present within spleen and blood. Results and the finding that memory lymphocytes in wild-type mice expressed L-selectin demonstrated a requirement for L-selectin in the regulation of memory lymphocyte migration. Therefore, L-selectin-dependent pathways of lymphocyte migration are important for the normal migration of both naive and memory lymphocytes (Steeber et al. 1996). Selectin deficient mice show impaired development of mucosal immunity in the gut-associated lymphoreticular tissue (Csencsits and Pascual 2002; Pascual et al. 2001; Csencsits and Pascual 2002). Using allogeneic skin graft rejection as a model of cutaneous inflammation, L-Sel$^{-/-}$ mice rejected both primary and secondary allogeneic skin grafts significantly more slowly than L-selectin$^{+/+}$ littermates. The delayed rejection of skin grafts by L-Sel$^{-/-}$ mice reflects impaired migration of effector cells into the graft rather than delayed or impaired generation of a CTL response (Tang et al. 1997).

Perhaps L-selectin is required on effector cells for local interactions in the CNS to cause myelin damage in experimental allergic encephalomyelitis as implied by the expression of ICAM-1, VCAM-1, L-selectin, and leukosialin in mouse CNS during the induction and remission stages of experimental allergic encephalomyelitis (Dopp et al. 1994; Grewal et al. 2001).

26.3.2.2 Humoral Immune Responses in L-Sel$^{-/-}$ mice

The altered distribution of lymphocyte subpopulations in L-Sel$^{-/-}$ mice resulted in significantly elevated serum IgM and IgG1 levels and augmented humoral immune responses to T cell-independent and T cell-dependent Ags following i.p. immunization. By contrast, s.c. immunization of L-selectin-deficient mice with a T cell-dependent Ag resulted in serum IgM responses that were lower when compared with wild-type littermates on d 7, while IgG responses were absent. Most serum Ig responses were normal by d 14 and secondary responses were higher in L-selectin-deficient

mice. These results indicate that lymphocyte migration plays an important role in the initiation of humoral responses and demonstrate complementary and overlapping roles for the spleen and other peripheral lymphoid tissues in the generation of immune responses (Steeber et al. 1996).

26.3.2.3 CD62L$^+$ Sub-Population of Regulatory T Cells Protect from Lethal Acute GVHD

The CD4$^+$CD25$^+$ T_{reg} cells are important in the regulation of immune responses in allogeneic bone marrow (BM) and solid organ transplantation. T_{reg} cells are recognized for their critical role in induction and maintenance of self-tolerance and prevention of autoimmunity. Reports indicate that L-Sel^{hi} T_{regs} interfere with the activation and expansion of GVHD effector T cells in secondary lymphoid organs early after BM transplantation. Ermann et al. (2005) examined the differential effect of CD62L$^+$ and CD25$^-$ subsets of CD4$^+$CD25$^+$ T_{reg} cells on aGVHD-related mortality. Both subpopulations showed the characteristic features of CD4$^+$CD25$^+$ T_{reg} cells in vitro and did not induce acute GVHD in vivo. However, in cotransfer with donor CD4$^+$CD25$^-$ T cells, only the CD62L$^+$ subset of CD4$^+$CD25$^+$ T_{reg} cells prevented severe tissue damage to the colon and protected recipients from lethal aGVHD (Ermann et al. 2005).

26.3.2.4 Human CD62L$^-$ Memory T Cells Are Less Responsive to Alloantigen

Human memory (CD45RO+) CD4+ T cells can be distinguished into two subpopulations on the basis of expression of the lymph node homing receptor, L-selectin. Human L-selectin-positive memory T-helper (Th) cells promote the maturation of IgG- and IgA-producing cells by naive B cells. Results suggest that the human L-selectin-negative and -positive subpopulations of human memory CD4$^+$ T cells contain Th1-like and Th2-like cytokine-producing cells, respectively (Kanegane et al. 1996). Memory T cells (CD62L$^-$) represent a population of T cells that have previously encountered pathogens and may contain fewer T cells capable of recognizing neoantigens including recipient alloantigen (aAg). Human naive (CD62L$^+$) or memory (CD62L$^-$) T cells have different capacities to respond to aAg (Foster et al. 2004). Physical fitness affects immune responses to a psychological but not a physical stressor (Hong et al. 2004).

26.3.2.5 Structural Requirements for Release of L-selectin

The cytoplasmic domain of L-selectin regulates leukocyte adhesion to endothelium independent of ligand recognition, by controlling cytoskeletal interactions and/or receptor avidity (Kansas et al. 1993). This was suggested by the strong amino acid sequence conservation of the cytoplasmic domain of L-selectin between humans and mice. L-selectin mediates leukocyte rolling on vascular endothelium at sites of inflammation and lymphocyte migration to PLN. L-selectin is rapidly shed from the cell surface after leukocyte activation by a proteolytic mechanism that cleaves the receptor in a membrane proximal extracellular region. The release of L-selectin is likely to be regulated by the generation of an appropriate tertiary conformation within the membrane-proximal region of the receptor, which allows recognition by a membrane-bound endoprotease with relaxed sequence specificity that cleaves the receptor at a specific distance from the plasma membrane. Studies suggest a generalized protein-processing pathway involved in the endoproteolytic release of specific transmembrane proteins which harbor widely differing primary sequences at/or neighboring their cleavage sites (Chen et al. 1995).

Effect of shedding of L-selectin: L-selectin shedding does not regulate constitutive T cell trafficking but controls the migration pathways of antigen-activated T lymphocytes. In other words, L-selectin shedding from antigen-activated T cells prevents re-entry into peripheral lymph nodes (Galkina et al. 2003). Expression of the L-selectin can be rapidly down-modulated by regulated proteolysis at a membrane-proximal site. Evidence suggested that the L-selectin secretase activity might involve a cell surface, zinc-dependent metalloprotease and might be related to the activity involved in processing of membrane-bound TNF-α (Feehan et al. 1996). In addition, the cytoplasmic domain of L-selectin may regulate shedding by a mechanism in which bound calmodulin may operate as a negative effector (Matala et al. 2001). Studies show that TNF-α-converting enzyme (TACE) is involved in the shedding of L-selectin by NSAIDS in human neutrophils (Gomez-Gaviro et al. 2002).

Role of Circulating L-Selectin: Leukocyte activation along inflamed vascular endothelium induces rapid endoproteolytic cleavage of L-selectin from the cell surface, generating soluble L-selectin (sL-selectin). Comparable with humans, sL-selectin is present in adult mouse sera at approximately 1.7 μg/ml. Adhesion molecule-deficient mice prone to spontaneous chronic inflammation and mice suffering from leukemia/lymphoma had increased serum sL-selectin levels. By contrast, serum sL-selectin levels were reduced in Rag-deficient mice lacking mature lymphocytes. The majority of serum sL-selectin had a molecular mass of 65–75 kDa, consistent with its lymphocyte origin. sL-selectin influences lymphocyte migration in vivo and that the increased sL-selectin levels present in certain pathologic conditions may adversely affect leukocyte migration (Tu et al. 2002).

26.4 L-Selectin: Carbohydrate Interactions

The binding specificity of L-selectin towards structurally defined sulphated oligosaccharides of the blood group Le^a and Le^x series, and of the glycosaminoglycan series heparin, chondroitin sulphate and keratan sulphate has been evaluated in several studies. L-selectin interacts with oversulfated chondroitin/dermatan sulfates containing GlcAβ1/IdoAα1-3GalNAc (4, 6-O-disulfate) (Kawashima et al. 2002). Leid et al. (2002) provided a comprehensive comparison of the L-selectin/cytoskeletal interaction with other functionally important surface antigens.

26.4.1 Glycan-Dependent Leukocyte Adhesion

Each selectin recognizes related but distinct counter-receptors displayed by leukocytes and/or the endothelium. These counter-receptors correspond to specific glycoproteins whose 'activity' is enabled by carefully controlled post-translational modifications. The characterization of the glycans associated with E- and P-selectin counter-receptors, and of mice with targeted deletions of glycosyltransferase and sulfotransferase genes, disclose that neutrophil E- and/or P-selectin counter-receptor activities derive, minimally, from essential synthetic collaborations amongst polypeptide N-acetylgalactosaminyl- transferase(s), a β-N-acetylglucosaminyltransferase that assembles core-2-type O-glycans, β-1,4-galactosyltransferase(s), protein tyrosine sulfotransferase(s), α-2,3-sialyltransferases, and a pair of α-1,3 fucosyltransferases (Lowe 2003).

26.4.1.1 Specificity of L-Selectin Towards Sulphated Oligosaccharides

L-selectin binds to 3′-sialyl-Le^x and –Le^a and to 3′-sulfo-Le^x and –Le^a sequences. Sulphated blood group Le^a/Le^x type sequences, with sulphate at the 3-position of galactose, have emerged as potent ligands for the E- and L-selectins. The recombinant soluble form of rat L-selectin (L-selectin-IgG Fc chimera) was shown to bind to lipid-linked oligosaccharides 3-O, 4-O and 6-O sulphated at galactose, such as sulphatides and a mixture of 3-sulphated Le^a/Le^x type tetrasaccharides isolated from ovarian cystadenoma, as well as to the HNK-1 glycolipid with 3-O sulphated glucuronic acid. The L-selectin was found to bind to the individual 3-sulphated Le^a and Le^x sequences (penta-, tetra- and trisaccharides), and with somewhat lower intensities to their non-fucosylated analogues. Green et al. (1995) proposed that the binding of the lymphocyte membrane L-selectin to endothelial glycosaminoglycans may provide a link between the selectin-mediated and integrin-mediated adhesion systems in leukocyte extravasation cascades. The possibility is also raised that lymphocyte L-selectin interactions with glycosaminoglycans may contribute to pathologies of glycosaminoglycan-rich tissues, e.g. cartilage loss in rheumatoid arthritis and inflammatory lesions of the cornea.

Conformational studies of selectins with NMR of the sulphated (Su) Le^a in comparison with the non-sulphated analogue, which is less strongly bound by E-selectin and not at all by L-selectin, revealed NMR parameters, which are in agreement for binding of both molecules. Molecular dynamics calculations for $SuLe^a$ lead to the conclusion that the conformation of $SuLe^a$ approximates to a single-rigid structure, as for Le^a molecule. Comparison of experimentally and theoretically obtained parameters for $SuLe^a$ with those for the non-sulphated Le^a molecule indicated no detectable changes in the three-dimensional structure of the trisaccharide upon sulphation. Thus, the enhanced selectin binding to the sulphated Le^a is most likely due to favourable electrostatic interactions between the charged sulphate group and corresponding charged groups on the selectin protein (Kogelberg and Rutherford 1994).

Sialyl-Lewis^x Sequence 6-O-Sulfated at N-Acetylglucosamine as Ligand for L-Selectin: Oligosaccharide sequences based on sialyl-Le^x with 6-O-sulfation at galactose (6′-sulfo) or at N-acetylglucosamine (6-sulfo) and expressed on high endothelial venules are considered likely endogenous ligands for L-selectin. In the course of high performance TLC of three hexaglycosylceramides 6′-sulfo sialyl Le^x, 6-sulfo sialyl Le^x, and 6′,6-bis-sulfo sialyl Le^x, synthesized chemically for selectin recognition studies, two minor byproducts were detected and isolated from each parent compound. These were identified as isomers containing a de-N-acetylated sialic acid or having a modified carboxyl group. Binding experiments with the parent compounds and the non-sulfated sialyl Le^x glycolipid show that 6-sulfation potentiates, whereas 6′-sulfation virtually abolishes L-selectin binding. Whereas modification of the sialic acid carboxyl group markedly impaired L-selectin binding, de-N-acetylation resulted in enhanced binding. The natural occurrence on high endothelial venules of this 'super-active' de-N-acetylated form of 6-sulfo sialyl Le^x, and related structures, now deserves investigation (Galustian et al. 1997). The binding to 3′-sialyl-Le^x is strongly affected by the presence of 6-O-sulfate as found on oligosaccharides of the counter receptor, GlyCAM-1; 6-O-sulfate on the N-acetylglucosamine (6-sulfation) enhances, whereas 6-O-sulfate on the galactose (6′-sulfation) virtually abolishes binding. Interactions with 3′-sulfo-Le^x based on the Le^x pentasaccharide sequence also showed that the 6-sulfation enhances where as 6′-sulfation suppresses L-selectin

binding. Thus, for synthetic strategies to design therapeutic oligosaccharide analogs as antagonists of L-selectin binding, those based on the simpler 3′-sulfo-Le^x (and also the 3′-sulfo-Le^a) would be most appropriate (Galustian et al. 1999, 2002).

Endothelial sLe^a and sLe^x During Cardiac Transplant Rejection: Acute organ transplant rejection is characterized by a heavy lymphocyte infiltration. Alterations in the graft endothelium result into increased lymphocyte traffic into the graft. Lymphocytes adhere to the endothelium of rejecting cardiac transplants, but not to the endothelium of syngeneic grafts or normal hearts. Concomitant with the enhanced lymphocyte adhesion, the cardiac endothelium begins to de novo express sialyl Le^a and sialyl Le^x epitopes, which overlap to the sequences of L-selectin counter-receptors. The endothelium of allografts, but not that of syngeneic grafts or normal controls, also reacted with the L-selectin-immunoglobulin G fusion protein, giving proof of inducible L-selectin counter-receptors (Turunen et al. 1995).

Heparin Sulfate Proteoglycans as L-Selectin Ligand: Heparin has been used clinically as an anticoagulant and antithrombotic agent for over 60 years. Calcium-dependent, heparin-like L-selectin ligands had been noticed in cultured bovine endothelial cells (Norgard-Sumnicht et al. 1993). Subsequently, these ligands were identified as heparin sulfate proteoglycans (HSPGs) associated either with the cultured cells or secreted into the medium and extracellular matrix. Studies showed that HSPGs from cultured endothelial cells, which can bind to L-selectin are enriched with unsubstituted amino groups on their GAG chains (Norgard-Sumnicht and Varki 1995). Presentltly, known L-selectin ligands include sulfated Lewis-type carbohydrates, glycolipids, and proteoglycans. L-selectin binding chondroitin/dermatan sulfate proteoglycans are present in cartilage, whereas L-selectin binding heparan sulfate proteoglycans are present in spleen and kidney. L-selectin binds only a subset of renal heparan sulfates, attached to a collagen type XVIII protein backbone and predominantly present in medullary tubular and vascular basement membranes. L-selectin does not bind other renal heparan sulfate proteoglycans such as perlecan, agrin, and syndecan-4, and not all collagen type XVIII expressed in the kidney bind L-selectin. This indicates that there is a specific L-selectin binding domain on heparan sulfate glycosaminoglycan chains. Based on the model of heparan sulfate domain organization, Celie et al. (2005) proposed a model for the interaction of L-selectin with heparan sulfate glycosaminoglycan chains. This opens the possibility of active regulation of L-selectin binding to heparan sulfate proteoglycans, e.g. under inflammatory conditions (Celie et al. 2005).

Heparan Sulfate Deficiency Impairs L-Selectin-Mediated Neutrophil Trafficking: The potent anti-inflammatory property of heparin results primarily from blockade of P-selectin and L-selectin. Mice deficient in P- or L-selectins showed impaired inflammation, which could be further reduced by heparin. However, heparin had no additional effect in mice deficient in both P- and L-selectins. The sulfate groups at C6 on the glucosamine residues play a critical role in selectin inhibition. Such analogs may prove useful as therapeutically effective inhibitors of inflammation (Wang et al. 2002). Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. L-selectin binding heparan sulfates attached to collagen type XVIII have been identified and a model for the interaction of L-selectin with heparan sulfate glycosaminoglycan chains has been proposed. Endothelial heparan sulfate is involved in the inflammation by inactivating N-acetyl glucosamine N-deacetylase-N-sulfotransferase-1 in endothelial cells and leukocytes, which is required for the addition of sulfate to the heparin sulfate chains. Mutant mice developed normally but showed impaired neutrophil infiltration in various inflammation models. Endothelial heparan sulfate has three functions in inflammation: by acting as a ligand for L-selectin during neutrophil rolling; in chemokine transcytosis; and by binding and presenting chemokines at the lumenal surface of the endothelium (Wang et al. 2005).

Some L-Selectin Ligand Not Recognized by MECA-79 in PLNs: In contrast to PLN addressin, which is recognized by mAb MECA-79, medullary venules expressed L-selectin ligands are not recognized by MECA-79. Both L-selectin ligands seem to be fucosylated by α1,3-fucosyltransferase (FucT)-IV or FucT-VII as rolling is absent in FucT-IV + $VII^{-/-}$ mice. Although MECA-79-reactive species predominate in HEVs, medullary venules express another ligand that is spatially, antigenically, and biosynthetically different from PLN addressin. The two distinct L-selectin ligands are segmentally confined to contiguous microvascular domains in PLNs (M'Rini et al. 2003).

26.4.1.2 Salivary MG2 Saccharides Function as Ligands for Neutrophil L-Selectin

The low-molecular-weight human salivary mucin (MG2) coats oral surfaces, where it is in a prime location for governing cell adhesion. Since oligosaccharides form many of the interactive facets on mucin molecules, Prakobphol et al. (1999) examined MG2 glycosylation in relation to the molecule's adhesive functions. Termini of MG2 oligosaccharide structures predominantly carry T, sialyl-T, Le^x, sialyl Le^x (sLe^x), lactosamine, and sialyl lactosamine. In addition, sLe^x determinants confer L-selectin ligand activity to this molecule. Adhesive interactions between

MG2 and cells that traffic in the oral cavity such as neutrophils and bacteria, revealed that under flow conditions, neutrophils tethered to MG2-coated surfaces at forces between 1.25 and 2 dyn/cm2. Together, results suggested that distinct subsets of MG2 saccharides function as ligands for neutrophil L-selectin and receptors for bacterial adhesion, a finding with interesting implications for both oral health and mucin function.

26.4.1.3 *H. pylori* Isolates Interact with L-, E- and P-Selectins

Carbohydrate components on *H. pylori* contribute to the persistent inflammation through interactions with leukocyte-endothelial adhesion molecules of the host. The LPS of most *H. pylori* strains contain sequences related to the Le^x or Le^a antigens, which are the ligands for the leukocyte-endothelium adhesion molecules of the host, namely, E, P- selectins. *H. pylori* isolates from patients with chronic gastritis, duodenal ulcer and gastric cancer for their interactions with the selectins provide evidence of interactions of isolates from each of the diagnostic groups with E- and L-selectins (Galustian et al. 2003). L-selectin interacts with *Helicobacter pylori* isolates from patients with chronic gastritis, duodenal ulcer and gastric cancer. Anaplasma phagocytophilum infected neutrophils showed reduced expression of P-selectin glycoprotein ligand 1 (PSGL-1, CD162) and L-selectin (CD62L) (Choi et al. 2003).

26.5 Cell Surface Ligands for L-Selectin

26.5.1 Subsets of Sialylated, Sulfated Mucins of Diverse Origins are Recognized by L-Selectin

Direct and indirect evidences indicate that L-selectin ligands required sulfate and sialic acid moieties for proper function. The mucin-type polypeptides GlyCAM-1, CD34, and MAdCAM-1 can function as ligands for L-selectin only when they are synthesized by the specialized HEV of lymph nodes. Since sialylation, sulfation, and possibly fucosylation are required for generating recognition, it was argued that other mucins known to have such components might also bind L-selectin. In support, it was shown that mucins secreted by human colon carcinoma cells, as well as those derived from human bronchial mucus can bind to human L-selectin in a calcium-dependent manner. As with Gly-CAM-1 synthesized by lymph node HEV, α2-3 linked sialic acids and sulfation seemed to play a critical role in generating this L-selectin binding. Taken together, studies indicated that a single unique oligosaccharide structure may not be responsible for high-affinity binding. Rather, diverse mucins with sialylated, sulfated, fucosylated lactosamine-type O-linked oligosaccharides can generate high-affinity L-selectin ligands, but only when they present these chains in unique spacing and/or clustered combinations, dictated by polypeptide backbone (Crottet et al. 1996; Tamatani et al. 1993) (Fig. 26.3).

26.5.2 GlyCAM-1

26.5.2.1 Mouse GlyCAM-1

A sulfated 50 kDa glycoprotein (Sgp50) called GlyCAM-1 has been identified as an HEV ligand for L-selectin. This endothelial ligand is an adhesion molecule that accomplishes cell binding by presenting carbohydrate(s) to the lectin domain of L-selectin, and the name GlyCAM 1 (**Gly**-cosylation-dependent **C**ell **A**dhesion **M**olecule 1) was proposed (Fig. 26.3). The GlyCAM-1 is present on HEV of LN and also in lactating mammary glands. The specifically glycosylated form of GlyCAM-1 plays an important role in leukocyte rolling along the inflamed endothelium. It is a secretory protein that is present in mouse serum. The mRNA encoding this glycoprotein is preferentially expressed in LN. The predicted sequence of a cDNA revealed a mucin-like molecule containing two serine/threonine-rich domains. The mucin-like endothelial glycoprotein appears to function as a scaffold that presents carbohydrates to the L-selectin lectin domain (Lasky et al. 1992). The GlyCAM-1 ligand contains a large percentage of serine and threonine residues, which are apparently O - glycosylated. The gene encoding GlyCAM-1 was found to map to murine chromosome 15 Dowbenko et al. (1993a). Adhesive interactions with the L selectin- lectin domain require that the GlyCAM 1 polypeptide chain be appropriately modified with carbohydrates. These carbohydrate modifications include the addition of sialic acid as well as sulfate residues to O-linked carbohydrate side chains that are clustered in two serine/threonine-rich domains of the mucin. An additional interesting structure that may have relevance to the association of GlyCAM 1 with the lumenal surface of the endothelium was a potential amphipathic helix at the C terminus of the glycoprotein. GlyCAM-1 has been shown to enhance β2-integrin function.

26.5.2.2 Rat GlyCAM

Dowbenko et al. 1993b cloned the rat homologue of GlyCAM 1. The sequence of this clone revealed a serine/threonine-rich protein that is highly homologous with the mouse GlyCAM 1. The mouse and the rat GlyCAM 1 homologues show a clustering of these potential O-linked carbohydrate acceptors in two domains of the protein.

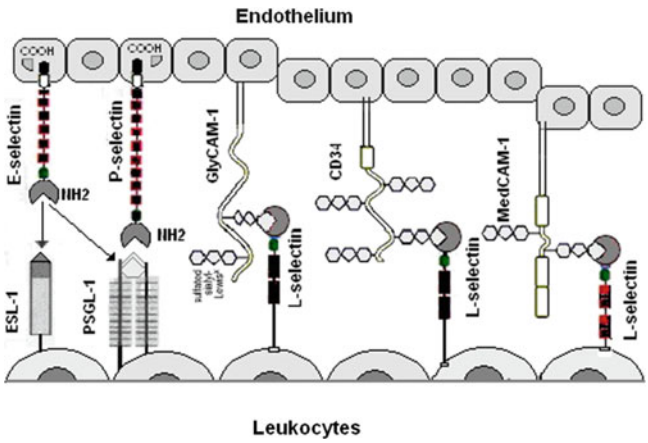

Fig. 26.3 Structures of selectins and their ligands. L-Selectin and the mucinlike vascular addressins direct naive lymphocyte homing to lymphoid tissues**.** E-selectin binds E-selectin ligand 1 (ESL-1) in mouse neutrophils, which is a chicken fibroblast receptor and not related to other adhesion receptor families. E-selectin also binds P-selectin glycoprotein 1 (PSGL-1) but with lower affinity than P-selectin. GlyCAM-1 lacks a transmembrane domain and may be secreted. L-Selectin is expressed on naive T cells, which bind to sulfated sialyl-Lewis^x moieties on the vascular addressins CD34 and GlyCAM-1 on high endothelial venules in order to enter lymph nodes. The relative importance of CD34 and GlyCAM-1 in this interaction is unclear. GlyCAM-1 is expressed exclusively on high endothelial venules but has no transmembrane region and it is unclear how it is attached to the membrane; CD34 has a transmembrane anchor and is expressed in appropriately glycosylated form only on high endothelial venule cells, although it is found in other forms on other endothelial cells. The addressin MAdCAM-1 is expressed on mucosal endothelium and guides entry into mucosal lymphoid tissue. The icon shown represents mouse MadCAM-1, which contains an IgA-like domain closest to the cell membrane; human MadCAM-1 has an elongated mucinlike domain and lacks the IgA-like domain. L-Selectin recognizes the carbohydrate moieties on the vascular addressins. EGF = epidermal growth factor; SCR = short consensus repeats. For selectins refer Fig. 26.1. ||||||||| in PSGL-1 referes to mucin domain

Interestingly, many of the serines and threonines are found to be spaced identically in the two homologues, consistent with the possibility that both density and position of the O-linked side chains may be important for appropriate L selectin-mediated adhesion. In support of its postulated functional importance, the C-terminal potential amphipathic helix is conserved in the rat homologue. Antibody against a relatively conserved portion of mouse GlyCAM 1 demonstrated an approximately 45-kDa sulfated ligand in rat lymph nodes that is analogous to that described for mouse lymph nodes (Dowbenko et al. 1993b; Samulowitz et al. 2002). The mC26 gene encoding GlyCAM-1 in the lactating mouse mammary gland comprises a 5,394 bp fragment of a genomic DNA, which codes for a protein highly expressed in the lactating mouse mammary gland (Nishimura et al. 1993). GlyCAM-1 is also expressed in the cochlea. GlyCAM-1 expressed in the cochlear region is heterogenous in terms of its glycosylation (Kanoh et al. 1999).

26.5.2.3 Complement Factor H as a Ligand for L-selectin in Human

There is no obvious GlyCAM-1 homologue in man. Malhotra et al. (1999) isolated three major glycoproteins of Mr 170, 70 and 50 kDa. The 170 kDa protein was identified as human complement protein Factor H. Human Factor H binds specifically to L-selectin in the presence of CaCl2, and binding was inhibited by anti-L-selectin antibodies, fucoidan and LPS. The interaction of Factor H with leukocyte L-selectin was shown to induce the secretion of TNF-α. It seems that a post-translationally modified form of human plasma Factor H is a potential physiological ligand for L-selectin (Malhotra et al. 1999).

26.5.3 CD34

CD34, a 90 kDa membrane-associated sialomucin, is expressed on the surface of hematopoietic stem/progenitor cells, stromal cells, and on the surface of HEV (Fig. 26.3). The CD34 binds L-selectin, which is important for leukocyte rolling on venules and lymphocyte homing to PLN. A predominant 105 kDa CD34 mucin-like protein has also been identified in human tonsil as peripheral addressin.

120 kDa Addressin Is L-Selectin-Binding Glycoform of CD34: A 120 kDa sialomucin has been identified as the predominant peripheral addressin in porcine lymph nodes. The 120 kDa porcine molecule has its ability to bind MECA-79 and an L-selectin-Fc chimera (LS-Fc). Whereas desialylation of 120 kDa ligand drastically reduced its binding to LS-Fc, this treatment appeared to enhance the binding of 120 kDa ligand to MECA-79. In contrast, the binding of both MECA-79 and LS-Fc to 120 kDa ligand was drastically reduced when de novo sulfation of this ligand was reduced by chlorate, a metabolic inhibitor of sulfation. N-Terminal amino acid sequences of the porcine 120 kDa protein revealed homology with human CD34. Taken together, these findings suggested that the porcine 120 kDa peripheral addressin is an L-selectin-binding glycoform of CD34 (Shailubhai et al. 1997).

Platelets Can Bind Reversibly to CD34$^+$ Cells from Human Blood and Bone: This interaction interferes with the accurate detection of endogenously expressed platelet glycoproteins (GPs). The interaction between these cells was dependent on divalent cations, and mediated by P-selectin. Enzymatic characterization showed the involvement of sialic acid residues. The presence of mRNA for PSGL-1 in the CD34$^+$ cells suggests that this molecule is present in these cells. Under conditions that prevent platelet adhesion, a small but distinct subpopulation of CD34$^+$ cells diffusely

expressed the platelet GPIIb/IIIa complex (Dercksen et al. 1995).

Functions in CD34-Deficient Mice: CD34-deficient mutant- and Wild-type animals do not show differences in lymphocyte binding to PLN HEV, in leukocyte rolling on venules or homing to PLN, in neutrophil extravasation into peritoneum in response to inflammatory stimulus, nor in delayed type hypersensitivity. However, eosinophil accumulation in the lung after inhalation of a model allergen, ovalbumin, was several-fold lower in mutant mice. Although CD34 was not expressed in these mice, a portion of its 90-kD band crossreactive with mAb MECA79 persisted on Western blot. Thus, CD34 seems an additional molecule(s) that might be involved in eosinophil trafficking into the lung (Suzuki et al. 1996). However, CD34 is clearly not the sole contributor of L-selectin ligand activity in HEVs, since CD34-null mice maintain virtually normal L-selectin-dependent lymphocyte homing activity.

Another HEV-expressed L-selectin ligand is the transmembrane sialomucin podocalyxin-like protein or PCLP. Like CD34, PCLP is expressed on some vascular endothelia, but it is also expressed on the foot processes of glomerular podocytes. HEV-derived PCLP interacts with recombinant L-selectin-Ig chimera and supports L-selectin-dependent lymphocyte adhesion under physiological conditions.

26.5.4 Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1)

The mucosal vascular addressin, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is an Ig family adhesion receptor preferentially expressed by venular endothelial cells at sites of lymphocyte extravasation in murine mucosal lymphoid tissues and lamina propria (Fig. 26.3). The MAdCAM-1 specifically binds to both human and mouse lymphocytes that express the homing receptor for Peyer's patches, the integrin α4β7. MAdCAM-1 interacts preferentially with the leukocyte β7 integrin LPAM-1 (α4β7), but also with L-selectin, and with VLA-4 (α4β7) on myeloid cells, and serves to direct leukocytes into mucosal and inflamed tissues. Expression of human MAdCAM-1 RNA is restricted to mucosal tissues, gut-associated lymphoid tissues and spleen. L-selectin counter-receptors in HEVs are recognized by mAb MECA-79, a surrogate marker for molecularly heterogeneous glycans termed PLN addressin (PNAd).

26.5.4.1 MAdCAM-1 Gene

Human MAdCAM-1 gene contains five exons where signal peptide, two Ig domains, and mucin domain are each encoded by separate exons. The transmembrane domain, cytoplasmic domain, and 3′ untranslated region are encoded together on exon 5. The mucin domain contains eight repeats in total that are subject to alternative splicing. Despite the absence of a human counterpart of the third IgA-homologous domain and lack of sequence conservation of the mucin domain, the genomic organizations of human and mouse MAdCAM-1 genes are similar. An alternatively spliced MAdCAM-1 variant that lacks exon 4 encoding the mucin domain, may mediate leukocyte adhesion to LPAM-1 without adhesion to the alternate receptor, L-selectin. The MAdCAM-1 gene was located at p13.3 on chromosome 19, in close proximity to the ICAM-1 and ICAM-3 genes (p13.2–p13.3) (Leung et al. 1997).

The mouse MAdCAM-1 gene is located on chromosome 10 and contains five exons. The signal peptide and each one of the three Ig domains are encoded by a distinct exon, whereas the transmembrane, cytoplasmic tail, and 3′-untranslated region of MAdCAM-1 are combined on a single exon. The mucin-like region and the third Ig domain are encoded together on exon 4. A short variant of MAdCAM-1 may be specialized to support α4β7-dependent adhesion strengthening, independent of carbohydrate-presenting function (Sampaio et al. 1995). The deduced amino acid sequence from full-length DNA encoding human brain MAdCAM-1 revealed an 18 amino acid signal peptide, two N-terminal Ig-like domains conserved (59–65%) in sequence with those of mouse homologue, an 86 amino acid mucin-like region rich in serine-threonine residues, a 20 amino acid transmembrane domain and a 43 amino acid charged cytoplasmic domain. No counterpart to the third IgA-like domain of mouse MAdCAM-1 was present; however, the serine-threonine-rich mucin domain was extended as two distinguishable major and minor mucin regions unrelated to the mouse domain. The major domain is formed from six tandem repeats of an eight amino acid sequence having the MUC-2-related consensus DTTSPEP/SP. Human MAdCAM-1 mRNA transcripts were restricted to small intestine, colon, spleen, pancreas and brain. Alternatively spliced MAdCAM-1 variants were identified that lack parts of the second Ig domain and all or part of the major mucin domain, indicating that the function of this vascular addressin is regulated by extensive modifications to its multi-domain structure (Leung et al. 1996).

Two Ig-like domains of human and macaque MAdCAM-1 are similar to the two amino-terminal integrin binding domains of murine MAdCAM-1. Human MAdCAM-1 exhibits considerable variation from murine MAdCAM-1 with respect to the length of the mucin-like sequence and the lack of a membrane proximal Ig/IgA-like domain. The MAdCAM-1 from different species demonstrated greater seleoffctive pressure for maintenance of amino acids involved in α4β7 binding than those sequences presumably involved in the presentation of carbohydrates for selectin binding (Shyjan et al. 1996).

26.5.4.2 Structural Requirement for MAdCAM-1 Binding to Lymphocyte Receptor α4β7

The murine MAdCAM-1 has two amino-terminal integrin binding Ig domains. A point mutation within the first Ig domain of MAdCAM-1 abolishes activation-independent α4β7 binding. This point mutation resides within an eight-amino acid motif with homology to sequences important for the integrin binding ability of the related vascular Ig family members, ICAM-1 and VCAM-1. The first domain of MAdCAM-1 is sufficient for interaction with α4β7, but sequences within the second domain support this interaction, either by providing additional contact points for integrin binding or by contributing to the conformation or presentation of the N-terminal domain. The second domain of MAdCAM-1 can also support activation-dependent LFA-1 binding to domain 1 of ICAM-1. These findings are similar to VCAM-1 binding to α4β7 and suggest that structural differences exist between vascular Ig-like ligands for α4 versus β2 integrins (Briskin et al. 1996). A crystal structure for two extracellular amino-terminal domains of human MAdCAM-1 confirmed their expected Ig superfamily topology. In second structure of this fragment, although the overall structure is similar to that previously reported, one edge strand in the amino-terminal domain is instead located on the opposite sheet. This alters the arrangement and conformation of amino acids in this region that had been shown to be crucial for ligand binding. MAdCAM-1 is also seen to form dimers within the crystal lattice raising the possibility that oligomerization may influence the biological role of this adhesion molecule (Dando et al. 2002).

26.5.5 PSGL-1 Binds L-Selectin

L-selectin binds with high affinity to the N-terminal region of P-selectin glycoprotein ligand-1 (PSGL-1) through cooperative interactions with three sulfated tyrosine residues and an appropriately positioned C2-O-sLe^x O-glycan (Leppanen et al. 2003). PSGL-1 binding to P-selectin controls early leukocyte rolling during inflammation. The L-selectin-dependent rolling after P-selectin blockade is completely absent in PSGL-1 deficient (PSGL-1$^{-/-}$) mice or wild-type mice treated with a PSGL-1 blocking mAb. It appeared that leukocyte-expressed PSGL-1 serves as the main L-selectin ligand in inflamed postcapillary venules (Sperandio et al. 2003). L-selectin binding to PSGL-1 initiates tethering events that enable L-selectin-independent leukocyte-endothelial interactions. Interestingly, antibodies and pharmacological inhibitors (e.g., rPSGL-Ig) that target the N-terminus of PSGL-1 reduce but do not abolish P-selectin-dependent leukocyte rolling in vivo whereas PSGL-1-deficient mice have almost no P-selectin-dependent rolling. However, Ridger et al. (2005) suggested that leukocytes can continue to roll in the absence of optimal P-selectin/PSGL-1 interaction using an alternative mechanism that involves P-selectin-, L-selectin-, and sLe^x-bearing ligands. L- and P-selectins collaborate to support leukocyte rolling in vivo when high-affinity PSGL-1 interaction is inhibited (Ridger et al. 2005) (see Chap 27 and Chap 28).

26.6 L-Selectin IN Pathological States

26.6.1 Gene Polymorphism in L-Selectin

LECAM-1 Genes in Ig A Nephropathy: Elucidating the genetic background of immunoglobulin A nephropathy (IgAN) and genetic factors associated with the pathogenesis of the disease, Takei et al. (2002) studied single-nucleotide polymorphisms (SNPs) in selectin gene cluster on chromosome 1q24–25 and found that two SNPs in the E-selectin gene (*SELE8* and *SELE13*) and six SNPs in the L-selectin gene (*SELL1*, *SELL4*, *SELL5*, *SELL6*, *SELL10*, and *SELL11*) were significantly associated with IgAN in Japanese patients. All eight SNPs were in almost complete linkage disequilibrium. *SELE8* and *SELL10* caused amino acid substitutions from His to Tyr and from Pro to Ser, and *SELL1* could affect promoter activity of the L-selectin gene. The TGT haplotype at these three loci was associated with IgAN (Fig. 26.3)

Polymorphism in Type 2 Diabetes Mellitus: Glomerular infiltration with monocytes/macrophages has been implicated in the pathogenesis of diabetic nephropathy. The LECAM-1 213PP genotype is a genetic risk factor for the development of nephropathy in type 2 diabetes mellitus (Kamiuchi et al. 2002). Strong relationship has been demonstrated between soluble L-selectin and diabetic retinopathy with strong correlation between sL-selectin and HbA1c. Soluble L-selectin is increased with poor glycemic control (Karadayi et al. 2003). In Finnish school children CAM is positive marker for precinical type 1diabetes in children (Toivonen et al. 2004).

Brucellosis: The 206Leu allele frequency occurred in 42% of the patients with coronary artery disease compared to % of the controls (Hajilooi et al. 2006). Higher frequency of L-selectin genotypes in patients with brucellosis than in controls and the association between the 206Leu allele and the occurrence of brucellosis relapse, suggest that the F206L polymorphism may make individuals more vulnerable to brucellosis (Rafiei et al. 2006).

26.6.2 Antitumor Effects of L-Selectin

26.6.2.1 Regulation of Host-Mediated Anti-Tumor Mechanisms by L-Selectin

Human tumor cells injected s.c. into mice lacking β3- or β3/β5-integrins or various selectins show enhanced tumor growth compared with growth in control mice. There was increased angiogenesis in mice lacking β3-integrins. Tumor growth also was affected by bone marrow-derived cells in mice lacking any one or all three selectins, implicating both leukocyte and endothelial selectins in tumor suppression. Cells of the innate immune system, macrophages or perhaps natural killer cells, seemed to be implicated in tumor suppression (Taverna et al. 2004). Malignant melanoma is often accompanied by a host response of inflammatory cell infiltration that is highly regulated by multiple adhesion molecules. L-selectin and ICAM-1 contribute co-operatively to the anti-tumor reaction by regulating lymphocyte infiltration to the tumor (Yamada et al. 2006). The plasma level of sL-selectin is possibly useful for early diagnosis of relapse and extramedullary infiltration in acute myeloid leukemia (Aref et al. 2002; Chang et al. 2003).

Suppression of Metastasis in Lymph Nodes by L-Selectin-Mediated NK Cells: The NK cells are known to reject certain tumors in vivo and prevent metastasis of tumors into secondary lymphoid organs. In tumor-bearing hosts, NK cells are recruited to regional lymph nodes in wild-type mice, but not in mice deficient for L-selectin or L-selectin ligands. L-selectin on NK cells and L-selectin ligands on endothelial cells seem to be essential for NK cell recruitment to lymph nodes. Although L-selectin-deficient NK cells efficiently lysed tumor cells in vitro, NK cell-dependent suppression of tumor metastasis was diminished in mice deficient for L-selectin or L-selectin ligands because of insufficient NK cell recruitment to lymph nodes. Evidences indicate that L-selectin-mediated NK cell recruitment plays a crucial role in the control of tumor metastasis into secondary lymphoid organs (Chen et al. 2005).

26.6.2.2 Can L-Selectin Facilitate Metastasis?

Transgenic mice that express rat insulin promoter regulate simian virus 40 Tag (RIP-Tag) develop large, local cancers that metastasize to liver but not LN. Mice, that developed insulinomas, specifically had LN metastases; metastasis was blocked by an anti L-selectin mAb. The highly vascularized islet carcinomas shed tumor cells into the bloodstream, which is a necessary but insufficient condition for metastasis to occur; L-selectin can facilitate homing of such tumor cells to LN, resulting in metastasis (Qian et al. 2001). Surprisingly, L-selectin expressed on endogenous leukocytes also facilitates metastasis in both the syngeneic and xenogeneic (T and B lymphocyte deficient) systems Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis (Borsig et al. 2002). It seems that L-selectin-mediated NK cell recruitment plays a crucial role in the control of tumor metastasis into secondary lymphoid organs (Chen et al. 2005). L-selectin deficiency also attenuates experimental metastasis. Although L-selectin deficiency did not affect platelet aggregation or initial tumor cell embolization, the association of leukocytes with tumor cells was reduced and tumor cell survival was diminished 24 h later. Inhibition of L-selectin by a function-blocking antibody also reduced metastasis. Therefore, L-selectin facilitation of metastasis progression involves leukocyte-endothelial interactions at sites of intravascular arrest supported by local induction of L-selectin ligands via fucosyltransferase-7. It provides an explanation for how L-selectin facilitates tumor metastasis (Laubli et al. 2006).

26.6.2.3 Adhesion Molecules in Kaposi's Sarcoma

Kaposi's sarcoma (KS) is a neoplasm with multifocal vascular lesions that is often seen in homosexual HIV-infected individuals. The products of leukocytes enhance the proliferation of KS cells in vitro and most likely are crucial for the development of KS lesions in vivo. Studies indicate that multiple proinflammatory agents can induce NF-kB binding activity and can enhance ICAM-1, VCAM-1, and E-selectin expression in KS cells. Thus, the induction of CAM expression could be an early event in the development of KS by recruiting leukocytes into KS lesions, thereby providing factors that could potentiate the development of KS (Sciacca et al. 1994; Yang et al. 1994).

Osteosarcomas and Rhabdomyosarcomas: Osteosarcomas and rhabdomyosarcomas are highly invading tumors. Before they can extravasate to the parenchymal organs and form metastases, they have to adhere to the endothelial cells lining the blood vessels and then penetrate through the endothelium. Human sarcoma cell lines and rhabdomyosarcoma RD express VLA-4 molecule on their surface and bind to the VCAM-I-expressing activated endothelial cell line Ea.hy 926. Sarcoma cells also adhere to recombinant sVCAM-I protein. On the other hand, these sarcoma cell lines do not express marked amounts of other ligands (such as CDII/18 or sialyl-Lex) for other endothelial adhesion molecules (ICAM-I, ICAM-2, E- and P-selectin) indicating that the VLA-4-VCAM-I dependent pathway might be of major importance in sarcoma extravasation (Mattila et al. 1992).

Selectins in the Triggering, Growth, and Dissemination of T-lymphoma Cells: The ICAM-1 expression by the host is essential for lymphoma dissemination. In selectin-deficient mice, though the absence of E-, P-, or L-selectins did not affect the triggering of radiation-induced thymic lymphoma, the absence of L-selectin on lymphoma cells reduced their capacity to grow in the thymus. This defect was overcome by altering the integrity of the L-selectin-mediated interactions in the thymus, as shown in L-selectin-deficient mice and by adoptive transfer experiments. Evidence shows that selectins play a significant role at different steps of T-cell lymphoma development (Belanger and St-Pierre 2005).

26.6.2.4 Role of CD62L$^+$ Cells and TGF-β in Patients with Gastric Cancer

CD62L$^+$ cells are decreased in the peripheral blood, but inversely increased in the spleen of gastric cancer patients. The increased CD62L$^+$ cells residing in the CD4$^+$ suppressor-inducer phenotype, and the removal of CD62L$^+$ cells from spleen cells results in a decrease of Con A-induced suppressor activity in vitro in one-way allogeneic MLR. The CD62L$^+$ cells included CD4$^+$CD25$^+$ regulatory T cells. The culture supernatant of CD62L$^+$ cells showed TGF-β activity, which was more significantly detectable in splenic vein than in the peripheral blood, and TGF-β mRNA was detectable in the spleen from advanced gastric cancer patients. These results suggest that CD62L$^+$ cells migrate into the spleen with disease progression of gastric cancer and serve as suppressor-inducer cells with TGF-β production to induce regulatory T cells, contributing to disease-associated immunosuppression in advanced gastric cancer patients (Noma et al. 2005).

26.6.2.5 Reduced Expression of CD62L Can Identify Tumor-Specific T Cells

Reduced expression of CD62L can identify tumor-specific T cells in lymph nodes draining murine tumors. This strategy could isolate tumor-specific T cells from vaccinated patients. Tumor vaccine-draining lymph node (TVDLN) T cells of patients were separated into populations with reduced (CD62L^{Low}) or high levels of CD62L (CD62L^{High}). Effector T cells generated from CD62L^{Low} cells maintained or enriched the autologous tumor-specific type 1 cytokine response compared to unseparated TVDLN T cells in four of four patients showing tumor-specific cytokine secretion. Effector T cells generated from CD62L^{Low} or CD62L^{High} TVDLN were polarized towards a dominant type 1 or type 2 cytokine profile respectively. For CD62L^{Low} T cells the type 1 cytokine profile appeared determined prior to culture. Since a tumor-specific type 1 cytokine profile appears critical for mediating anti-tumor activity in vivo, this approach can be used to isolate T cells for adoptive immunotherapy (Meijer et al. 2004).

It was shown that tumor vaccine-sensitized draining lymph node (vDLN) cells activated ex vivo with bryostatin and ionomycin (B/I) are capable of inducing antigen-specific regression of a murine mammary tumor, 4T07. The vDLN cells not activated with B/I, were ineffective. It was suggested that B/I selectively activates tumor-sensitized (CD62L^{low}) lymphocytes, to account for the highly potent and tumor-specific activity. It was also hypothesized that CD8$^+$ CD62L^{low} cells may be preferentially activated by B/I treatment, infiltrate the tumors and mediate tumor regression in mice. It was suggested that CD62L^{low} cells are preferentially activated by B/I, leading to a highly effective anti-tumor T cell population (Chin et al. 2004).

26.6.2.6 CD44 Glycoform as E- and L-Selectin Ligand on Colon Carcinoma Cells

Engagement of E-selectin and L-selectin with relevant counter-receptors expressed on tumor cells contributes to the hematogenous spread of colon carcinoma. The LS174T colon carcinoma cell line expresses CD44 glycoform known as hematopoietic cell E-/L-selectin ligand (HCELL), which functions as a high affinity E- and L-selectin ligand on these cells. Expression of HCELL confers robust and predominant tumor cell binding to E- and L-selectin, highlighting a central role for HCELL in promoting shear-resistant adhesive interactions essential for hematogenous cancer dissemination (Burdick et al. 2006).

26.6.3 Autoimmune Diseases

26.6.3.1 L-Selectin in Autoimmune Diabetes

Administration of anti-L-selectin (CD62L) mAb to neonatal non-obese diabetic (NOD) mice mediates long term protection against the development of insulitis and overt diabetes, suggesting that CD62L has a key role in the general function of beta cell-specific T cells.

CD4$^+$CD62L$^+$ Regulatory T Cells in Controlling Onset of Diabetes in Mice: Autoimmune diabetes is characterized by an early mononuclear infiltration of pancreatic islets and later selective autoimmune destruction of insulin-producing β cells. Lymphocyte homing receptors are the candidate targets to prevent autoimmune diabetes. L-selectin (CD62L), expressed in naive T and B cells can be blocked in vivo with specific antibodies such as Mel-14, which partially impairs insulitis and diabetes in autoimmune diabetes-prone NOD mice. Genetic blockade of leukocyte

homing into peripheral lymph nodes can prevent the development of diabetes. Though L-selectin plays a small role in the homing of autoreactive lymphocytes to regional (pancreatic) lymph nodes in NOD mice (Mora et al. 2004), experimental evidences indicate that $CD4^+$ regulatory T cells control progression of autoimmune insulitis in NOD mice. You et al. (2004) showed that diabetes onset is prevented in such mice by infusion of polyclonal $CD4^+$ T cells expressing L-selectin (CD62L) but not prevented or only marginally prevented by $CD4^+CD25^+$ T cells. Report argues for the role of $CD4^+CD62L^+$ T cells present within the polyclonal diabetogenic population in mediating this apparently paradoxical effect. The central role of $CD4^+CD62L^+$ regulatory T cells in controlling disease onset was confirmed in a transgenic model of autoimmune diabetes and possibly through intervention of homeostatic mechanisms as part of their mode of action (You et al. 2004). Despite these observations, the patterns of T cell activation, migration, and beta cell-specific reactivity were similar in NOD mice in different genotypes, suggesting that CD62L expression is not essential for the development of type 1diabetes in NOD mice (Friedline et al. 2002). Nevertheless, CD62L expression is necessary for the diabetes-delaying effect of transfer of $CD4^+CD25^+$ splenocytes in vivo, but not for their suppressor function in vitro (Szanya et al. 2002).

26.6.3.2 L-Selectin Deficiency Reduces Immediate-Type Hypersensitivity

Antigen-sensitized $CD4^+CD62L^{low}$ memory/effector T helper 2 cells can induce airway hyperresponsiveness in an antigen free setting (Nakagome et al. 2005). Dermal and pulmonary inflammatory disease in E-selectin and P-selectin double-null mice is reduced in triple-selectin-null mice (Collins et al. 2001). P- and E-selectin mediate CD4+ Th1 cell migration into the inflamed skin in a murine contact hypersensitivity model. In this model, not only CD4+ T cells but also CD8+ T cells infiltrate the inflamed skin, and the role of CD8+ type 1 cytotoxic T (Tc1) cells as effector cells has been demonstrated. In mice deficient in both P- and E-selectin, the infiltration of CD8+ T cells in the inflamed skin is reduced, which suggests that these selectins participate in CD8+ T cell migration. Tc1 cells are able to migrate into the inflamed skin of wild-type mice. This migration is partially mediated by P- and E-selectin, as shown by the reduced Tc1 cell migration into the inflamed skin of mice deficient in both, P- and E-selectin or wild-type mice treated with the combination of anti-P-selectin and anti-E-selectin Abs. During P- and E-selectin-mediated migration of Tc1 cells, P-selectin glycoprotein ligand-1 appears to be the sole ligand for P-selectin and one of the ligands for E-selectin. P- and E-selectin-independent migration of Tc1 cells into the inflamed skin was predominantly mediated by L-selectin. These observations indicate that all three selectins can mediate Tc1 cell migration into the inflamed skin (Hirata et al. 2002).

The deposition of immune complexes (IC) induces an acute inflammatory response with tissue injury. IC-induced inflammation is mediated by inflammatory cell infiltration, a process highly regulated by expression of multiple adhesion molecules. The cutaneous reverse passive Arthus reaction examined in mice lacking L-selectin (L-selectin$^{-/-}$), ICAM-1 (ICAM-1$^{-/-}$), or both (L-selectin/ICAM-1$^{-/-}$), indicates that ICAM-1 and L-selectin cooperatively contribute to the cutaneous Arthus reaction by regulating neutrophil and mast cell recruitment (Kaburagi et al. 2002). Repeated Ag exposure in wild-type littermates resulted in increased levels of serum L-selectin, also observed in atopic dermatitis patients (Shimada et al. 2003). These studies demonstrates that L-selectin and ICAM-1 cooperatively regulate the induction of the immediate-type response by mediating mast cell accumulation into inflammatory sites and suggests that L-selectin and ICAM-1 are potential therapeutic targets for regulating human allergic reactions

26.6.3.3 L-Selectin Is Required for Early Neutrophil Extravasation

L -selectin (CD62L) and CD44 are major adhesion receptors that support the rolling of leukocytes on endothelium, the first step of leukocyte entry into inflamed tissue. The requirement for these receptors for inflammatory cell recruitment during Ag-induced arthritis was studied in CD44-deficient, L-selectin-deficient, and CD44/L-selectin double knockout mice. Study suggested a greater requirement for L-selectin than for CD44 for neutrophil extravasation during the early phase of Ag-induced arthritis (Szanto et al. 2004). Verdrengh et al. (2000) described a dual role for selectins in *S. aureus*-induced arthritis: on the one hand, blockade of these selectins leads to less severe arthritic lesions in the initial stage of the disease; on the other, delayed recruitment of phagocytes decreases the clearance of bacteria (Verdrengh et al. 2000).

26.6.3.4 L-Selectin in Mouse CNS During EAE

Adhesion molecules facilitate infiltration of leukocytes into CNS of mice with experimental allergic encephalomyelitis (EAE). Cellular infiltration and expression of the adhesion molecules ICAM-1 (CD54), VCAM-1 (CD106), L-selectin (CD62L), and leukosialin (CD43) in EAE-susceptible SWXJ mice preceded the EAE clinical symptoms by a minimum of 3 days, suggesting a causal role of adhesion molecules in the initiation of CNS inflammation. However, prophylactic injections of mAbs against either of ICAM-1, L-selectin, or CD43, did not ameliorate the clinical severity of EAE in these mice (Dopp et al. 1994). In order to study the role of CD62L in the immunopathology of EAE, Grewal et al. (2001) crossed

CD62L-deficient mice with myelin basic protein-specific TCR (MBP-TCR) transgenic mice. CD62L-deficient MBP-TCR transgenic mice failed to develop antigen-induced EAE, and, despite the presence of leukocyte infiltration, damage to myelin in the CNS was not seen. EAE could, however, be induced in CD62L-deficient mice upon adoptive transfer of wild-type macrophages. Study indicated that CD62L is not required for activation of autoimmune CD4 T cells but is important for the final destructive function of effector cells in the CNS and supports a novel mechanism whereby CD62L expressed on effector cells is important in mediating myelin damage (Grewal et al. 2001).

26.6.3.5 Central and Effector Memory T Cells to Recall Responses

The absolute number of memory $CD8^+$ T cells in the spleen following antigen encounter remains stable for many years. However, the relative capacity of these cells to mediate recall responses was studied by Roberts et al. (2005). A dual adoptive transfer approach demonstrated a progressive increase in the quality of memory T cell pools in terms of their ability to proliferate and accumulate at effector sites in response to secondary pathogen challenge. This temporal increase in efficacy occurred in $CD62L^{low}$ (effector memory) and $CD62L^{high}$ (central memory) subpopulations, but was most prominent in the CD62L hi subpopulation. Data indicated that the contribution of effector memory and central memory T cells to the recall response changes substantially over time (Roberts et al. 2005).

During infection with lymphocytic choriomeningitis virus, $CD8^+$ T cells differentiate rapidly into effectors ($CD62L^{low}CD44^{high}$) that differentiate further into the central memory phenotype ($CD62L^{high}CD44^{high}$) gradually. Van Faassen et al. (2005) indicated that the potency of the pathogen can influence the differentiation and fate of $CD8^+$Tcells enormously, and the extent of attrition of primed $CD8^+$ T cells correlates inversely to the early differentiation of $CD8^+$ T cells primarily into the central $CD8^+$ T cell subset (van Faassen et al. 2005).

26.6.4 CD62L in Other Conditions

Adhesion Molecules in Inflammatory Bowel Disease: Mucosal endothelium has become one of the major areas of investigation in gut inflammation. It is now well recognised that it plays an active role in the pathogenesis of both forms of inflammatory bowel disease, Crohn's disease and ulcerative colitis, since endothelial cells regulate mucosal immune homeostasis, acting as "gatekeepers", controlling leukocyte accumulation in the interstitial compartment. This process is mediated by leukocyte-endothelial adhesion molecules. The major molecules that mediate leukocyte-endothelial interactions, have been reviewed and the results of the most recent clinical trials targeting adhesion molecules in inflammatory bowel disease summarized (Danese et al. 2005). Compared to controls, the total amount of platelet P-selectin (tP-selectin) does not change in patients with inflammatory bowel disease (IBD) and 5-aminosalicylic acid medication. However, on gender basis, the male patients showed higher levels of total P-selectin compared to male controls. Increased total P-selectin levels may alter the inflammatory response and susceptibility to thromboembolic disease. As found with sP-selectin, total P-selectin showed gender dependent differences, which are important to consider for future studies (Fagerstam and Whiss 2006).

Appendix plays an important role in the pathogenesis of colitis (Farkas et al. 2005). Since appendectomy can protect against development of ulcerative colitis and Crohn's disease and since. T cells affect the appendix in the development of colitis, Farkas et al. (2005) demonstrated the preferential migration of $CD62L^+CD4^+$ cells into the appendix as compared to the colon. This migration pattern correlated with up-regulation of integrin $\alpha4\beta7$ and CD154 (CD40 ligand) on T cells. Thus In a chronic ileitis model, pathogenic $CD4^+$ T cells alternatively engage L-selectin in order to recirculate to the chronically inflamed small intestine (Rivera-Nieves et al. 2005). L-selectin is important in experimental abdominal aortic aneurysm (AAA) formation in rodents. L-selectin-mediated neutrophil recruitment may be a critical early step in AAA formation (Hannawa et al. 2005). The resident flora plays a critical role in initiation and perpetuation of intestinal inflammation, as demonstrated in experimental models of colitis where animals fail to develop disease under germ free conditions. Strauch et al. (2005) indicated that bacterial antigens are crucial for the generation and/or expansion of T_{reg} cells in a healthy individual. Therefore, bacterial colonisation is of great importance in maintaining the immunological balance.

Other Conditions: During acute familial Mediterranean fever (FMF) there was no change in L-selectin expression, but there was an increased neutrophil surface CD11b compared with normal controls. Neutrophils of FMF patients regulate CD11b and L-selectin expression induced by chemoattractant (FMLP) stimulation, similar to that in controls (Molad et al. 2004). Considering that the inflammation is the basic stage in the early period of ^{131}I therapy (RAI) of hyperthyroidism, E- and L-selectins are not useful indicators in thyroid, while ICAM-1 and IL-6 may be important in the thyroid during radioiodine therapy, especially Graves' disease (Jurgilewicz et al. 2002). Inflammatory

cells play a crucial role in wound healing. Although ICAM-1 contributes to wound repair to a greater extent than L-selectin, a role for L-selectin was revealed in the absence of ICAM-1. The impaired wound repair was associated with reduced infiltration of neutrophils and macrophages in ICAM-$1^{-/-}$ and L-selectin/ICAM-$1^{-/-}$ mice. Results suggested a distinct role of ICAM-1 and L-selectin in wound healing and the delayed wound healing in the absence of these molecules is likely because of decreased leukocyte accumulation at the wound site (Nagaoka et al. 2000). Soluble L-selectin increases in serum samples from subjects with *cryptococcosis* than in those from uninfected subjects (Jackson et al. 2005).

26.7 Oligonucleotide Antagonists IN Therapeutic Applications

Selectin-directed therapeutic agents are now proven to be effective in blocking many of the pathological effects resulting from leukocyte entry into sites of inflammation. Studies are being focused on how the selectins interact with the increasing array of other adhesion molecules and inflammatory mediators. L-selectin expression on CD3+, CD4+ and CD8+ T cells was significantly lower in HIV infected children than in the control group. The percentage of neutrophils expressing CD62L was significantly reduced in patients with severe immunologic suppression. Altered leukocyte functions such as migration and homing resulting from reduced expression of CD62L may be an important contributor of the progressive dysfunction of the immune system in HIV infected children (Gaddi et al. 2005).

26.7.1 Monomeric and Multimeric Blockers of Selectins

Selectins bind to carbohydrate ligands in a calcium-dependent manner and play critical roles in host defense and possibly in tumor metastasis. It is possible to isolate peptides mimicking carbohydrate ligands by screening the peptides for binding to anticarbohydrate antibodies and then use them to inhibit carbohydrate-dependent experimental tumor metastasis (Fukuda et al. 2000). The monomeric and polymeric compounds, 4-nitrophenylthiomannoside, phenylmannoside, conjugated with polyacrylic acid, and α-mannose, conjugated with polyacrylamide, inhibited the binding of the model ligand to P- and L-selectins (but not to E-selectin). These compounds (i.v.) caused a dose-dependent reduction of neutrophil accumulation in rat peritoneum. The polysaccharide mannan was inactive in both types of experiments. The conjugate of phenylmannoside with polyacrylic acid was the most effective blocker in vitro as well as in vivo. The inhibitory effect of (s.c) 4-nitrophenylthiomannoside was indicated on mouse peritonitis (Ushakova et al. 2001). Ikegami-Kuzuhara et al. (2001) investigated the ability of a synthetic sugar derivative, OJ-R9188, [N-(2-tetradecylhexadecanoyl)-O-(L-alpha-fucofuranosyl)-D-seryl]-L-glutamic acid 1-methylamide 5-L-arginine salt, to block binding of selectins to their ligands in vitro and inhibit the infiltration of leukocytes in vivo. OJ-R9188 prevented the binding of human E-, P- and L-selectin-IgG fusion proteins to immobilized sialyl Le^x-pentasaccharide glycolipid. In a mouse model of thioglycollate-induced peritonitis, OJ-R9188 inhibited neutrophil accumulation in the peritoneal cavity. Thus blocking selectin-sLe^x binding site is a promising strategy for the treatment of allergic skin diseases.

The potency of oligosaccharides $SiaLe^x$, Sia Le^a, HSO_3 Le^x, and HSO_3 Le^a, their conjugates with polyacrylamide (PAA), and other monomeric and polymeric selectin inhibitors has been compared with that of the polysaccharide fucoidan. PAA-conjugates, containing tyrosine-O-sulfate (sTyr) in addition to one of the sialylated oligosaccharides, are the potent synthetic blockers in vitro. Compared with fucoidan, the bi-ligand glycoconjugate HSO_3 Le^a-PAA-sTyr displayed similar inhibitory activity in vitro towards L-selectin and about ten times lower activity towards P-selectin. All of the synthetic polymers displayed a similar ability to inhibit neutrophil extravasation in the peritonitis model (in vivo). Evidence indicates that monomeric $SiaLe^x$ is considerably more effective as a selectin blocker in vivo than in vitro, whereas the opposite is true for fucoidan and the bi-ligand neoglycoconjugate HSO_3 Le^a-PAA-sTyr (Ushakova et al. 2005).

26.7.2 Synthetic and Semisynthetic Oligosaccharides

The SELEX (systematic evolution of ligands by exponential enrichment) yields high affinity oligonucleotides with unexpected binding specificities. Nuclease-stabilized randomized oligonucleotides subjected to SELEX against recombinant L-selectin yielded calcium-dependent antagonists which shared a common consensus sequence. Unlike sialyl Le^x, these antagonists were specific to L-selectin since they showed little binding to E- or P-selectin. However, they showed binding to native L-selectin on peripheral blood lymphocytes and blocked L-selectin-dependent interactions with the natural ligands on HEV (O'Connell et al. 1996).

While high affinity recognition of natural ligands is associated with α1-3(4)fucosylated, α2-3sialylated (and/or sulfated) lactosamine sequences, small oligosaccharides that potentialy inhibit the selectins were found by Koenig et al. (1997). Using synthetic and semisynthetic oligosaccharides

related to those on natural ligands Koenig et al. (1997) confirmed that α2-3-linked sialic acids, and α1-3(4) fucosylation are important for recognition. Immobilized targets for the three selectins indicated that the binding sites for sialic acid and sulfate are very close, or identical. While O-sulfate esters mostly improved L- and P-selectin recognition, effects depended upon their position and number. Of particular note, the "major capping group" of GlyCAM-1 was not an unusually potent or highly selective inhibitor of L-selectin, even when studying the interaction of L-selectin with native GlyCAM-1 itself (Koenig et al. 1997).

Nishida et al. (2000) described a synthetic approach for artificial L- and P-selectin blockers. This synthesis involves radical bi- and terpolymerizations of p-(N-acrylamido)phenyl 3- or/and 6-sulfo-β-D-galactoside with allyl α-L-fucoside in the presence of acrylamide. Each of the two glycosyl monomers constructs a key carbohydrate module responsible for selectins/sulfated sialyl Lewis^x bindings. Whereas an acrylamide copolymer carrying 3-sulfo-galactoside showed no activity for any selectins, the fucosylated terpolymer showed a potent activity to block both of P- and L-selectins/sLe^x binding at a concentration of a few μg/ml. The enhanced activity is apparently ascribed to the cooperative binding effects of the fucoside and the 3-sulfo-galactoside residues.

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P-Selectin and Its Ligands

27

G.S. Gupta

27.1 Platelet Adhesion and Activation

When a blood vessel is injured, control of bleeding starts with the rapid adhesion of circulating platelets to the site of damage. Within seconds, the adhered platelets are activated, secrete the contents of storage organelles, spread out over the damaged area and recruit more platelets to the developing thrombus. However, if this process occurs in a diseased, sclerotic or occluded vessel, the resulting platelet thrombus may break away and block the coronary artery, causing a heart attack, or restrict blood supply to the brain, causing a stroke. The glycoprotein (GP) Ib-IX-V complex, a member of the leucine-rich protein family, is a constitutive platelet membrane receptor for von Willebrand Factor (vWF), a multimeric adhesive glycoprotein found in the matrix underlying the endothelial cell lining of the blood vessel wall and in the plasma. Binding of vWF to the Ib-IX-V complex regulates adhesion of platelets to the sub-endothelium at high shear flow, and initiates signal transduction leading to platelet activation. The GP Ib-IX-V complex also constitutes a binding site for α-thrombin, an interaction that facilitates thrombin-dependent platelet activation. Analysis of GP Ib-IX-V complex and vWF has identified discrete amino acid sequences that mediate their interaction. An anionic/sulfated tyrosine sequence of the GP Ib α-chain that is critical for binding of Ib-IX-V complex to both vWF and α-thrombin is analogous to sulfated anionic amino acid sequences mediating interactions of other adhesive proteins, including P-selectin binding to PSGL-1 and Factor VIII binding to vWF (Andrews et al. 1997).

27.2 P-Selectin (GMP-140, PADGEM, CD62): A Member of Selectin Adhesion Family

27.2.1 Platelets and Vascular Endothelium Express P-Selectin

Selectins are adhesion receptors that mediate leukocyte adhesion to platelets or endothelial cells through Ca^{2+}-dependent interactions with cell surface oligosaccharides. The selectin family consists of three closely related cell-surface molecules with differential expression by leukocytes (L-selectin), platelets (P-selectin), and vascular endothelium (E- and P-selectin). The forms, functions and some clinical applications of L-selectins have been discussed in previous chapter (Chap. 26). P-selectin and E-selectin are adhesion receptors for monocytes and neutrophils and expressed by stimulated endothelial cells. P-selectin is stored in Weibel-Palade bodies, and rapidly delivered to plasma membrane upon exocytosis of these secretory granules. E-selectin is not stored, and its synthesis is induced by cytokines. The fate of the two proteins has been studied after their surface expression by following the intracellular routing of internalized antibodies to the selectins. After a brief surface exposure, internalized E-selectin is degraded in the lysosomes, whereas P-selectin returns to the storage granules from where it can be reused. E-selectin and P-selectin are two closely related vascular cell adhesion proteins. P-selectin is a 140-kDa glycoprotein expressed on endothelial cells and platelets and expressed on the surface of these cells in response to inflammatory stimuli. P-selectin mediates the tethering and rolling of leukocytes along the endothelium during early step of leukocyte extravasation. Each selectin has an amino-terminal C-type lectin domain that possesses carbohydrate binding site, which recognizes certain vascular mucin-type glycoproteins bearing the carbohydrate structure sialyl-Lewis^x (sLe^x or CD15s) (Neu5Acα2-3Galβ1-4(Fucα1-3) GlcNAc). In addition to sLex carbohydrate, P-selectin binds sulfated proteoglycan, 3-sulfated galactosyl ceramide (sulfatide), and heparin. The clinical prognosis and metastatic progression of many epithelial carcinomas has been correlated independently with production of tumor mucins and with enhanced expression of sialyl-Lewis^x.

P-selectin is also expressed on macrophages in the arterial wall after carotid denudation injury and spontaneous atherosclerosis in atherosclerosis-prone apoE-deficient (apoE$^{-/-}$) mice. Furthermore, P-selectin mRNA expression

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was readily detectable in macrophage-rich plaques of atherosclerotic innominate arteries and blood monocyte-derived macrophages from apoE$^{-/-}$ mice. Thus, macrophages in carotid injury-induced neointimal lesions and spontaneous atherosclerotic plaques of the innominate artery acquire the ability to express P-selectin, as does regenerating endothelium. These findings provide a potential new paradigm in macrophage-mediated vascular inflammation, atherosclerosis, and neointimal hyperplasia after arterial injury (Li et al. 2005) (Fig. 27.1).

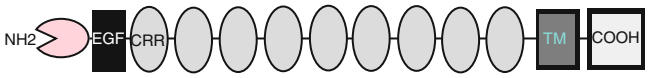

Fig. 27.1 Domain organization of selectin-P (CD62) also called granule membrane protein 140 (GMP-140). The extracellular portion of P-selectin contains an amino terminal domain homologous to C-type lectins and an adjacent epidermal growth factor (*EGF*)-like domain. The single EGF element that is found in P-selectin is followed by nine repetitive elements, each ~60 amino acids long, which resemble protein motives found in complement regulatory repeats (*CRR*) and a transmembrane (*TM*) sequence. A short cytoplasmic sequence is at the carboxyl terminus (*COOH*) of P-selectin. The number of amino acids present in the mature P-selectin as deduced from the cDNA sequences is 789

27.2.2 Human Granule Membrane Protein 140 (GMP-140)/P-Selectin

A structurally and functionally related group of genes, lymph node homing receptor (LHR), granule membrane protein 140 (GMP-140), and endothelial leukocyte adhesion molecule 1 (ELAM-1) constitute a gene cluster on mouse and human chromosome 1. GMP-140 is an inducible granule membrane protein of the selectin family of cell surface receptors that mediate interactions of leukocytes with the blood vessel wall. After cellular activation, it is rapidly redistributed to the plasma membrane. The cDNA-derived primary structure of GMP-140 predicted a cysteine-rich protein with multiple domains, including a "lectin" region, an "EGF:" domain, nine tandem consensus repeats related to those in complement-binding proteins, a transmembrane domain, and a short cytoplasmic tail. Some cDNAs also predicted a soluble protein with a deleted transmembrane segment. The domain organization of GMP-140 is similar to that of ELAM-1, which binds neutrophils. This similarity suggests that GMP-140 belongs to a new family of inducible receptors with related structure and function on vascular cells. In situ hybridization mapped GMP-140 to human chromosome 1 bands 21–24 consistent with chromosomal localization of LHR. Gene linkage analysis in the mouse indicated that these genes and serum coagulation factor V (FV) all map to a region of distal mouse chromosome 1 that is syntenic with human chromosome 1, with no crossovers identified between these four genes in 428 meiotic events. Moreover, long range restriction site mapping demonstrated that these genes map to within 300 kb in both the human and mouse genomes. Studies suggest that LHR, ELAM-1, and GMP-140 comprise the selectins that arose by multiple gene duplication events before divergence of mouse and human. The location of these genes on mouse and human chromosome 1 is consistent with a close evolutionary relationship to the complement receptor-related genes, which also are positioned on the same chromosomes in both species and with which these genes share a region of sequence homology (Johnston et al. 1989; Watson et al. 1990).

Two variant cDNAs for GMP-140 have been identified, one predicting a soluble form of the molecule lacking the transmembrane domain and the other predicting a molecule containing eight instead of nine consensus repeats. The human gene encoding GMP-140, which spans over 50 kbp, contains 17 exons. Almost all exons encode distinct structural domains, including the lectin-like domain, the EGF-like domain, each of the nine consensus repeats, and the transmembrane region. Each of the two deletions found in the variant cDNAs is precisely encoded by an exon, suggesting that these forms of GMP-140 are derived from alternative splicing of mRNA. Transcripts encoding the putative soluble form of GMP-140 can be amplified from both platelet and endothelial cell RNA by PCR (Johnston et al. 1990).

27.2.3 Murine P-Selectin

P-selectin contains a NH2-terminal carbohydrate-recognition domain, an epidermal growth factor motif, nine consensus repeats, a transmembrane domain, and a cytoplasmic tail. E-selectin (ELAM-1) and P-selectin (PADGEM) have both been described as human endothelial cell adhesion molecules for neutrophils and monocytes. The molecular cloning and sequencing of murine cDNA for P-selectin revealed the presence of lectin, EGF-like, transmembrane, and cytoplasmic domains, which are highly conserved between mouse and human, with an overall amino acid identity of 79% (Sanders et al. 1992; Weller et al. 1992). cDNA clones covering the full-length coding sequence of the homologous mouse proteins for both endothelial selectins were isolated by Weller et al. (1992). Rat P-selectin cDNA shows a significant nucleotide and amino-acid identity with human and mouse P-selectin. Similar to mouse P-selectin, the rat sequence lacks the equivalent of human complement regulatory protein-like repeat 2 (CR2). Seven potential N-linked glycosylation sites are conserved between the three species, suggesting that carbohydrate modification

may play an important role in P-selectin function. P-selectin mRNA is undetectable in tissues of vehicle-treated animals.

Mouse P-selectin, like E-selectin, is transiently induced by TNF-α in several endothelioma cell lines derived from mouse tissues. The TNF induced, newly synthesized P-selectin protein is detected on the cell surface. Thus, P-selectin can be regulated on two different levels. The transport of stored P-selectin to the cell surface is controlled by regulated secretion of storage granules. Cell surface appearance of E an d P selectins on human umbilical vein endothelial cells (HUVEC) can be regulated by different mechanisms: E-selectin is transcriptionally induced, within hours, by TNF-α while P-selectin is transported from storage granules to the plasma membrane within minutes upon induction by various stimulating agents. After injection of endotoxin, the cellular response in vivo includes a rapid increase in the level of mRNA, presumably for new synthesis of P-selectin. After administration of endotoxin, the highest levels of mRNA expression were detected in liver, lung, kidney, thymus, spleen, and small intestine and heart (Sanders et al. 1992; Auchampach et al. 1994).

27.2.3.1 Rabbit P-Selectin

In rabbit platelets, P-selectin is an alpha-granule membrane protein that mediates leukocyte adhesion and thrombus propagation. The sequences of tryptic peptides of rabbit P-selectin showed an overall sequence identity of 74% with human P-selectin, and 69–77% identity with cow, dog, mouse, rat and sheep P-selectins. The apparent molecular mass of reduced rabbit P-selectin is 117 + 7 kDa which is approximately 8 kDa larger than the unreduced protein (109 + 5 kDa). Rabbit P-selectin appears smaller than human P-selectin, but is comparable to other species P-selectins, that have fewer 'complement regulatory protein' repeat domains. Rabbit P-selectin is nearly absent from the surface of platelets (290 + 30 molecules/cell). However, cellular activation with thrombin causes nearly a 30-fold increase in expression to 14,200 + 1,100 molecules/cell. P-selectin is expressed on the surface of rabbit platelets activated by other agonists like ADP, A23817 and epinephrine. P-selectin in rabbit platelets is similar in structure, cell localization and expression to human and other species P-selectins (Reed et al. 1998).

27.2.4 P-Selectin Promoter

27.2.4.1 Multiple Initiation Sites in 5′-Flanking Region of P-Selectin Gene

The 5′-flanking region of the human P-selectin gene shows multiple transcriptional initiation sites from -95 to -25 nucleotides relative to the start of protein-coding sequence. P-selectin gene is regulated by a combination of cis elements and their cognate transcription factors. Transfection experiments indicated that the sequence from -249 to -13 was sufficient to promote high level gene expression. Deletions to -197, -147, and -128 gradually reduced expression to basal levels, and further deletion to -100 abolished expression. The putative regulatory elements in short 5′-flanking sequence included a CACCC sequence, two inverted repeats similar to binding sites for the Ets and NF-kB/rel families, a GATA motif, and a sequence related to the GT-IIC element of the SV40 enhancer. The GATA element was functional, as it bound recombinant GATA-2 (Pan and McEver 1993).

27.2.4.2 Species-Specific Mechanisms for Transcriptional Regulation in Endothelial Cells

The 5′-flanking region of murine P-selectin gene was compared with the human gene. The murine and human genes shared conserved Stat-like, Hox, Ets, GATA, and GT-IIC elements. In the murine gene, a conserved GATA element bound to GATA-2 and functioned as a positive regulatory element, whereas a conserved Ets element bound to GA-binding protein and functioned as a negative regulatory element. However, the murine P-selectin gene had several features not found in the human gene. These included an insertion from -987 to -649 that contained tandem GATA and tandem AP1-like sequences, which resembled enhancers in β-globin locus control regions. Both tandem elements bound specifically to nuclear proteins. The murine gene lacked the unique κB site specific for p50 or p52 homodimers found in the human gene. Instead, it contained two tandem κB elements and a variant activating transcription factor/cAMP response element site, which closely resembled sites in the E-selectin gene that are required for TNF-α or LPS-inducible expression. Deletional analysis of the murine 5′-flanking region revealed several sequences that were required for either constitutive or inducible expression (Pan et al. 1998a).

27.2.4.3 kB Site in P-Selectin Promoter

Pan and McEver (1995) identified and characterized a kB site (-218GGGGGTGACCCC-207) in the promoter of the human P-selectin gene. The κB site is unique in that it binds constitutive nuclear protein complexes containing p50 or p52, but not inducible nuclear protein complexes containing p65. Furthermore, the element bound recombinant p50 or p52 homodimers, but not p65 homodimers. The p50 or p52 homodimers contacted the guanines at positions -218 to -214 on the coding strand and at -210 to -207 on the noncoding strand. Changes in the three central residues at -213 to -211 altered binding specificity for members of the NF-kB/Rel family. Data suggest that Bcl-3 differentially regulates the effects of p50 and p52 homodimers bound to kB site of the

P-selectin promoter. This site may be a prototype for kB elements in other genes that bind specifically to p50 and/or p52 homodimers.

kB Sites and an ATF/CREB for TNF-α- or LPS-Induced Expression of Murine P-Selectin Gene: The ATF-a_o cDNA, a variant of the ATF/CREB transcription factor family contains a large in-frame deletion of 525 bp that removes the P/S/T-rich putative transactivation domain and binds E-selectin promoter, NF-ELAM1/δ A. The putative mRNAs for ATF-a_o and ATF-a are present at varying ratios in different tissues. ATF-a_o is an important member of the ATF family with a negative regulatory role in transactivation (Pescini et al. 1994). The TNF-α and LPS augment the expression of a reporter gene driven by murine, but not the human P-selectin promoter in transfected endothelial cells. The regions from -593 to -474 and from -229 to -13 in the murine P-selectin promoter are required for TNF-α or LPS to stimulate reporter gene expression. Within these regions, there are two tandem kB elements, a reverse-oriented kB site and a variant ATF/cAMP response element (ATF/CRE), that participate in TNF-α or LPS-induced expression. The tandem kB elements bound to NF-kB heterodimers and p65 homodimers, the reverse-oriented kB site bound to p65 homodimers, and the variant ATF/CRE bound to nuclear proteins that included ATF-2. Co-overexpression of p50 and p65 enhanced murine P-selectin promoter activity in a kB site-dependent manner. Results indicated that kB sites and the variant ATF/CRE are required for TNF-α and LPS to optimally induce the expression of murine P-selectin gene. The presence of these elements in the murine, but not the human, P-selectin gene may explain in part why TNF-α or LPS stimulates transcription of P-selectin in a species-specific manner. Thus, both species-specific and conserved mechanisms regulate transcription of the human and murine P-selectin genes (Pan et al. 1998b).

Interference of Sp1 and NF-kB: Gene activation by NF-kB/Rel transcription factors is modulated by synergistic or antagonistic interactions with other promoter-bound transcription factors. For example, Sp1 sites are often found in NF-kB-regulated genes, and Sp1 can activate certain promoters in synergism with NF-kB through nonoverlapping binding sites. It was found that Sp1 acts directly through a subset of NF-kB binding GC box sites. In contrast, NF-kB does not bind to a GC box Sp1 site. Sp1 can activate transcription through immunoglobulin k-chain enhancer or P-selectin promoter NF-kB sites. The p50 homodimers replace Sp1 from the P-selectin promoter by binding site competition and thereby either inhibit basal Sp1-driven expression or, in concert with Bcl-3, stimulate expression. The interaction of Sp1 with NF-kB sites thus provides a means to keep an elevated basal expression of NF-kB-dependent genes in the absence of activated nuclear NF-kB/Rel (Hirano et al. 1998).

27.2.4.4 Stat6 Activation Is Essential for IL-4 Induction of P-Selectin Transcription

Agonists of P-selectin expression fall into two categories: those that induce a very rapid, transient increase, lasting only hours, and those that induce prolonged upregulation lasting days. The latter group includes IL-4 that is a mediator of chronic P-selectin upregulation. The increase in P-selectin expression induced by IL-4 results from increased transcriptional activation of the P-selectin gene. Khew-Goodall et al. (1999) demonstrated the existence of two functional signal transducers and activator of transcription 6 (Stat6) binding sites on the P-selectin; binding of at least one site at activated Stat6 is essential for IL-4-induction in P-selectin transcription. Site 1 (nt -142) bound Stat6 with a higher affinity than did site 2 (nt -229). IL-4 also induced prolonged activation of Stat6, which was contingent on the continuous presence of IL-4. The sustained activation of Stat6 induced by IL-4 is likely to be a key factor leading to the prolonged activation of the P-selectin promoter, thereby resulting in prolonged P-selectin upregulation.

27.2.4.5 c-GMP Dependent Down-Regulation of P-Selectin Expression

The NO receptor, soluble guanylate cyclase (sGC), is expressed in endothelial cells. Fluorescence-activated cell sorting analysis in vitro and a specific P-selectin antibody in vivo revealed that selective down-regulation of P-selectin expression accounted the anti-adhesive effects of sGC activation. Thus, sGC plays a key anti-inflammatory role by inhibiting P-selectin expression and leukocyte recruitment (Ahluwalia et al. 2004).

27.2.5 Structure-Function Studies

27.2.5.1 Amino Acid Residue in E- and P-Selectin EGF Domains and Carbohydrate Specificity

Both E- and P-selectin have an EGF domain that is immediately adjacent to C-terminal lectin domain. The mutagenic substitution of single amino acid residues in either the P- or E-selectin EGF domain can dramatically alter selectin binding to sLe^x, heparin, or sulfatide. Substitution of E- and P-selectin EGF domain residue Ser128 with an arginine results in E- and P-selectin proteins that have lost the requirement for α1-3-linked fucose and are thus able to bind to sialyllactosamine (Graves et al. 1994). Additionally, conservative substitution of EGF domain residues 124 and 128 can alter E-selectin binding such that it is able to adhere to heparin or sulfatide and reduce P-selectin adherence to

these ligands. The distance between the substituted EGF domain amino acid residues and the primary carbohydrate binding site within the lectin domain and their relative positioning as determined by crystal structure analysis of the E-selectin lectin and EGF domains suggest that there is little direct contact between the two domains. Selectin oligosaccharide binding may be modulated by both domains and that wild-type E- and P-selectin/sLe^x binding interactions may be significantly different from those previously hypothesized (Revelle et al. 1996a).

27.2.5.2 Monomeric Soluble- and Membrane P-Selectins

Ushiyama et al. (1993) expressed two soluble forms of P-selectin, one truncated after the ninth repeat (tPS) and the other lacking the transmembrane domain due to alternative RNA splicing (asPS). When visualized by electron microscopy, each was a monomeric rod-like structure with a globular domain at one end, whereas membrane P-selectin (mPS) from platelets formed rosettes with the globular domains facing outward. While tPS and asPS are asymmetric monomers, mPS is oligomeric. HL-60 cells adhered to immobilized tPS and asPS, although less efficiently than to mPS. 125I-Labeled tPS and asPS bound to approximately 25,000 sites/neutrophil and approximately 36,000 sites/HL-60 cell with an apparent K_D of 70 nM. Treatment of HL-60 cells with O-sialoglycoprotease eliminated the binding sites for asPS. Thus; (1) P-selectin is a rigid, asymmetric protein; (2) monomeric soluble P-selectin binds to high affinity ligands with sialylated O-linked oligosaccharides on leukocytes; and (3) oligomerization of mPS enhances its avidity for leukocytes (Ushiyama et al. 1993).

27.2.5.3 Cytoplasmic Domain of P-Selectin

The cytoplasmic domains of many membrane proteins have short sequences, usually including a tyrosine or a di-leucine, that function as sorting signals. The cytoplasmic domain of P-selectin was sufficient to confer rapid turnover on LDL-R. The 35-residue cytoplasmic domain of P-selectin contains signals for sorting into regulated secretory granules, for endocytosis, and for movement from endosomes to lysosomes. The domain has a membrane-distal sequence, YGVFTNAAF that resembles some tyrosine-based signals. Deletion of 10 amino acids from the cytoplasmic domain of P-selectin implicates this sequence as a necessary element of a lysosomal targeting signal. Sorting of P-selectin away from efficiently recycled proteins occurs in endosomes. This sorting event represents a constitutive equivalent of receptor down regulation, and may function to regulate the expression of P-selectin at the surface of activated endothelial cells (Green et al. 1994). However, mutations and deletions in the putative tyrosine-based motif in the cytoplasmic tail of human P-selectin did not clearly implicate these residues as critical components of a short internalization signal in transfected CHO cells, and it was not possible to identify a short internalization signal in the cytoplasmic tail of P-selectin. Residues throughout the cytoplasmic domain, and perhaps the transmembrane sequence to which the domain is attached, affect the efficiency of internalization (Setiadi et al. 1995).

Norcott et al. (1996) reported that P-selectin contains signals that target a chimera composed of horseradish peroxidase (HRP) and P-selectin, to the synaptic-like microves. Mutagenesis of the chimera followed by transient expression showed that at least two different sequences within the carboxy-terminal cytoplasmic tail of P-selectin are necessary, but that neither is sufficient for trafficking to the synaptic-like microvesicles (SLMV). One of these sequences is centred on the 10 amino acids of the membrane-proximal C1 exon that is also implicated in lysosomal targeting. The other sequence needed for trafficking to the SLMV includes the last four amino acids of the protein. Blagoveshchenskaya et al. (1998b) discovered a lysosomal targeting signal, KCPL, located within the C1 domain of the cytoplasmic tail. Alanine substitution of this tetrapeptide reduced lysosomal targeting to the level of a tailless HRP-P-selectin chimera, which was previously found to be deficient in both internalization and delivery to lysosomes. A proline residue within this lysosomal targeting signal makes a major contribution to the efficiency of lysosomal targeting. The sequence KCPL within the cytoplasmic tail of P-selectin is a structural element that mediates sorting from endosomes to lysosomes. A balance of positive and negative signals is required for proper lysosomal sorting of P-selectin. Within the sequence KCPL, Cys-766 plays a major role along with Pro-767, whereas Lys-765 and Leu-768 make no contribution to promoting lysosomal targeting. In addition, HRP-P-selectin chimeras were capable of acylation in vivo with ^{3H}palmitic acid at Cys-766, since no labeling of a chimera in which Cys-766 was replaced with Ala was detected (Blagoveshchenskaya et al. 1998a). The cytoplasmic domain is acylated at Cys766 through a thioester bond. Fatty acid acylation may regulate intracellular trafficking or other functions of P-selectin (Fujimoto et al. 1993).

P-selectin mutants expressed in AtT-20, a murine cell line with secretory granules containing the hormone corticotropin ('ACTH') revealed that wild-type P-selectin and mutants with alanine substitutions at 14 different positions in the cytoplasmic tail were concentrated in the tips of the cellular processes, which contained the majority of corticotropin granules. However, targeting to the cell tips was greatly decreased for Tyr^{777}—>Ala, Tyr^{777}—>Phe, Gly^{778}—>Ala, Phe^{780}—>Ala and Leu^{768}/Asn^{769}—>Ala/Ala mutants. Results indicated that Tyr^{777}, Gly^{778} and Phe^{780} form part of an atypical tyrosine-based motif, which also requires the

presence of Leu768 and/or Asn769 to mediate sorting of P-selectin to secretory granules (Modderman et al. 1998).

Within the cytoplasmic domain of P-selectin, Tyr^{777} is needed for the appearance of P-selectin in immature and mature dense core granules (DCG), as well as for targeting to synaptic-like microvesicles (SLMV). The latter destination also requires additional sequences (Leu^{768} and $^{786}DPSP^{789}$) which are responsible for movement through endosomes en route to the SLMV. Leu^{768} also mediates transfer from early transferrin (Trn)-positive endosomes to the lysosomes; i.e., operates as a lysosomal targeting signal. Together, results are consistent with a model of SLMV biogenesis which involves an endosomal intermediate in neuroendocrine PC12 cells. In addition, the impairment of SLMV or DCG targeting results in a concomitant increase in lysosomal delivery, illustrating the entwined relationships between routes leading to regulated secretory organelles (RSO) and to lysosomes (Blagoveshchenskaya et al. 1999). The addition of the cytoplasmic domain of P-selectin to FIX modifies the cellular fate of the FIX molecule by directing the recombinant protein toward regulated-secretory granules without altering its coagulant activity (Plantier et al. 2003).

27.2.6 P-Selectin-Sialyl Lewis^x Binding Interactions

P-selectin adhesion to leukocytes is mediated by the amino-terminal lectin domain that binds sLe^x carbohydrate (Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc). Using known binding interactions that occur between the rat MBP and its ligand (oligomannose) as a template (Weis et al. 1992), and substituting Ala-77 with lysine, it was observed that P-selectin-carbohydrate binding specificity changed from sLe^x to oligomannose. Results indicated that P-selectin binds sLe^x in a shallow cleft that is similar to the MBP saccharide-binding cleft. Additionally, Lys-113 has been implicated earlier in P-selectin binding to both sLe^x and 3-sulfated galactosylceramide (sulfatide). But Revelle et al. (1996b) demonstrated that Lys-113 is probably not involved in P-selectin binding to either sulfatide or sLe^x. Functionally, it appeared that P-selectin has retained a conserved carbohydrate and calcium coordination site that enables it to bind carbohydrate in a manner that is quite similar to that which has been determined for the rat mannose-binding protein (Revelle et al. 1996b). Several peptides corresponding to amino acid sequences within the lectin domains of selectins inhibit neutrophil (PMN) adhesion to P-selectin. One of the active regions, 109–118 aa contains residues, which are critical for E-selectin binding to sLe^x counter receptor. Peptide sequences which inhibited PMN binding to the fusion proteins were not necessarily those that inhibited fusion protein binding to sLe^x. In addition, various amino acid substitutions could be tolerated at the 111 and 113 positions without altering inhibitory activity. Modeling suggests that structural conformations of peptide analogues could explain the differences in biological activity of peptide analogues compared to mutants of the native protein (Tam et al. 1996).

Compared to weak binding of selectins to sLe^x-like glycans, selectins bind with high-affinity to specific glycoprotein counterreceptors, including PSGL-1. Somers et al. (2000) reported crystal structures of human P- and E-selectin constructs containing the lectin and EGF (LE) domains co-complexed with s-Le^x the crystal structure of P-selectin LE co-complexed with the N-terminal domain of human PSGL-1 modified by both tyrosine sulfation and s-Le^x. These structures reveal differences in how E- and P-selectin bind SLe^x and the molecular basis of the high-affinity interaction between P-selectin and PSGL-1.

27.2.7 Functions of P-Selectin

27.2.7.1 Mediation of Leukocyte and Platelet Rolling on the Vessel Wall

Selectins not only mediate leukocyte rolling, but also platelet rolling on the vessel wall. The functional significance of platelet rolling has not been established. The process could be important for hemostasis leading to firm platelet adhesion at sites of denuded endothelium and/or in inflammation. After activation, platelets may help in leukocyte recruitment as shown by studies of lymphocyte homing to peripheral lymph nodes. Surprisingly, work with the P-selectin mutant mice has also revealed an anti-inflammatory aspect of platelet P-selectin. P-selectin binding to leukocytes promoted the transcellular production of an anti-inflammatory mediator limiting the extent of acute glomeluronephritis. In addition, soluble P-selectin was shown to be shed from both activated platelets and endothelium and there are strong indications that it too could have an attenuating effect on inflammatory disease progression. The selectin-deficient mice demonstrated crucial role of P- and E-selectins in the homing of hematopoietic progenitor cells to the bone marrow. The selectin mutant mice have taught us a great deal about the role of selectins in normal physiology and in pathology. Further studies are needed to explore the regulation of shedding of the selectins and the function of soluble selectins in vivo. Exploring new territories of selectin-mediated interactions may provide a basis for developing

new interventions and treatments for diseases in which the role of selectins has not yet been suspected (Hartwell and Wagner 1999).

27.2.7.2 Migration of T Lymphocyte Progenitors to Thymus

It has been shown that P-selectin is expressed by thymic endothelium and that lymphoid progenitors in bone marrow and thymus bind P-selectin. Parabiosis, competitive thymus reconstitution and short-term homing assays indicated that P-selectin and its ligand PSGL-1 are functionally important components of the thymic homing process. Accordingly, thymi of mice lacking PSGL-1 contained fewer early thymic progenitors and had increased empty niches for prothymocytes compared with wild-type mice. Furthermore, the number of resident thymic progenitors controling thymic expression of P-selectin suggested that regulation of P-selectin expression by a thymic 'niche occupancy sensor' may be used to direct progenitor access (Rossi et al. 2005).

27.2.7.3 Acute Emigration of Neutrophils

Whereas P-selectin is known to be involved in early stages of an inflammatory response, Johnson et al. (1995) indicated that it is additionally responsible for leukocyte rolling and macrophage recruitment in more prolonged tissue injury. P-Selectin also mediates rolling or slowing of neutrophils, while ICAM-1 contributes to the firm adhesion and emigration of neutrophils. Removing the function of either molecule partially prevents neutrophil emigration. P-selectin-deficient mice exhibit peripheral neutrophilia (Mayadas et al. 1993). This is not caused by changes in bone marrow precursors nor by a lack of neutrophil margination. Bullard et al. (1995) generated a line of mice with mutations in both of these molecules. While mice with either mutation alone show a 60–70% reduction in acute neutrophil emigration into the peritoneum during *S. pneumoniae*-induced peritonitis, double mutant mice show a complete loss of neutrophil emigration. In contrast, neutrophil emigration into the alveolar spaces during acute *S. pneumoniae*-induced pneumonia is normal in double mutant mice. These data demonstrate organ-specific differences, since emigration into the peritoneum requires both adhesion molecules while emigration into the lung requires neither. In the peritoneum, P-selectin-independent and ICAM-1-independent adhesive mechanisms permit reduced emigration when one of these molecules is deficient, but P-selectin-independent mechanisms cannot lead to ICAM-1-independent firm adhesion and emigration (Bullard et al. 1995).

27.2.7.4 P-Selectin Mediates Adhesion of Leukocytes, Platelets, and Cancer Cells

The P-selectin has long been known to support leukocyte rolling and emigration at sites of inflammation. Rolling under the hydrodynamic drag forces of blood flow is mediated by the interaction between selectins and their ligands across the leukocyte and endothelial cell surfaces. The high strength of binding combined with force-dependent rate constants and high molecular elasticity are tailored to support physiological leukocyte rolling (Fritz et al. 1998). Multiple-targeted deficiencies in selectins revealed a predominant role for P-selectin in leukocyte recruitment (Robinson et al. 1999). P-selectin is also a key molecule in hemostasis and thrombosis, mediating platelet rolling, generating procoagulant microparticles containing active tissue factor and enhancing fibrin deposition. Elevated levels of plasma P-selectin are indicative of thrombotic disorders and predictive of future cardiovascular events. Because the interaction between P-selectin and its receptor P-selectin glycoprotein ligand-1 (PSGL-1/CD162) represents an important mechanism by which P-selectin induces the formation of procoagulant microparticles and recruits the microparticles to thrombi, anti-thrombotic strategies are currently aimed at inhibiting this interaction. Recent developments also suggest that the procoagulant potential of P-selectin could be used to treat coagulation disorders such as hemophilia A (Cambien and Wagner 2004).

Stimulated endothelial cells and activated platelets express P-selectin, which interacts with PSGL-1 for leukocyte rolling on stimulated endothelial cells and heterotypic aggregation of activated platelets onto leukocytes. Cross-linking of PSGL-1 by P-selectin also primes leukocytes intracellularly for cytokine and chemoattractant-induced β2-integrin activation for firm adhesion of leukocytes. Furthermore, P-selectin mediates heterotypic aggregation of activated platelets to cancer cells and adhesion of cancer cells to stimulated endothelial cells. P-selectin blockade significantly inhibits inflammation and neointimal formation after arterial injury. In atherosclerosis, both platelet and endothelial cell P-selectins are important. Platelet P-selectin expression, but not endothelial P-selectin, plays a crucial role in the development of neointimal formation after arterial injury, and therapeutic strategies targeting leukocyte-platelet interactions could be effective in inhibiting restenosis (Wang et al. 2005). Chen and Geng (2006) provided a comprehensive summary of the functional roles and the biological importance of P-selectin-mediated cell adhesive interactions in the pathogeneses of inflammation, thrombosis, and the growth and metastasis of cancers (Chen and Geng 2006). Crystal structures of lectin and EGF–like domains of P-selectin show 'bent' and 'extended' conformations. An extended conformation would be 'favored' by forces exerted on a selectin bound at one end to a ligand and at the other end to a cell experiencing hydrodynamic drag force. The introduction of an *N*-glycosylation site to 'wedge open' the interface between the lectin and EGF-like domains of P-selectin increased the affinity of

P-selectin for its ligand PSGL-1, and thereby the strength of P-selectin-mediated rolling adhesion. Similarly, an asparagine-to-glycine substitution in the lectin-EGF-like domain interface of L-selectin enhanced rolling adhesion under shear flow. These results demonstrate that force, by 'favoring' an extended selectin conformation, can strengthen selectin-ligand bonds (Phan et al. 2006).

The selectins must resist applied forces to mediate leukocyte tethering and rolling along the endothelium and have 2 conformational states. Selectin–ligand bond dissociation increases only modestly with applied force, and exhibits catch bond behavior in a low-force regime where bond lifetimes counterintuitively increase with increasing force. Both allosteric and sliding–rebinding models have emerged to explain catch bonds. Waldron TT and Springer (2009) suggest a large residue into a cleft that opens within the lectin domain to stabilize the more extended, high-affinity selectin conformation. This mutation stabilizes the high-affinity state, but surprisingly makes rolling less stable. The position of the mutation in the lectin domain provides evidence for an allosteric pathway through the lectin domain, connecting changes at the lectin–EGF interface to the distal binding interface (Fig. 27.2).

Superoxide Anion Release by Monocytes and Neutrophils Through P-Selectin: The leukocytes are functionally modified by their adhesion to activated platelets. The levels of superoxide anion production were found to be markedly elevated when thrombin-activated platelets were used. The extent of this enhancement was much smaller when leukocytes were cultured with resting platelets than activated platelets. The membranes prepared from activated platelets also induced superoxide anion production, but the culture supernatant of activated platelets did not. This indicated that the adhesion of activated platelets to the leukocytes through P-selectin is a crucial step for the activation of leukocyte function, and support the idea that activated platelets are actively involved in inflammation processes (Nagata et al. 1993).

Two Sites on P-Selectin Are Involved in Adhesion of Monocytes to Thrombin-Activated EC: Peptides derived from both the lectin (residues 19–34 and 51–61) and EGF-like (residues 127–139) domains inhibit the adhesion of peripheral blood mononuclear cells (PBMC), elutriated monocytes and a monocytic cell line (U937) to thrombin-activated EC. All three peptides, when conjugated to BSA and coated on plastic plates, mediated U937 cell adhesion. This study shows that two sites on P-selectin, the lectin and EGF-like domains, are involved in the adhesion of monocytes to thrombin-activated EC (Murphy and McGregor 1994).

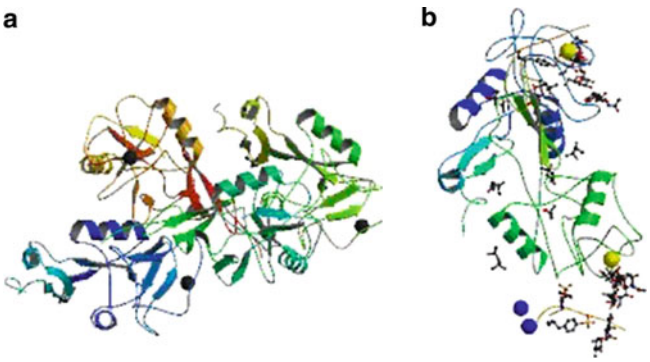

Fig. 27.2 (**a**) Crystal structure of human P-selectin lectin/EGF domains (PDB ID: 1G1Q); (**b**) crystal structure of P-selectin lectin/EGF domains complexed with PSGL-1 peptide (PDB ID:1G1S) (Somers et al. 2000)

27.2.7.5 P-Selectin Regulates MCP-1 and TNF-α Secretion

Adhesion of human monocytes to P-selectin, the most rapidly expressed endothelial tethering factor, increased the secretion of monocyte chemotactic protein-1 (MCP-1) and TNF-α by the leukocytes when they were stimulated with platelet-activating factor (PAF). Tethering by P-selectin specifically enhanced nuclear translocation of NF-kB required for expression of MCP-1, TNF-α, and other immediate-early genes. Results demonstrate that P-selectin, through its ligands on monocytes, may locally regulate cytokine secretion in inflamed tissues (Weyrich et al. 1995). P-selectin plays an important role in PAF-induced injury in mice, and the selectins and the integrin-ICAM-1 system work in concert to mediate the inflammatory response to PAF in vivo (Sun et al. 1997).

27.2.7.6 B Lymphocyte Binding to E- and P-Selectins

Activated but not resting B cells are able to interact with E and P selectins. This binding capacity of activated B cells paralleles the induction of different carbohydrate epitopes (Lewis^x, sialyl-Lewis^x, CD57 and CDw65) as well as other molecules bearing these or related epitopes in myeloid cells (L-selectin, αLβ2 and αXβ2 integrins, and CD35) involved in the interaction of different cell types with selectins. B cells infiltrating inflamed tissues like in Hashimoto's thyroiditis, also express these selectin-binding carbohydrates in parallel with the expression of E-selectin by surrounding follicular dendritic cells. Thus, in addition to the involvement of integrins, E- and P-selectins could play an important role in the interaction of B lymphocytes with the endothelium during B cell extravasation into lymphoid tissues and inflammatory foci as well as in their organization into lymphoid organs (Postigo et al. 1994).

27.2.7.7 Nonrolling Interaction Mediated by P-Selectin

The renal glomerulus is one of the few sites within the microvasculature in which leukocyte recruitment occurs in capillaries. Following infusion of anti-glomerular basement membrane (GBM) Ab, leukocytes became adherent in glomerular capillaries via a process of immediate arrest, without undergoing prior detectable rolling. However, despite the absence of rolling, this recruitment involved nonredundant roles for the P-selectin/P-selectin glycoprotein ligand-1 and β2 integrin/ICAM-1 pathways, suggesting that a novel form of the multistep leukocyte adhesion cascade occurs in these vessels. Perhaps, anti-GBM Ab-induced leukocyte adhesion in glomeruli occurs via a pathway that involvs a nonrolling interaction mediated by platelet-derived P-selectin (Kuligowski et al. 2006).

27.2.7.8 P-Selectin-Dependent, PSGL-1-Independent Rolling

Interestingly, antibodies and pharma-cological inhibitors (e.g., rPSGL-Ig) that target the N-terminus of PSGL-1 reduce but do not abolish P-selectin-dependent leukocyte rolling in vivo whereas PSGL-1-deficient mice have almost no P-selectin-dependent rolling. Therefore, Ridger et al. (2005) investigated mechanisms of P-selectin-dependent, PSGL-1-independent rolling. Data suggest that leukocytes can continue to roll in the absence of optimal P-selectin/PSGL-1 interaction using an alternative mechanism that involves P-selectin-, L-selectin-, and sialyl Le^x-bearing ligands.

27.3 P-Selectin Glycoprotein Ligand-1 (PSGL-1)

27.3.1 PSGL-1: The Major Ligand for P-Selectin

Based on site-directed mutagenesis, blocking monoclonal antibodies, and biochemical analyses, the extreme amino terminal extracellular domain of PSGL-1 is critical for interactions with selectins The current hypothesis is that for high affinity interactions with P-selectin, PSGL-1 must contain O-glycans with a core-2 branched motif containing the s-Lewis X antigen (NeuAc α2—>3Gal β1—>4[Fuc α1—>3]GlcNAc β1—>R). In addition, high affinity interactions require the co-expression of tyrosine sulfate on tyrosine residues near the critical O-glycan structure. This review addresses the biochemical evidence for this hypothesis and the evidence that PSGL-1 is an important in vivo ligand for cell adhesion (Cummings 1999; Moore 1998; Kum et al. 2009).

27.3.1.1 Human (PSGL-1, CD162)

Human P-selectin glycoprotein ligand-1 (PSGL-1, CD162) is expressed on the surface of myeloid cells and serves as the high affinity counter receptor for P-selectin. PSGL-1 is a disulfide-bonded, homodimeric mucin (approximately 250 kDa) on leukocytes that binds to P-selectin on platelets and endothelial cells during the initial steps in inflammation. PSGL-1 is a transmembrane glycoprotein, which is constitutively expressed on leukocytes, binds selectins. It mediates the initial tethering of leukocytes to activated platelets and endothelium. PSGL-1 is an essential adhesive molecule mediating the rolling of leukocytes on the endothelial cells and the recruitment of leukocytes to the inflamed tissue. In addition to its direct role in capture of leukocytes from the blood stream, PSGL-1 also functions as a signal-transducing receptor and initiates a series of intracellular signal events during the activation of leukocytes. PSGL-1 shares common features with platelet glycoprotein Ibα. A recently described polymorphism in this receptor that results in a variable number of tandem repeats (VNTR) sequence present either 16, 15 or 14 times (alleles A, B or C) could, similar to GP Ibα, be functionally relevant. PSGL-1 is a disulfide-bonded, homodimeric mucin (approximately 250 kDa) on leukocytes that binds to P-selectin on platelets and endothelial cells during the initial steps in inflammation. Genes involved in inflammatory processes are candidates for predisposition to prothrombotic syndromes. Moreover, adhesion to E-selectin inhibits the proliferation of human $CD34^+$ cells isolated either from blood, or steady-state bone marrow (Winkler et al. 2004). A dimeric sialoglycoprotein from myeloid cells with subunits of Mr of 120 kDa is selectively recognized by P-selectin. This P-selectin ligand carries α2-3-linked sialic acids and the sialyl-$Lewis^x$ (sialyl Le^x) tetrasaccharide motif and contains < 1% of the total membrane-bound sialic acids and a very small fraction of the total sialyl Le^x on neutrophil membranes. The predominant form of sialic acid on the ligand is N-acetylneuraminic acid. The P-selectin ligand carries large numbers of closely spaced sialylated O-linked oligosaccharides. The 120-kDa ligand is the major determinant of P-selectin: myeloid cell interaction in vivo (Norgard et al. 1993). Platelet PSGL-1 expression is 25–100-fold lower than that of leukocytes. Presence of a functional PSGL-1 on platelets suggests one of the mechanism by which selectins and their ligands participate in inflammatory and/or hemostatic responses (Frenette et al. 2000).

The predicted amino acid sequence of a functional ligand for P-selectin from an HL-60 reveals a novel mucin-like transmembrane protein. Binding of transfected COS cells to P-selectin requires coexpression of both the protein ligand and a fucosyltransferase. This binding is calcium dependent and can be inhibited by mAb to P-selectin. Cotransfected COS cells express the ligand as a homodimer of 220 kDa. A soluble ligand construct, when coexpressed with fucosyltransferase in COS cells, also mediates P-selectin binding and is immunocrossreactive with the

major HL-60glycoprotein that specifically binds P-selectin (Sako et al. 1993). In vitro studies have suggested that PSGL-1 may also be a ligand for E- and L-selectins. However, P-selectin-PSGL-1 interaction alone is sufficient to mediate rolling in vivo and that E-selectin-PSGL-1 interaction supports slow rolling (Norman et al. 2000).

27.3.1.2 The Mouse Homolog of PSGL-1

The *mouse* homolog of PSGL-1, flanking the entire ORF of 397 amino acids is composed of a single exon. Mouse and human PSGL-1 show an overall similarity of 67% and an identity of 50% and contain a similar domain organization. However, there are 10 threonine/serine-rich decameric repeats in mouse PSGL-1 as compared to 15 threonine-rich repeats in human PSGL-1. The mouse PSGL-1 gene, *Selpl,* was mapped to a position on mouse chromosome 5 (Chr 5). Moderate expression of a PSGL-1 mRNA occurs in species in most tissues and high levels of expression in blood, bone marrow, brain, adipose tissue, spleen, and thymus. Whereas certain mouse myeloid cell lines including PU5-1.8, WEHI-3B, and 32 DC13 express high levels of PSGL-1 mRNA, only WEHI-3B and 32 DC13 bind to P-selectin. WEHI-3B cells bind significantly better to P-selectin than to E-selectin. Thus, mouse PSGL-1 has structural and functional homology to human PSGL-1 but is characterized by differences in the composition and number of the decameric repeats. PSGL-1 on mouse myeloid cells is critical for high-affinity binding to P-selectin but not E-selectin (Yang et al. 1996).

27.3.1.3 PSGL-1 in Equine

A high baseline level of P-selectin expression in circulating equine platelets suggests a primed state toward inflammation and thrombosis via P-selectin/PSGL-1 adhesion. Equine PSGL-1 (ePSGL-1) subunit is predicted to be 43 kDa and composed of 420 amino acids with a predicted 18-amino-acid signal sequence showing 78% homology to hPSGL-1. Though earlier work has shown that binding of P-selectin requires sulfation of at least one of three tyrosines and O-glycosylation of one threonine in the N-terminus of human PSGL-1, the corresponding domain in ePSGL-1, spanning residues 19–43, contains only one tyrosine in the vicinity of two threonines at positions 25 and 41. The ePSGL-1 contains 14 threonine/serine-rich decameric repeats as compared to hPSGL-1 which contains 14–16 threonine-rich decameric repeats. The transmembrane and cytoplasmic domains display 91% and 74% homology to corresponding human PSGL-1 domains, respectively. Overall, there is 71% homology between ORF of ePSGL-1 and hPSGL-1. The greatest homologies between species exist in the transmembrane domain and cytoplasmic tail while substantial differences exist in the extra-cellular domain (Xu et al. 2005).

27.3.1.4 Bovine PSGL-1

The ORF of bovine PSGL-1 (bPSGL-1) cDNA is 1,284 bp in length, predicting a protein of 427 amino acids including an 18-amino-acid signal peptide, an extra-cellular region with a mucin-like domain, and transmembrane and cytoplasmic domains. The amino acid sequence of bPSGL-1 demonstrated 52%, 49% and 40% overall homology to equine, human and mouse, respectively. A single extracellular cysteine, at the transmembrane and extracellular domain junction, suggests a disulfide-bonding pattern. Alignment of bovine with equine, human and mouse PSGL-1 demonstrates high conservation of transmembrane and cytoplasmic domains, but diversity of the extracellular domain, especially in the anionic NH_2-terminal of PSGL-1, the putative P-selectin binding domain. In the NH_2-terminal of bPSGL-1, there are three potential tyrosine sulfation sites and three potential threonine O-glycosylation sites, all of which are required for P-selectin binding in human PSGL-1. The bPSGL-1 shares only 57% homology in amino acid sequence with the corresponding epitope region in human PSGL-1 without cross-reactivity with bovine leukocytes (Xu et al. 2006).

27.3.1.5 Eosinophil Versus Neutrophil PSGL-1

Studies have indicated an important role for P-selectin in eosinophil adhesion. Eosinophils bound to twofold more blood vessels within the nasal polyp tissue than neutrophils. The eosinophil P-selectin ligand is a sialylated, homodimeric glycoprotein consistent with the known structure of PSGL-1. However, expression of PSGL-1 by eosinophils was greater than on neutrophils. The eosinophil ligand had a molecular mass of approximately 10 kDa greater than the neutrophil ligand, which was not due to differences in N-glycosylation. Eosinophils expressed the 15-decapeptide repeat form of PSGL-1 compared with neutrophils that have the 16-decapeptide repeat form. The increased binding of eosinophils, compared with neutrophils, to P-selectin in both an ex-vivo and in vitro assay suggests that P-selectin may have a role directing the specific migration of eosinophils in diseases such as asthma (Symon et al. 1996). While the roles of P- and E-selectin in neutrophil recruitment are well established, the mechanisms of L-selectin-mediated neutrophil recruitment remain elusive. One proposal is that tethering is mediated by L-selectin on flowing neutrophils interacting with PSGL-1 on adherent neutrophils. To clarify this point, Shigeta et al. (2008) examined the impact of L-selectin deficiency in PSGL-1-deficient mice. In L-selectin and PSGL-1 double-knockout mice, Shigeta et al. (2008) provided evidence for the existence of another L-selectin ligand distinct from PSGL-1 in inflammation and indicated that such a ligand is expressed on endothelial cells, that promotes neutrophil rolling in vivo.

27.3.2 Genomic Organization

27.3.2.1 The PSGL-1Gene

The PSGL-1 gene from human placenta contains a single intron of approximately 9 kb in the 5′-untranslated region; the complete coding region resides in exon 2. The genomic clone differs from the cDNA clone isolated from HL-60 cells in that it encodes an extra copy of the decameric repeat located in the extracellular domain of PSGL-1. Further analysis indicated that the PSGL-1 genes of HL-60 and U-937 cells contain 15 repeats, whereas the PSGL-1 genes of polymorphonuclear leukocytes, monocytes, and several other cell lines contain 16 repeats. There was no functional difference among these two variants of PSGL-1. The organization of the PSGL-1 gene closely resembles those of CD43 and human platelet glycoprotein GPIb α, both of which have an intron in the 5′-noncoding region, a long second exon containing the complete coding region, and TATA-lesspromoters. The gene for human PSGL-1, designated SELPLG, was mapped to chromosome 12q24 (Veldman et al. 1995).

27.3.3 Specificity of PSGL-1 as Ligand for P-Selectin

P-, L, and E- Selectins Have Overlapping Leukocyte Ligand Specificities: Although, L-selectin binds multiple ligands expressed on endothelial cells and P-selectin is proposed to interact exclusively with PSGL-1 on leukocytes, one study showed that L-selectin binds leukocytes through PSGL-1, although at lower levels than P-selectin. L-selectin binding to PSGL-1 appeared to be specific since it was blocked by Abs to L-selectin or PSGL-1, and required appropriate glycosylation of PSGL-1. The lectin and EGF domains of L- and P-selectin contributed significantly to binding through similar, if not identical, regions of PSGL-1. The different chimeric selectins revealed that the lectin domain was the dominant determinant for ligand binding, while cooperative interactions between the lectin, EGF, and short consensus repeat domains of the selectins also modified ligand binding specificity. It seems that L-selectin binding to PSGL-1 expressed by leukocytes may mediate neutrophil rolling on stationary leukocytes bound to cytokine-induced endothelial cells, which is a L-selectin-dependent process (Tu et al. 1996). The proposed ligands for P- and E-selectin receptors contain the Le^x core and sialic acid. Since both E-selectin and P-selectin bind to sialylated Lex, Larsen et al. (1992) evaluated whether E-selectin and P-selectin recognize the same counter-receptor on leukocytes. Although sialic acid and Le^x are components of the P-selectin ligand and the E-selectin ligand, results indicated that the ligands are related, having overlapping specificities, but are structurally distinct. A protein component containing sialyl Le^x in proximity to sialyl-2,6 βGal structures on the P-selectin ligand may contribute to its specificity for P-selectin.

27.3.4 Structural Polymorphism in PSGL-1 and CAD Risk

27.3.4.1 PSGL-1 Is Highly Polymorphic and Shows Structural Polymorphism

P-selectin and P-selectin glycoprotein ligand constitutes a receptor/ligand complex that is likely to be involved in the development of atherosclerosis and its complications. PSGL-1 is highly polymorphic and contains a structural polymorphism that potentially indicates functional variation in the human population (Afshar-Kharghan et al. 2001; Tregouet et al. 2003). The functional relevance of a variable number of tandem repeats polymorphism affecting the PSGL-1 has been demonstrated. Platelet glycoprotein (GP) Ibα and leukocyte PSGL-1 are membrane mucins with a number of structural and functional similarities. Like GP Ibα, PSGL-1 is affected by a variable number of tandem repeat polymorphism in its mucin-like region. By PCR amplification of the genomic region encoding the PSGL-1 repeats, three allelic variants were identified in the human population. The three alleles-A, B, and C-from largest to smallest, contained 16, 15, and 14 decameric repeats, respectively, with the B variant lacking repeat 2 and the C variant retaining repeat 2 but lacking repeats 9 and 10. Allele frequencies were highest for the A variant and lowest for the C variant in the two populations studied (frequencies of 0.81, 0.17, and 0.02 in white persons and 0.65, 0.35, and 0.00 in Japanese). The coding and regulatory sequences of the PSGL-1 showed nine polymorphisms. The identified polymorphisms were genotyped in the AtheroGene study. Haplotype analysis revealed that two polymorphisms of PSGL-1, the M62I and the VNTR, independently influence plasma PSGL-1 levels. Conversely, haplotypes of PSGL-1 are not associated with CAD risk (Tregouet et al. 2003).

Hancer et al. (2005) determined the allele and genotype frequencies of polymorphisms GP Ibα Kozak and PSGL-1 in the Turkish population. Allele frequencies of T and C were calculated to be 0.873 and 0.127 for the GPIbα Kozak polymorphism and no significant difference was found between Turkish and French populations. In contrast, the difference between Turkish and Japanese population was highly significant. In the PSGL-1 group, allele frequencies of A, B and C were calculated as 0.818, 0.160, 0.022 respectively. Thus, for the PSGL-1, although the difference between Turkish and French populations was not significant, the difference between the Turkish and Japanese was extremely significant. A Turkish population database has

been established for these gene polymorphisms (Hancer et al. 2005).

Short Alleles Protect Against Premature Myocardial Infarction: Neutrophils, carrying short alleles, exhibit a significantly lower capacity to bind activated platelets. These alleles consistently protect against transient ischemic attack, probably because of their lesser adhesive capacity. The role of this polymorphism in premature myocardial infarction was evaluated for the fact that genetic risk factors are more relevant in the development of disease in young patients. Hence, Roldan et al. (2004) genotyped 219 Caucasian patients who had suffered a premature myocardial infarction (MI) and 594 control subjects from Mediterranean area. The frequency of the short alleles (B and C) was significantly lower in patients than in controls. Multiple regression analysis revealed that B and C alleles had an independent protective effect on the development of premature MI.

In Cerebro-Vascular Disease, CHD, and Venous Thrombosis: The variable number of tandem repeat (VNTR) polymorphism in the PSGL-1 gene has been associated with ischemic cerebro-vascular disease (CVD) and with CHD. A recently described polymorphism in this receptor that results in VNTR sequence present 16, 15 or 14 times (alleles A, B or C) could, similar to GPIbα be functionally relevant. The allelic frequency of this polymorphism in 469 individuals from the south of Spain, was similar to that described in other Caucasian populations: 85% A, 14% B and 1% C alleles. Lozano et al. (2001) identified two new polymorphisms genetically linked to the C isoform, resulting in the Ser273Phe and Met274Val changes. Neutrophils carrying the shortest C allele and the amino acid variations in residues 273 and 274 exhibited a significantly lower capacity to bind activated platelets than A/B and A/A samples. The distribution of VNTR was analyzed in three case-control studies including 104 patients of CVD, 101 patients of CHD and 150 deep venous thrombosis (DVT) patients. Smaller (B and C) alleles seemed to be associated with a lower risk of developing CVD but not to be related to CHD or DVT (Lozano et al. 2001).

In another study involving low number of CHD patients (281) and 397 healthy blood donors, the prevalence of homozygous carriers of the PSGL-1 VNTR allele with 15 repeat units was significantly higher in the CHD patients (5.3% vs. 1.5%) than in controls, suggesting an effect of this marker in CHD. But genotyping, performed in a larger sample size including 2,578 CHD patients, 731 patients without CHD, and 1,084 healthy blood donors, failed to confirm the putative role of PSGL-1 VNTR polymorphism in CHD. Frequencies of the PSGL-1 VNTR 15 repeats for homozygous carriers were 2.2% in healthy blood donors, 2.3% in patients without CHD and 2.7%, in CHD cases, respectively. Based on these results, the PSGL-1 VNTR polymorphism is not a genetic risk factor for CHD (Bugert et al. 2003). Thus, polymorphisms of the PSGL-1 may influence the neutrophil-platelet binding, and may represent a risk factor for CVD

Multiple Sclerosis and PSGL-1: PSGL-1 represents a crucial step in the pathogenesis of multiple sclerosis (MS). Three hundred twenty-one MS patients and 342 controls were genotyped for the presence of a polymorphism in the PSGL-1 gene, consisting of a VNTR originating three alleles: A, B and C, in order to test whether they influence the susceptibility and the course of the disease. No significant differences among allelic frequencies of A, B and C alleles in MS as compared with controls were observed. However, the C allele of the VNTR polymorphism in PSGL-1 is likely to be associated with PP-MS. As this allele has been demonstrated to have a very low efficiency in mediating lymphocyte binding to brain endothelium during attacks, its high frequency in PP-MS could be related to the absence of exacerbations in such patients (Scalabrini et al. 2005).

27.3.5 Carbohydrate Structures on PSGL-1

27.3.5.1 Primitive Human Hematopoietic Progenitors Adhere to P-Selectin

P-selectin binds human hematopoietic progenitors (colony-forming unit-granulocyte-macrophage [CFU-GM] and burst-forming unit-erythroid [BFU-E]) as identified by their expression of CD34 antigen. In addition, P-selectin binds all precursors (pre-CFU) of committed myeloid progenitors. This suggested that PSGL-1 comprises a P-selectin ligand expressed by primitive hematopoietic cells, but did not preclude the existence of additional P-selectin ligands on these cells. It has been proposed that only covalently dimerized PSGL-1 can bind P-selectin. Epperson et al. (2000) examined whether covalent dimers of PSGL-1 are required for binding to P-selectin. Recombinant forms of PSGL-1, in which the single extracellular Cys (Cys-320) was replaced with either Ser (C320S-PSGL-1) or Ala (C320A-PSGL-1), suggested that Cys(320)-dependent dimerization of PSGL-1 is not required for binding to P-selectin and that a monomeric fragment of PSGL-1 is sufficient for P-selectin recognition (Epperson et al. 2000).

27.3.5.2 Structure Containing GalNAc-Lewis^x and Neu5Acα2-3Galβ1-3GalNAc Sequences on PSGL-1

The PSGL-1-P-selectin interaction is calcium dependent and requires presentation of sialyl-Le^x -type structures on the

O-linked glycans of PSGL-1. The selectins interact under normal and pathological situations with certain sialylated, fucosylated glycoconjugate ligands containing sialyl-Le^x (Neu5Acα2-3Galβ1-4(Fucα1-3)GlcN Ac). Much effort has gone into the synthesis of sialylated and sulfated Le^x analogs as competitive ligands for the selectins. Other studies have shown that sulfate esters can replace sialic acid in some selectin ligands (Yuen et al. 1992; Imai et al. 1993). Since the natural selectin ligands GlyCAM-1 and PSGL-1 carry sialyl-Le^x as part of a branched Core 2 O-linked structure, Koenig et al. (1997) synthesized Galβ1-4(Fucα1-3) GlcNAcβ1-6(SE-3Galβ1^{+++}-3)GalNAc1αOMe and found it to be a moderately superior ligand for L and P-selectin. Jain et al. (1998) synthesized Galβ1-4(Fucα1-3)GlcNAcβ1-6 (Neu5Acα2^{+++} 3Galβ1-3)-GalNAc α1-OB, which was found to be 2- to 3-fold better than sialyl Le^x for P and L selectin, respectively. The unusual structure GalNAcβ1-4(Fucα1-3)GlcNAcβ1-OMe (GalNAc-Le^x-O-methyl glycoside) also proved to be a better inhibitor of L- and P-selectin than sialyl-Le^x-OMe. In view of Core 2 branched structures, Jain et al. (1998) synthesized a molecule that is 5- to 6-fold better at inhibiting L- and P-selectin than sialyl-Le^x-OMe. By contrast to un-branched structures, substitution of a sulfate ester group for a sialic acid residue in such a molecule resulted in a considerable loss of inhibition ability. Thus, the combination of a sialic acid residue on the primary (β1-3) arm, and a modified Le^x unit on the branched (β1-6) arm on an O-linked Core 2 structure generated a monovalent synthetic oliogosaccharide inhibitor superior to Sialyl-Le^x for both L- and P-selectin (Jain et al. 1998). The density of α-Gal epitopes on PSGL-1 is dependent on the expression of O-linked glycans with core 2 structures and lactosamine extensions. Hence, the structural complexity of the terminal Gal-Gal expressing O-glycans with both neutral as well as sialic acid-containing structures is likely to contribute to the high adsorption efficacy (Liu et al. 2005). Moore et al. (1994) suggested that PSGL-1 from human neutrophils displays complex, sialylated, and fucosylated O-linked poly-N-acetyllactosamine that promote high affinity binding to P-selectin, but not to E-selectin.

27.3.5.3 Carbohydrate and Non-Carbohydrate Moieties of PSGL-1 in Binding P-Selectin

In order to define the modifications required for PSGL-1 to bind P- and E-selectin, CHO cells were transfected with cDNAs for PSGL-1 and specific glycosyltransferases. CHO cells synthesized only core 1 O-linked glycans (Galβ1-3GalNAc α1-Se r/Thr), which lacked core 2 O-linked glycans (Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAc α1 -Ser/Thr). PSGL-1 expressed on transfected CHO cells bound P- and E-selectin only when it was co-expressed with both C2GnT and a α1,3 fucosyltransferase (Fuc-TIII, Fuc-TIV, or Fuc-TVII). Results indicated that PSGL-1 requires core 2 O-linked glycans that are sialylated and fucosylated to bind P- and E-selectin. PSGL-1 also requires tyrosine sulfate to bind P-selectin but not E-selectin (Li et al. 1996). Sako et al. (1995) reported the identification of a non-carbohydrate component of the binding determinant that is critical for high affinity binding to P-selectin. Located within the first 19 amino acids, this anionic polypeptide segment contains at least one sulfated tyrosine residue. This sulfotyrosine-containing segment of PSGL-1, in conjunction with sLex presented on O-linked glycans, constitutes the high affinity P-selectin-binding site (Sako et al. 1995).

27.3.6 Role of PSGL-1

Selectin-dependent rolling is the earliest observable event in the recruitment of leukocytes to inflamed tissues. Several glycoproteins decorated with sialic acid, fucose, and/or sulfate, have been shown to bind the selectins. The best-characterized selectin ligand is PSGL-1 that supports P-selectin- dependent rolling in vitro and in vivo.

27.3.6.1 PSGL-1 Mediates Rolling of Neutrophils on P-Selectin

Neutrophils roll on P-selectin expressed by activated platelets or endothelial cells under the shear stresses in the microcirculation. PSGL-1 accounts for the high affinity binding sites for P-selectin on leukocytes, and it must interact with P-selectin for neutrophils to roll on P-selectin at physiological shear stresses (Moore et al. 1995). Activated T cells regulate inflammatory diseases in the intestinal tract. P-selectin and PSGL-1 are dominating molecules in supporting adhesive interactions of CD8 T cells in inflamed colonic venules and may be useful targets to protect against pathological inflammation in the large bowel (Asaduzzaman et al. 2009). Human PSGL-1 interacts with CCL27, a skin-associated chemokine that attracts skin-homing T lymphocytes. Hirata et al. (2004) suggest a role for PSGL-1 in regulating chemokine-mediated responses, in addition to its role as a selectin ligand. The aspartyl protease BACE1 cleaves the amyloid precursor protein and the sialyltransferase ST6Gal I and is important in the pathogenesis of Alzheimer's disease. It was shown that BACE1 acts on PSGL-1, which mediates leukocyte adhesion in inflammatory reactions and that PSGL-1 is an additional substrate for BACE1 (Lichtenthaler et al. 2003).

Memory T cells expressing Cutaneous lymphocyte-associated antigen (CLA) occur in humans and accumulate in normal and inflamed skin. These cells uniformly bind to E-selectin, yet only a subset binds to P-selectin. Findings indicate that unlike memory CLA^+ $CD4^+$ T cells, when activated these cells can broadly bind to P-selectin,

suggesting a more diverse tissue trafficking capacity (Ni and Walcheck 2009).

27.3.6.2 Interaction of ERM Proteins with Syk Mediates Signaling by PSGL-1

In addition to its direct role in capture of leukocytes from the bloodstream, PSGL-1 also functions as a signal-transducing receptor and initiates a series of intracellular signal events during activation of leukocytes. Antibody engagement of PSGL-1 up-regulates the transcriptional activity of CSF-1 promotor and increases the endogenous expression of CSF-1 mRNA in Jurkat cell. The PSGL-1 associates with Syk. This association is mediated by the actin-linking proteins moesin and ezrin, which directly interact with Syk in an ITAM-dependent manner. PSGL-1 engagement induces tyrosine phosphorylation of Syk and SRE-dependent transcriptional activity. Study revealed a new role for ERMs (ezrin/radixin/moesin) as adaptor molecules in the interactions of adhesion receptors and intracellular tyrosine kinases and showed that PSGL-1 is a signaling molecule in leukocytes (Urzainqui et al. 2002). Studies suggest that signal transduced by PSGL-1 up-regulates the transcriptional activity of CSF-1, and non-receptor tyrosine kinase Syk participates in this pathway (Ba et al. 2005). The PSGL-1 peptide corresponding to the 18-residue juxtamembrane region from the mouse PSGL-1 cytoplasmic tail (residues 2–19 in Fig. 27.3) binds to the radixin FERM domain with a dissociation constant K_D of 201 nm.

Structural Basis of PSGL-1 Binding to ERM Proteins: Proteins involved in mediating cell migration and attachment have been identified using molecular genetic and biochemical approaches and many of these proteins contain highly conserved protein interaction domains. One such family of proteins is: which contains a FERM (Four.1 protein, ezrin, radixin, moesin) domain that functions as a protein docking surface with the cytosolic tail of transmembrane proteins such as CD44. On activation, ERM proteins mediate the redistribution of PSGL-1 on polarized cell surfaces to facilitate binding to target molecules. ERM proteins recognize a short binding motif, Motif-1, conserved in cytoplasmic tails of adhesion molecules, whereas PSGL-1 lacks Motif-1 residues important for binding to ERM proteins. The crystal structure of the complex between the radixin FERM domain and a PSGL-1 juxtamembrane peptide reveals that the peptide binds the groove of FERM subdomain C by forming a β-strand associated with strand β5C, followed by a loop flipped out towards the solvent (Takai et al. 2007) (Fig. 27.4).

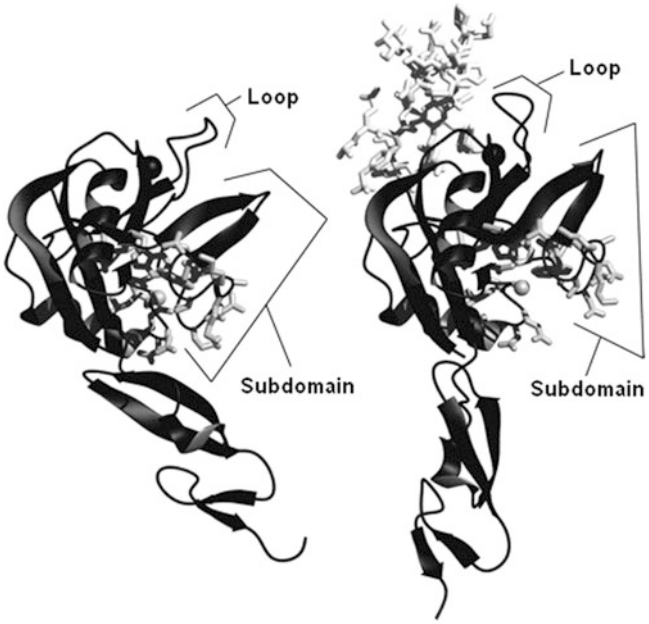

Fig. 27.3 Opening of a cleft in the lectin domain in the extended selectin conformation. (*Left*) P-selectin in the unliganded conformation (PDB ID code 1G1Q). (*Right*) P-selectin in the liganded, extended conformation (1G1S). In each structure, the Cβ atom of Ala-28 in the cleft is shown as a *gray sphere*, and other side chains that line the cleft are shown as *white sticks*. The divalent cation in the ligand binding site is shown as a larger *black sphere*. Molecules are shown as *ribbons* in identical orientations after superposition on the lectin domain (Adapted with permission from Waldron and Springer 2009 © National Academy of Sciences, USA)

27.4 Other Ligands of P-Selectin

CD24 as a Ligand for P-Selectin in Carcinoma Cells: P-selectin can also bind to breast and a small cell lung carcinoma cell line, which are negative for PSGL-1. But CD24, a mucin-type glycosyl phosphatidyl-inositol-linked cell surface molecule on human neutrophils, pre B lymphocytes, and many tumors can promote binding to P-selectin. Results establish a role of CD24 as a ligand for P-selectin on tumor cells. The CD24/P-selectin binding pathway could be important in the dissimination of tumor cells by facilitating the interaction with platelets or endothelial cells (Aigner et al. 1997).

CD44v as Ligands on Colon Carcinoma Cells: Variant isoforms of CD44 (CD44v) on LS174T colon carcinoma cells possess P-/L-/E-selectin binding activity, in contrast to the standard isoform of CD44 (CD44s) on hematopoietic-progenitor cells (HPCs), which is primarily

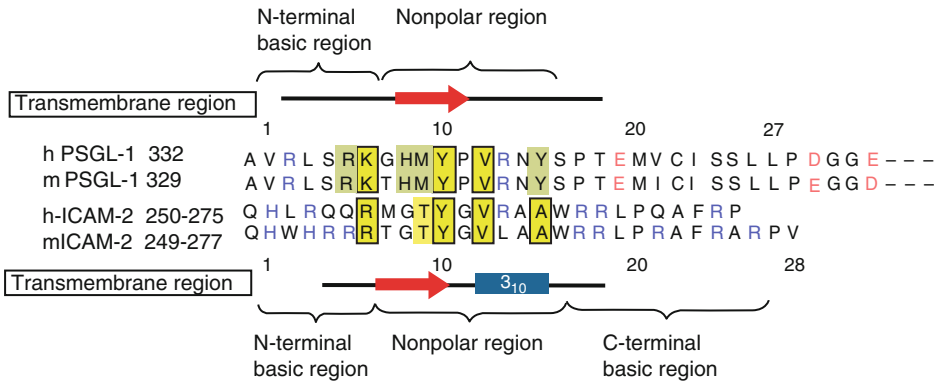

Fig. 27.4 Comparison of FERM-binding sequences in PSGL-1 and ICAM-2. The juxtamembrane region of the cytoplasmic tail of PSGL-1 is compared with that of ICAM-2, a member of the Ig superfamily of adhesion molecules that bind the FERM domain of ERM proteins. Basic and acidic residues are in *blue* and *red*, respectively. Compared with the ICAM-2 tail that displays three characteristic regions which include the N-terminal basic region, the middle nonpolar region and the C-terminal basic region, the PSGL-1 juxtamembrane region lacks the corresponding C-terminal basic region and instead contains acidic residues. PSGL-1-specific residues that are important in stabilizing conformation and binding are highlighted in *green*. The residue numbering for PSGL-1 follows that of ICAM-2. Part of the nonpolar region forms a short β-strand (residues 8–11; a *red arrow*) in FERM–PSGL-1 complex. Of the 18 PSGL-1 peptide residues, 17 (residues 2–18; indicated with *bold lines*) were defined in the current electron density map. The secondary structure of the mouse ICAM-2 cytoplasmic tail is shown at the *bottom* of the alignment. Conserved Motif-1 residues (RXXTYXVXXA) present in ICAM-2 are highlighted in *yellow* and the most important residues for binding to the FERM domain are *boxed*. PSGL-1 lacks Thr and Ala of Motif-1 (Reprinted with permission from Takai et al. 2007 © John Wiley and Sons)

an L-/E-selectin ligand. Moreover, the selectin-binding determinants on CD44v from LS174T cells are sialofucosylated structures displayed on O-linked glycans, akin to those on PSGL-1, but distinct from the HECA-452-reactive N-glycans on CD44s expressed on HPCs. Hanley et al. (2006) suggested that CD44v selectin ligands offer a unifying perspective on the apparent enhanced metastatic potential associated with tumor cell CD44v over-expression and the critical role of selectins in metastasis.

E-Selectin Promotes Growth Inhibition and Apoptosis of Hematopoietic Progenitor Cells Independent of PSGL-1: In human bone marrow, PSGL-1 is expressed by primitive bone marrow $CD34^+$ cells, mediates their adhesion to P-selectin, and inhibits their proliferation. Moreover, adhesion to E-selectin inhibits the proliferation of human $CD34^+$ cells isolated either from blood, or steady-state bone marrow. Furthermore, a subpopulation, which does not contain the most primitive hematopoietic progenitor cells, undergoes apoptosis following E-selectin-mediated adhesion. Studies suggest that an E-selectin ligand(s) other than PSGL-1 transduces growth inhibitory and pro-apoptotic signals and requires post-translational fucosylation to be functional (Winkler et al. 2004).

Lysosomal Membrane Glycoprotein-1 Can Present Ligands for E-Selectin: Lysosomal membrane glycoprotein (lamp)-1 and -2 are the most abundant glycoproteins in lysosomal membrane. A small amount of lamp-1 and lamp-2 molecules, however, can be present on the cell surface. The extent of adhesion to E-selectin and cell surface sialyl-Le^x determinants is proportional to the amount of cell surface lamp-1. Moreover, it was demonstrated that such adhesion can be inhibited by soluble lamp-1 generated from CHO cells expressing sialyl-Le^x structures. Results indicate that lamp-1 can efficiently present ligands for E-selectin and at the same time can be a useful reagent for inhibition of E-selectin (and possibly P-selectin)-mediated interaction (Sawada et al. 1993).

Sulfatides: Leukocytes may be important in the development of intimal hyperplasia. Sulfatides (3-sulfated galactosyl ceramides) are native ligands of L- and P-selectin. Findings suggest that neutrophil accumulation on the subendothelial matrix or adherence of platelets mediated by adhesive interactions between L- or P-selectin and sulfatides may contribute to the development of intimal hyperplasia. The neutrophil accumulation may be mediated by an increase in Mac-1 caused by the agonistic effects of sulfatides on the neutrophil membrane surface, or by an increase in L- and P-selectin ligands resulting from the binding of sulfatides onto the exposed subendothelial matrix (Shimazawa et al. 2005).

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E-Selectin (CD62E) and Associated Adhesion Molecules

28

G.S. Gupta

28.1 E-Selectin (Endothelial Leukocyte Adhesion Molecule 1: ELAM-1)

28.1.1 Endothelial Cells Express E-Selectin (ELAM-1/CD62E)

E-selectin, also known as CD62 antigen-like family member E (CD62E), endothelial-leukocyte adhesion molecule 1 (ELAM-1), or leukocyte-endothelial cell adhesion molecule 2 (LECAM2), is a cell adhesion receptor expressed on endothelial cells activated by cytokines. Like other selectins, it plays an important part in inflammation. In humans, E-selectin is encoded by the *E-sel* gene. E-selectin or ELAM-1 is a member of the lectin/epidermal-growth-factor/complement-regulatory-protein-like cell-adhesion molecule family, which includes structurally related molecules referred to as selectins. Whereas P-selectin is stored in Weibel-Palade bodies, and it reaches the plasma membrane after exocytosis of these granules, E-selectin is not stored, and its synthesis is induced by cytokines. The fate of E-selectin has been studied after its surface expression following the intracellular routing of internalized antibodies to the selectin. After a brief surface exposure, internalized E-selectin is degraded in the lysosomes, whereas P-selectin returns to the storage granules from where it can be reused (Subramaniam et al. 1993). The presence of selectins on the cell surface is tightly regulated and abnormal appearance is associated with a number of inflammatory disease conditions. Multiple studies indicate that the selectins mediate neutrophil, monocyte, and lymphocyte rolling along the venular wall (Tedder et al. 1995).

28.1.1.1 E-Selectin Expression Is Not Confined to Endothelium

Proper differentiation and maturation of trophoblast contributes to the fetal-maternal vascular interface of the mature placenta and is required for all subsequent stages of embryogenesis. E-selectin is expressed in a unique pattern in secondary trophoblast giant cells, trophoblast lining the central artery, and a subpopulation of labyrinthine trophoblast all located at the fetal-maternal interface of the murine placenta. Placentae lacking E-selectin show increased trophoblast glycogen cells and fewer labyrinthine neutrophils compared with normal placentae, suggesting that recognition of E-selectin on trophoblast by counter-receptors on other cells contributes to placental development (Milstone et al. 2000). The E-selectin-specific monoclonal antibody H4/18 to keratinocytes in inflamed human oral mucosa, particularly gingival epithelium shows that E-selectin expression is not confined to endothelium (Pietrzak et al. 1996). The E-selectin and ICAM-1 are also localized to subfoveal choroidal neovascular membranes (CNVMs) in patients with age-related macular degeneration (AMD). Mesothelial cells can express a set of adhesion molecules overlapping with, but distinct from those expressed in vascular endothelium (ICAM-1, ICAM-2, VCAM-1, E-selectin), which are functionally relevant for interacting with mononuclear phagocytes (Jonjic et al. 1992).

28.2 E-Selectin Genomic DNA

28.2.1 Human E-Selectin

A full-length cDNA for E-selectin was isolated by transient expression in COS cells. The ELAM-1 clone express a surface structure recognized by E-selectin specific monoclonal antibodies and supported the adhesion of human neutrophils and the promyelocytic cell line HL-60. The primary sequence of E-selectin predicts an amino-terminal lectin-like domain, an EGF domain, and six tandem repetitive motifs (about 60 amino acids each) related to those found in complement regulatory proteins. A similar domain structure is also found in L-selectin homing receptor, and in P-selectin, a membrane glycoprotein of platelet and endothelial secretory granules (Bevilacqua et al. 1989) (Fig. 28.1).

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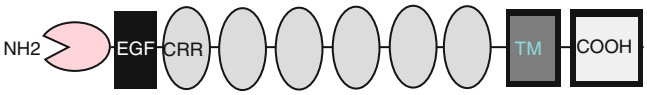

Fig. 28.1 Domain composition of human E-selectin. The extracellular portion of E-selectin contains an amino terminal domain homologous to C-type lectins and an adjacent epidermal growth factor (*EGF*)-like domain. The single EGF element, found in E-selectin is followed by six repetitive elements, each ~60 amino acids long, which resemble protein motives found in complement regulatory proteins (*CRR*), and a transmembrane (*TM*) sequence. A short cytoplasmic sequence is at the carboxyl terminus of E-selectin. The number of amino acids present in the mature E-selectin as deduced from the cDNA sequences is 589

The *E-sel* gene is present in a single copy in the human genome and contains 14 exons spanning about 13 kb of DNA. The positions of exon-intron boundaries correlate with the putative functional subdivisions of the protein. Introns are found at similar positions in all of the six complement regulatory repeats suggesting that these elements arose by internal gene duplication. A consensus TATAA element is located upstream of the transcriptional start site. The E-selectin promoter contains an inverted CCAAT box and consensus NF-kB- and AP-1-binding sites. The *E-sel* gene was assigned to the q12 greater than qter region of human chromosome. Two other members of the selectin gene family, the LAM-1/L-selectin and CD62P/P-selectin have been localized to the long arm of chromosome 1, as have the structurally related complement binding proteins, suggesting that these genes may share a common evolutionary history (Collins et al. 1995). Furthermore, the location of these genes on mouse and human chromosome 1 is consistent with a close evolutionary relationship to the complement receptor-related genes, which also are positioned on the same chromosomes in both species and with which these genes share a region of sequence homology (Watson et al. 1990).

28.2.2 E-Selectin Gene in Other Species

Murine E-selectin is encoded by a single-copy gene, spanning about 13 kb, which is structurally organized into 14 exons and 13 introns; very similar to its human counterpart. The exon/intron architecture exactly parallels the domain structure of the encoded protein. The nucleotide sequence of murine *E-sel* from heart tissue of an IL-1-treated mouse shows an overall similarity of 70% to human *E-sel* cDNA. Highest expression of murine *E-sel* gene occurs in heart and only low expression in lung tissue. Within the promoter, most of the identified regulatory elements are conserved. An exception is the NF-kB box sequence, which, in the murine *E-sel* promoter, does not correspond to the consensus NF-kB sequence (Becker-Andre et al. 1992). Transient expression in COS cells demonstrated that the lectin and EGF domains were sufficient to mediate the binding of mouse and human neutrophils as well as HL60 cells (Norton et al. 1993). The porcine gene comprises 12 exons and 11 introns. Two pseudoexons are contained within intron 4 and intron 6. These sequences are similar to the corresponding exons in the human E-selectin sequence; however, they are not present in the porcine E-selectin-encoding cDNA. Transcription starts at position −498 relative to the translation initiation site. The first ATG is located within exon 2. Translation stops in exon 11 leaving exon 12 untranslated in its entirety (Winkler et al. 1996).

28.3 E-Selectin Gene Regulation

28.3.1 Transcriptional Regulation of CAMs

The innate immune system plays an important role as a first defense against pathogens and involves the recognition of bacteria and viruses, and byproducts thereof, by Toll receptors on immunecompetent cells. Activated cells synthesize and secrete cytokines, which in turn activate systemic responses directed at clearing the pathogen. The "inflammatory triad" of IL-1, TNF-α and LPS are potent stimulators of the endothelial cell (EC) activation/adhesion molecules: ICAM-1, E-selectin (ELAM-1), and VCAM-1 (Bender et al. 1994; Fries et al. 1992). These inflammatory triad molecules, as well as the Toll receptors, initiate intracellular signaling cascades that activate nuclear factor κB (NF-κB), activator protein-1 (AP-1), cAMP responsive element binding proteins (CREBs), and various other transcription factors, which are essential for the regulation of numerous genes, many of which play important roles in immunological processes. The positive regulatory elements required for maximal levels of cytokine induction have been defined in the promoters of all three genes of cell adhesion molecules (CAMs). DNA binding studies reveal a requirement for NF-κB and other transcriptional activators. The organization of the cytokine-inducible element in the E-selectin promoter is remarkably similar to that of the virus-inducible promoter of the human IFN-β gene in that both promoters require NF-κB, ATF-2, and high mobility group protein I(Y) for induction. Based on this structural similarity, a model was proposed for the cytokine-induced E-selectin enhancer that is similar to the stereospecific complex proposed for the IFN-β gene promoter. In these models, multiple DNA bending proteins facilitate the assembly of higher order complexes of transcriptional activators that interact as a unit with the basal transcriptional machinery. The assembly of unique enhancer complexes from similar sets of transcriptional factors may provide the specificity required to regulate complex patterns of gene expression and correlate with the distinct patterns of expression of the leukocyte adhesion

molecules. Not only IL-1 but also weakly oxidized LDL, glycated LDL, and hypoxia may be possible factors that cause the expression of ELAM-1 (Collins et al. 1995).

28.3.2 Induction of E-Selectin and Associated CAMs by TNF-α

28.3.2.1 Cell Surface Expression of CAMs Can be Regulated by Different Mechanisms

Expression of some of the endothelial-leukocyte adhesion molecules is dynamically regulated at sites of leukocyte recruitment. For example, endothelial expression of E-selectin and VCAM-1 is dramatically induced, and expression of ICAM-1 is substantially increased at sites of inflammation. These dynamic changes in surface proteins provide the endothelial cell with a mechanism of regulating cell-cell interactions. The three endothehial cell-surface proteins have different patterns of expression, and are structurally and functionally distinct. Transcription of all three of these genes is substantially increased when endothelial cells are exposed to cytokines. Cell surface appearance of E-selectin and P-selectin on human umbilical vein endothelial (HUVE) cells can be regulated by different mechanisms. E-selectin is transcriptionally induced, within hours, by TNF-α while P-selectin is transported from storage granules to the plasma membrane within minutes upon induction by various stimulating agents (Weller et al. 1992).

PKC Pathway in Expression of CAMs: A protein kinase C agonist, 12-deoxyphorbol 13 phenylacetate 20-acetate (dPPA), selective for the β I isozyme, induces NF-kB-like binding activity and surface expression of E-selectin and VCAM-1 in human umbilical vein endothelial cells (HUVECs), similar to TNF-α. Induction of E-selectin and VCAM-1 expression by dPPA was completely inhibited by the PKC inhibitors staurosporine and Ro31-7549. The PKC inhibitors also reduced TNF-α-induced VCAM-1 expression. However, neither dPPA nor TNF translocated PKC from cytosolic to plasma/or nuclear membrane particulate fractions in HUVEC. These results indicated that activation of the βI PKC isozyme is sufficient for expression of E-selectin and VCAM-1, and suggested that PKC might mediate the effects of TNF-α and dPPA without requiring the translocation normally associated with activation of PKC (Deisher et al. 1993b). Inhibitors of topoisomerase II prevent cytokine-induced expression of VCAM-1, while augmenting the expression of ELAM-1 on human HUVECs. It appeared that topoisomerase II activity may differentially regulate the expression of adhesion molecules on HUVEC (Deisher et al. 1993a). The lower classic PKC activity on pretreatment with phorbol ester (phorbol 12-myristate 13-acetate (PMA) markedly decreased IL-1β-induced E-selectin mRNA expression in HUVEC in the presence of fetal calf serum and bFGF, although the induction of ICAM-1 mRNA expression was only influenced a little by the PKC down-regulation. On the other hand, TNFα-induced gene expression of these adhesion molecules was unaffected by such PKC modulation. Promoter analysis of E-selectin indicated that the NF-ELAM1/ATF element is critical for the synergistic effect of the cotreatment with IL-1β and PMA (Tamaru and Narumi 1999).

Tyrosine Phosphorylation in Endothelial Adhesion Molecule Induction: Induction of endothelial adhesion molecules by TNFα can occur independently of PKC. Activation of a protein tyrosine kinase (PTK) has been implicated in the upregulation of VCAM-1 by IL-4 on endothelial cells. The PTK inhibitors herbimycin A or genistein suppress induction of endothelial VCAM-1 and E-selectin, as well as subsequent monocytic cell adhesion to endothelial cells stimulated by TNF. Inhibition studies indicated that specific tyrosine phosphorylation following PTK activation is involved in the mobilization of NF-kB, and VCAM-1 mRNA expression. This may have implications for pathophysiological conditions that involve the upregulation of these molecules (e.g. inflammation and atherosclerosis) (Weber 1996)

E-Selectin Expression by Double-Stranded RNA and TNF-α: Since the double stranded RNA (dsRNA)-activated kinase (PKR) mediates dsRNA induction of NF-kB, murine aortic endothelial (MuAE) cells from wild-type and PKR-null mice were investigated for the role of PKR in the induction of E-selectin expression by dsRNA (pIC) and TNF-α. Study indicated that PKR is required for full activation of E-selectin expression by pIC and TNF-α in primary MuAE cells identifying activating transcription factor 2 as a new target for PKR-dependent regulation and suggested a role for PKR in leukocyte adhesion (Bandyopadhyay et al. 2000).

TNF-α Activates Two Signaling Pathways, NF-kB and JNK/p38, Both Required for Expression of E-Selectin: Transcriptional regulation of the E-selectin promoter by TNFα requires multiple NF-kB binding sites and a cAMP-responsive element/activating transcription factor-like binding site designated positive domain II (PDII). Read et al. (1997) while studying the role of MAP kinases in induced expression of E-selectin, suggested that TNFα activates two signaling pathways, NF-kB and JNK/p38, which are both required for maximal expression of E-selectin (Xu et al. 1998). Moreover, transient overexpression of catalytically inactive JNK or truncated TRAF2 (TNF receptor-associated

factor 2) partially inhibits endogenous E-selectin protein expression in human endothelial cells. Min and Pober (1997) suggest that TNF activates parallel TRAF-NF-kB and TRAF-RAC/CDC42-JNK-c-Jun/ATF2 pathways to initiate E-selectin transcription. Furthermore, endogenous inhibitors of the MAPK cascade, such as the dual-specificity phosphatases may be important for the postinduction repression of MAPK activity and E-selectin transcription in endothelial cells. These inhibitors may play an important role in limiting the inflammatory effects of TNF-α and IL-1β (Wadgaonkar et al. 2004).

IL-4 Suppression of TNFα-Stimulated E-Selectin Gene: IL-4, secreted by activated T-helper 2 lymphocytes, eosinophils, and mast cells, stimulates the expression of pyrogen-induced upregulation of ICAM-1, E-selectin and VCAM-1 in cultured HUVEC (Kapiotis et al. 1994). IL-4 stimulates a number of immune system genes via activation of STAT6. However, IL-4 can concomitantly suppress the expression of other immune-related gene products, including k light chain, FcγRI, IL-8, and E-selectin (Bennett et al. 1997).

Regulation by Cyclosporin A and Transcription Factor NFAT: Nuclear factor of activated T cells (NFAT) supports the activation of cytokine gene expression and mediates the immunoregulatory effects of cyclosporin A (CsA). Activated endothelial cells also express NFAT. CsA completely suppressed the induction of NFAT in endothelial cells and inhibited the activity of GM-CSF gene regulatory elements. CsA also suppressed E-selectin, but not VCAM-1 expression in endothelial cells, even though E-selectin promoter is activated by NF-kB rather than NFAT. Hence, induction of cell surface expression of E-selectin by TNF-α is reduced in presence of CsA, and this was reflected by a decrease in neutrophil adhesion. This suggests a mechanism by which CsA could function as an antiinflammatory agent (Cockerill et al. 1995).

28.3.2.2 TNF-α-Induced Cell Adhesion to Human Endothelial Cells Through TNF-Receptors

Mackay et al. (1993) reported that TNF-α triggers cell responses through two distinct membrane receptors. The HUVEC express both TNF receptor types, TNF-R55 and TNF-R75. But the TNF-α-induced cell adhesion to HUVEC is controlled almost exclusively by TNF-R55, but not through TNF-R75. This finding correlated with the exclusive activity of TNF-R55 in the TNF-α-dependent regulation of the expression of the ICAM-1, E-selectin, and VCAM-1. However, both TNF-R55 and TNF-R75 upregulate α2 integrin expression in HUVEC. The predominant role of TNF-R55 in TNF-α-induced adhesion in HUVEC may correlate with its specific control of NF-kB activation, since kB elements are known to be present in ICAM-1, E-selectin, and VCAM-1 gene regulatory sequences (Mackay et al. 1993).

Fish TNF-α Displays Different Sorts of Bioactivity to Their Mammalian Counterparts: In two complementary fish models, gilthead seabream and zebrafish, the proinflammatory effects of fish TNF-α are mediated through the activation of endothelial cells leading to the expression of E-selectin and different chemokines in endothelial cells, thus explaining the recruitment and activation of phagocytes observed in vivo in both species. Results indicate that fish TNF-α displays different sorts of bioactivity to their mammalian counterparts and point to the complexity of the evolution that has taken place in the regulation of innate immunity by cytokines (Roca et al. 2008).

28.3.3 IL-1-Mediated Expression of CAMs

Stimulation of E-selectin, VCAM-1, and ICAM-1 after treatment with IL-1β, TNF-α, and LPS, in a dose-dependent fashion was observed in cultured human intestinal microvascular endothelial cells (HIMEC). Each molecule displayed a time-related response comparable to those obtained with HUVEC (Haraldsen et al. 1996). Sphingomyelin hydrolysis to ceramide did not trigger, but rather enhanced cytokine-induced E-selectin expression. Sphingomyelin hydrolysis to ceramide did not mediate all the effects of IL-1β, although it might play important roles in IL-1β signal transduction in HUVECs (Masamune et al. 1996).

The infiltration of leukocytes into the CNS is associated with many pathologic conditions of the brain. The elevated levels of IL-1 in brain appear to accompany the pathogenesis. The evidences suggest that IL-1 can induce the expression of adhesion molecules for leukocytes on glial cells and a role for NF-kB in the induction process. Thus, the expression of adhesion molecules for leukocytes on glial cells in response to IL-1 may represent an important mechanism for retention of immune cells in the CNS that may be a prologue to inflammatory conditions in the brain (Moynagh et al. 1994; Couffinhal et al. 1994).

28.4 Factors in the Regulation of CAMs

28.4.1 NF-kB: A Dominant Regulator

A growing body of evidence demonstrates that nuclear transcription factor NF-kB acts as a dominant regulator of transcription of endothelial CAMs, E-selectin, VCAM-1 and

ICAM-1 genes. This molecular pathway may be involved in the pathogenesis of acute inflammatory diseases. Pharmacologic antagonism of transcription from these genes therefore represents a novel approach to the development of anti-inflammatory therapeutics and NF-kB may represent a prime target for therapeutic intervention in pathologic conditions associated with EC activation such as allo- and xenograft rejection, atherosclerosis, ischemic reperfusion injury and vasculitis.

NF-kB Is Required for TNF-α-Induced Expression of CAMs in Endothelial Cells: In response to inflammation stimuli, TNF-α induces expression of CAMs in ECs. Studies suggested that NF-kB and p38 MAP kinase (p38) signaling pathways play central roles in this process. However, subsequent analysis revealed that p38 activity is not essential for TNF-α-induced CAMs. Rajan et al. (2008) demonstrated that NF-kB, but not p38, is critical for TNF-α-induced CAM expression. In addition to TNF-α, Tie-1, an endothelial specific cell surface protein upregulates VCAM-1, E-selectin, and ICAM-1, partly through a p38-dependent mechanism (Chan et al. 2008). Similarly, LPS can activate p38 MAPK in equine ECs and that both neutrophil adhesion to LPS-activated EDVEC and prostacyclin (PGI_2) release are dependent upon p38 MAPK phosphorylation. Results reveal that inhibition of this kinase may reduce inflammatory events in the endotoxemic horse (Brooks et al. 2009).

NF-kB and IkBα: An Inducible Regulatory System in Endothelial Activation: Endothelial cells encode transcripts encoding the p50/p105 and p65 components of NF-kB and the rel-related proto-oncogene c-rel. These transcripts are transiently increased by TNF-α. Stimulation of endothelial cells with TNF-α resulted in nuclear accumulation of the p50 and p65 components of NF-kB and their binding to the E-selectin kB site. Endothelial cells also express I kB-α (MAD-3), an inhibitor of NF-kB activation. Level of I kB-α inhibitor falls rapidly after TNF-α stimulation. In parallel, p50 and p65 accumulate in the nucleus, where as RNA transcripts for IkB-α are dramatically upregulated. Studies suggest that NF-kB and IkB-α system may be an inducible regulatory mechanism in endothelial activation (Read et al. 1994, 1996). TNF causes persistent activation of NF-κB in human EC and that this may result from sustained reductions in I kB-β levels (Johnson et al. 1996).

Nuclear NF-kB DNA-binding activity was rapidly increased within lung and heart tissues of rats administered endotoxin, consistent with the translocation of NF-kB complexes from the cytoplasm to the nucleus. NF-kB activation preceded the transcriptional activation of E-selectin, VCAM-1, and ICAM-1 genes. These molecular events were temporally associated with the sequestration of leukocytes and the development of pulmonary inflammation. Report supported that NF-κB activation is the underlying molecular mechanism for constitutive expression of E-selectin, VCAM-1, and ICAM-1 on human B lymphocytes, plasma cells and in hepatic vascular lining cells though the hepatic parenchymal cells, despite NF-kB activation do not express E- selectin mRNA. This indicated that NF-kB activation alone is not sufficient for E-selectin gene transcription in vivo (Essani et al. 1996; Manning et al. 1995; Wang et al. 1995).

Platelet factor 4 (PF4), a platelet-specific chemokine, has been localized to atherosclerotic lesions, including macrophages and endothelium. The PF4, which is able to increase expression of E-selectin by endothelial cells, represents one of the potential mechanisms by which platelets may participate in atherosclerotic lesion progression (Yu et al. 2005). E-selectin and its RNA are upregulated in HUVE cells exposed to PF4. It appeared that activation of NF-kB is critical for PF4-induced E-selectin expression. The LDL receptor-related protein as the cell surface receptor may mediate this effect (Yu et al. 2005). The POZ domain of FBI-1 (factor that binds to the inducer of short transcripts of HIV-1) interacts with the Rel homology domain of the p65 subunit of NF-kB in in vivo and in vitro protein-protein interactions. FBI-1 enhanced NF-kB-mediated transcription of E-selectin genes in HeLa cells upon stimulation and overcame gene repression (Lee et al. 2005).

Glucocorticoid-Mediated E-Selectin Repression: Induction of E-selectin expression by stimuli such as TNF-α or LPS is reduced markedly in the presence of dexamethasone, a potent anti-inflammatory agent. E-selectin promoter analysis revealed that induction by proinflammatory stimuli as well as repression by dexamethasone are mediated by the same promoter region containing three closely spaced binding sites for NF-kB and an element, NF-ELAM-1, constitutively occupied by ATF and c-Jun. NF-ELAM-1 contributes to maximal promoter activity, but does not confer glucocorticoid inhibition, as demonstrated by site-directed mutagenesis. In contrast, transcription directed by the E-selectin NF-kB elements is reduced strongly in the presence of dexamethasone, thus identifying NF-kB as the primary target for glucocorticoid-mediated E-selectin repression. The interference by glucocorticoids receptor (GR) with the transcriptional activation potential of DNA-bound NF-kB complexes might contribute to mechanisms underlying the anti-inflammatory effects of glucocorticoids (Brostjan et al. 1997; Ray et al. 1997).

28.4.2 Cyclic AMP Inhibits NF-kB-Mediated Transcription

Cytokines induce the expression of E-selectin, VCAM-1, and ICAM-1 on HUVECs. Expression of these surface

proteins is differently affected by cAMP. Increased cAMP levels decrease E-selectin and VCAM-1 but increase ICAM-1 expression. The cAMP repression of E-selectin occurs at the transcription level. This effect is abolished by protein kinase A (PKA) inhibition, suggesting that repression is mediated by PKA-driven phosphorylation. A minimal E-selectin promoter sequence necessary to confer cytokine inducibility is also sufficient to mimic the cAMP effect in transfected HUVECs. There are two regions (NF-kB and NF-ELAM1) of the minimal promoter that bind transcription factors necessary for E-selectin induction. Increased cAMP did not alter the binding of the complexes formed on either the NF-kB or NF-ELAM1 site. In contrast, in IL-1-treated HUVECs transactivity due to an NF-kB site was reduced by elevated cAMP. Increased cAMP in HUVECs appears to induce a protein kinase activity that reduces the cytokine signal for E-selectin and VCAM-1 expression (Ghersa et al. 1994). Ollivier et al. (1996) examined the molecular mechanism by which agents that elevate intracellular cAMP inhibit the expression of TNFα, tissue factor, ELAM-1, and VCAM-1 genes. Both forskolin and dibutyryl cAMP, which elevate intracellular cAMP by independent mechanisms, inhibited TNFα and tissue factor expression at the level of transcription. Induction of NF-kB-dependent gene expression in transiently transfected human monocytic THP-1 cells and HUVEC was inhibited by elevated cAMP and by over-expression of the catalytic subunit of PKA. This indicated that activation of PKA reduced the induction of a distinct set of genes in monocytes and endothelial cells by inhibiting NF-kB-mediated transcription.

Cilostazol Inhibits Cytokine-Induced NF-kB Activation Via AMP-Activated Protein Kinase Activation: Cilostazol is a selective inhibitor of phosphodiesterase-3 that increases intracellular cAMP levels and activates PKA, thereby inhibiting platelet aggregation and inducing peripheral vasodilation. Cilostazol also inhibited TNFα-induced NF-kB activation and TNFα-induced I κB kinase activity. Furthermore, cilostazol attenuated the TNFα-induced gene expression of various pro-inflammatory and cell adhesion molecules, such as VCAM-1, E-selectin, ICAM-1, MCP-1, and PECAM-1 in HUVEC. Hattori et al. (2009) suggested that cilostazol might attenuate the cytokine-induced expression of adhesion molecule genes by inhibiting NF-κB following AMP-activated protein kinase.

28.4.3 Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors which down-regulate inflammatory signaling pathways. Three peroxisome proliferator-activated receptors (PPARs) have been identified: PPARα, PPARβ/δ, and PPARγ, all of which have multiple biological effects, especially the inhibition of inflammation. The expressions of 3 PPAR isoforms and PPAR-responsive genes are markedly upregulated in spontaneously hypertensive rats (SHR) compared with those of Wistar-Kyoto rats (WKY). An enhanced inflammatory response in the organs of SHR might play a key role in pathogenesis of hypertension and secondary organ complications. Changes (increases) in PPARs expression may reflect a compensatory mechanism to the inflammatory status of hypertensive rats (Sun et al. 2008). PPAR-γ has an essential role in adipogenesis and glucose homeostasis. The PPAR-γ is expressed in vascular tissues including endothelial cells. Its activity is regulated by many pharmacological agonists. PPAR-γ might be involved in the control of inflammation and in modulating the expression of various cytokines. PPAR-γ activation suppresses the expression of vascular adhesion molecules in ECs and the ensuing leukocyte recruitment. Evidence shows that constitutive activation of PPAR-γ is sufficient to prevent ECs from converting into a pro-inflammatory phenotype (Wang et al. 2002).

The PPAR activators have been shown to inhibit the expression of E-selectin of HVECs in response to TNF-α. Troglitazone, pioglitazone, α-clofibrate, and 15-deoxy-δ 12,14-prostaglandin J2 inhibited the TNF-α-stimulated E-selectin gene transcription. The activators caused a significant induction of liver regenerating factor 1 (LRF1)/ATF3, which bound to NF-ELAM1 site and repressed the TNF-α-induced E-selectin gene expression. It appears that the effect of PPAR activators is mediated, in part, through the induction of LRF1/ATF3 (Nawa et al. 2000). In another study, pioglitazone inhibited the expression of VCAM-1 protein and mRNA on HUVEC after IL-1β stimulation, though it showed little effect on the expression of ICAM-1 and E-selectin. The study revealed that pioglitazone can influence monocyte-EC binding by inhibiting VCAM-1 expression on activated EC and neutrophil-EC binding by inhibiting upregulation of CD11b/CD18 on activated neutrophils. This might provide a molecular basis of anti-inflammatory effect of PPAR activators (Nawa et al. 2000; Imamoto et al. 2004).

The role of PPAR-δ in endothelial activation remains poorly understood. In human umbilical vein endothelial cells (HUVECs), the synthetic PPAR-δ ligands GW0742 and GW501516 significantly inhibited TNF-α-induced expression of VCAM-1 and E-selectin, as well as ensuing endothelial-leukocyte adhesion. Activation of PPAR-δ upregulated the expression of antioxidant genes: SOD-1, catalase, and thioredoxin and decreased ROS production in ECs. This shows that ligand activation of PPAR-δ in ECs has a potent antiinflammatory effect, probably via a binary mechanism involving the induction of antioxidative genes and the release of nuclear corepressors (Fan et al. 2008).

PPAR-δ agonists may have a potential for treating inflammatory diseases such as atherosclerosis and diabetes. Alterations of PPAR functions can contribute to HIV-1-induced dysfunction of brain endothelial cells. Treatment with HIV-1 transactivator of transcription (Tat) protein decreased PPAR transactivation in brain endothelial cells. Tat-induced up-regulation of inflammatory mediators, such as IL-1β, TNF-α, CCL2, and E-selectin were markedly attenuated in hCMEC/D3 over-expressing PPARα or PPARγ. Data suggest that targeting PPAR signaling may provide a novel therapeutic approach to attenuate HIV-1-induced local inflammatory responses in brain endothelial cells (Huang et al. 2008).

28.4.4 Endogenous Factors Regulating CAM Genes

In order to understand the role of complement components as regulators of expression of endothelial adhesive molecules in response to immune complexes (ICs), Lozada et al. (1995) showed that ICs stimulate both the endothelial adhesiveness for leukocytes and expression of E-selectin, ICAM-1, and VCAM-1. ICs (BSA-anti-BSA) stimulated EC adhesiveness for added leukocytes in the presence of complement-sufficient human serum. Studies showed that ICs stimulate ECs to express adhesive proteins for leukocytes in presence of a heat-labile serum factor, C1q (Lozada et al. 1995).

Native LDL (n-LDL) increases HUVEC adherence of mononuclear cells. Such phenotypic changes suggest that n-LDL alters the usual expression of cell adhesion molecules to enhance the adhesive properties of the endothelium. n-LDL increased ICAM-1 protein expression corresponded with increased ICAM-1 mRNA levels. n-LDL also appeared to increase E-selectin and VCAM-1 message levels, but these changes were not statistically significant. n-LDL increase of ICAM-1 expression seems to enhance the adhesive properties of the endothelium. Such perturbations in HUVEC function likely represent a proinflammatory response to protracted n-LDL exposure and one of the early steps in atherogenesis (Smalley et al. 1996).

Adrenomedullin (AM) and corticotrophin (ACTH) are both vasoactive peptides produced by a variety of cell types, including ECs. Their role in inflammation and the immune response was studied by Hagi-Pavli et al. (2004). AM and ACTH induce cell surface expression of E-selectin, VCAM-1, and ICAM-1 on HUVEC. This effect appears to be mediated in part via elevation of cAMP, and that both peptides elevate cAMP. The effect of AM and ACTH is inhibited by the adenylyl cyclase inhibitor SQ-22528. This demonstrates a role for AM and ACTH in the regulation of the immune and inflammatory response through adhesion molecules (Hagi-Pavli et al. 2004).

Methylation May Play a Role in Blocking E-Selectin Expression in Non-Endothelial Cells: The E-selectin promoter in cultured endothelial cells is under-methylated in comparison with non-expressing HeLa cells. Plasmid constructs carrying a reporter driven by E-selectin promoter and methylated in vitro are not transcribed in either an in vitro transcription system or in transiently transfected cells. The NF-kB site in the promoter of E-selectin is the likely target for this methylation-mediated repression in a minimal promoter carrying only this and an associated element. Thus, methylation is likely to play a role in blocking E-selectin expression in non-endothelial cells (Smith et al. 1993).

28.4.5 Tat Protein Activates Human Endothelial Cells

Tat protein, an HIV gene product, functions as a transactivator for HIV replication and is known to be secreted extra-cellularly by infected cells. Tat stimulates endothelial cells. This is evidenced by the expression of E-selectin, critical for the initial binding of leukocytes to the blood vessel wall, and its effect on increased synthesis of IL-6, a cytokine known to enhance endothelial cell permeability. Furthermore, tat acts synergistically with low concentrations of TNF-α to enhance IL-6 secretion. Tat activates human CNS-derived endothelial cells (CNS-EC) by the increase in the expression of E-selectin, the synthesis of IL-6, and the secretion of plasminogen activator inhibitor-1. It was suggested that secreted tat protein may increase leukocyte binding, and alter the blood-brain barrier permeability to enhance dissemination of HIV-infected cells into the CNS (Hofman et al. 1994). Treatment of HUVEC with Tat induces the cell surface expression of ICAM-1, VCAM-1, and E-selectin-1 and the effect on expression of adhesion molecules was potentiated by TNF. Like TNF, Tat also enhanced the adhesion of human promyelomonocytic HL-60 cells to EC (Dhawan et al. 1997). Both Tat and TNF activated p65 translocation and binding to an oligonucleotide containing the E-selectin kB site 3 sequence. A super-repressor adenovirus (AdIkBαSR) that constitutively sequesters IkB in the cytoplasm as well as cycloheximide or actinomycin D inhibited Tat- or TNF-mediated kB translocation and E-selectin up-regulation (Cota-Gomez et al. 2002).

28.4.6 Reactive Oxygen Species (ROS)

ROS is critical signal in the activation of NF-kB and in E-selectin expression: Decreased NO activity, the formation of ROS and increased endothelial expression of the redox-

sensitive VCAM-1 gene in the vessel wall are early and characteristic features of atherosclerosis. Through this mechanism, NO may function as an immunomodulator of the vessel wall and thus mediate inflammatory events involved in the pathogenesis of atherosclerosis (Khan et al. 1996). Reactive oxygen intermediates may act as second messengers in the activation of NF-kB.

Antioxidants and NO Inhibit Adhesion Molecule Expression: Using the antioxidant pyrrolidine dithiocarbamate (PDTC) for the study of the effect of NF-kB inhibition in TNF-α-induced EC activation in vitro, Ferran et al. (1995) showed that PDTC strongly reduces the TNF-α-mediated induction of E-selectin, VCAM-1, and ICAM-1. The N-acetyl-L-cysteine (NAC) is a thiol-containing antioxidant that inhibits agonist-induced monocytic cell adhesion to endothelial cells. Unlike PDTC, which specifically inhibits VCAM-1, NAC inhibits IL-1β -induced mRNA and cell surface expression of both E-selectin and VCAM-1. Although NAC reduced NF-kB activation in EC, the antioxidant had no appreciable effect when an oligomer corresponding to the consensus NF-kB binding site of the E-selectin gene was used. (Faruqi et al. 1997). Trans fatty acid intake could also affect biomarkers of inflammation and endothelial dysfunction including C-reactive protein (CRP), IL-6, sTNFR-2, E-selectin, sICAM-1 and sVCAM-1. Higher intake of trans fatty acids could adversely affect endothelial function, which might partially explain why the positive relation between trans fat and cardiovascular risk is greater than one would predict based solely on its adverse effects on lipids (Lopez-Garcia et al. 2005). Findings support that TNF-α induces NF-kB activation of the resultant E-selectin gene by a pathway that involves formation of ROS and that the E-selectin expression can be inhibited by the antioxidant action of NAC or PDTC. The results support the hypothesis that generation of ROS in endothelial cells induced by proinflammatory cytokines such as TNF-α is a critical signal mediating E-selectin expression (Rahman et al. 1998; Spiecker et al. 2000). The inhibitory effects of NO on adhesion molecule expression differ from that of antioxidants in terms of the mechanism by which NF-kB is inactivated (Spiecker et al. 2000). NO inhibited IL-1α-stimulated VCAM-1 expression in a concentration-dependent manner. NO also decreased the endothelial expression of E-selectin and to a lesser extent ICAM-1 and secretable cytokines (IL-6 and IL-8) (De Caterina et al. 1995).

28.4.7 Role of Hypoxia

Anoxia/reoxygenation (A/R) leads to a biphasic increase in neutrophil adhesion to HUVECs, with peak responses occurring at 30 min (phase 1) and 240 min (phase 2) after reoxygenation. It was indicated that A/R elicits a two-phase neutrophil-endothelial cell adhesion response that involves transcription-independent and transcription-dependent surface expression of different endothelial CAMs. Tissue injury that accompanies hypoxemia/reoxygenation shares features with the host response in inflammation, suggesting that cytokines, such as IL-1, may act as mediators in this setting. Human endothelial cells subjected to hypoxia elaborated IL-1 activity into conditioned media in a time-dependent manner. Production of IL-1 activity by hypoxic ECs was associated with an increase in the level of mRNA for IL-1 alpha, and was followed by induction of ELAM-1 and enhanced ICAM-1 during reoxygenation. This suggests that hypoxia is a stimulus, which induces EC synthesis and release of IL-1 alpha, resulting in an autocrine enhancement in the expression of adhesion molecules (Shreeniwas et al. 1992). Findings make the use of inducible enhancers a promising strategy for increasing tissue specific gene expression.

28.5 Binding Elements in E-Selectin Promoter

28.5.1 Transcription Factors Stimulating *E-sel* Gene

As seen, expression of E-selectin is cell and stimulus specific, as it is mainly expressed on endothelial cells in response to induction by IL-1 and TNF-α as well as LPS and phorbol myristate acetate. IL-1 and TNF-α initiate intracellular signaling cascades that activate NF-kB, activator protein-1 (AP-1), and cAMP responsive element binding proteins (CREBs), which are transcription factors essential for the regulation of numerous genes, many of which play important roles in immunological processes. In addition, E-selectin gene activity is transient. Expression is maximal 2–4 h following cytokine induction and returns to the basal level by 24 h. This tight regulation of gene activity is likely to require complex control mechanisms (Jensen and Whitehead 2003) .

Factors in addition to NF-kB appear to play a central role in determining the specific expression of ELEM-1 gene in response to cytokines. In support of this, Whelan et al. (1991) have shown that NF-kB alone, although essential, is not sufficient to mediate IL-1-induced activation of E-selectin gene. Hooft van Hujsduinen et al. (1992) identified two additional factors, which were referred to as NF-ELAM1 and NF-ELAM2 (binding at positions −153 to −144 and −104 to −100, respectively), that play critical roles in controlling cytokine-induced expression of the E-selectin gene. While neither of these elements alone is sufficient to confer enhancer activity on a heterologous promoter, NF-ELAM1 was shown to cooperate with NF-kB to augment cytokine-induced expression to levels

significantly above that observed with NF-kB alone. Hooft van Hujsduinen et al. (1992) demonstrated that NF-ELAM1 functionally cooperates with NF-kB in IL-1 induction of the E-selectin gene.

Multiple NF-kB Binding Sites in Human *E-sel* Gene: Promoter binding factors responsible for induced ELAM-1 gene expression include NF-kB, which binds at three sites within the E-selectin promoter; two of these NF-kB binding sites are partially overlapping, that have been reported to be required for full induction by cytokines. In band shift assays, Hooft van Huijsduijnen et al. (1993) detected two distinct NF-kB complexes in nuclear extracts from several cytokine-induced cells. A putative AP-1 site at position −499 to −493 within the promoter has been shown not to affect cytokine induction (Schindler and Baichwal 1994). The presence of a TNF-α-inducible element close to the transcriptional start site. 170 bp of upstream sequences was sufficient to confer TNF-α induction. Site-directed mutagenesis of this region revealed two regulatory elements (−129 to −110 and −99 to −80) that are essential for maximal promoter activity following cytokine treatment. Binding studies with crude nuclear extracts and recombinant proteins showed that the two elements correspond to three NF-kB binding sites (site 1, −126; site 2, 116; and site 3, −94). Thus induction of E-selectin gene requires the interaction of NF-kB proteins at multiple regulatory elements (Schindler and Baichwal 1994). Consequently, this promoter is often used to drive expression of luciferase in reporter assays and is frequently considered to be an "NF-kB-specific" promoter. Whitley et al. identified four positive regulatory domains (PDI to PDIV) in the E-selectin promoter that are required for maximal induction by TNF-α in endothelial cells. Two of these domains contain adjacent binding sites for NF-kB (PDIII and PDIV), where as the third corresponds to CRE/ATF site (PDII), and a fourth is a consensus NF-kB site (PDI) (Fig. 28.2). Mutations that decrease the binding of NF-kB to any one of the NF-kB binding sites in vitro abolish cytokine-induced E-selectin gene expression in vivo. A similar correlation was demonstrated between ATF binding to PDII and E-selectin gene expression (Whitley et al. 1994).

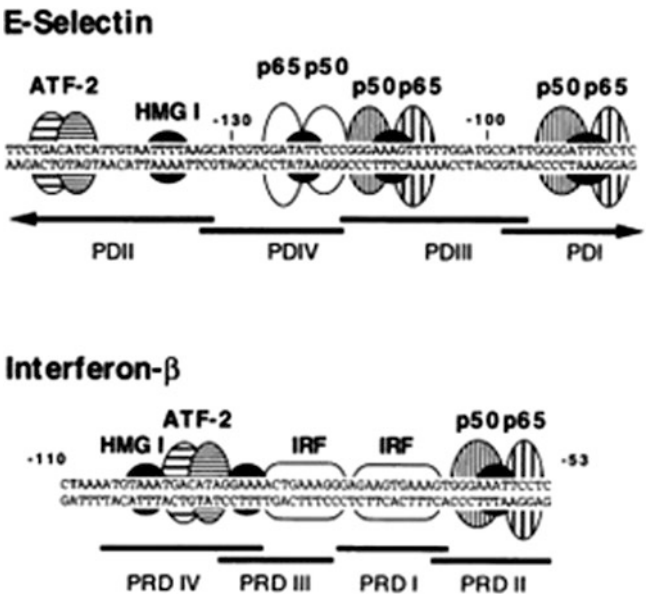

Fig. 28.2 Comparison of the human promoters for the IFN-β and E-selectin genes. The double-stranded DNA sequences of the IFN-β promoter from −110 to −53 and of the human E-selectin from −156 to −83, upstream of the transcriptional start site, are shown. Both regions contain four regulatory domains, designated PRDI through PRDIV, in the human IFN-P gene and PDI through PDIV in the E-selectin gene. Transcription factors that bind to each of the elements are shown for both genes. Those for the IFN-l gene are as follows (reviewed in reference 19): NF-KB binds to PRDII, IFN regulatory factor 1 (*IRF*) binds to PRDI and PDIII, and ATF-2 binds to PRDIV. Binding sites for HMG I(Y) are found in PRDII and PRDIV and are indicated. Transcription factors that bind to the E-selectin cytokine response region are as follows: NF-kB binds to PDI, PDIII, and PDIV, and ATF-a or ATF-2 homodimer or the corresponding c-Jun heterodimer binds to PDII. HMG I(Y) binds to a region within PDII as well as to the AT-rich regions in the kB sites contained in PDI, PDIII, and PDIV (Adapted with permission from Whitley et al. 1994 © American Society for Microbiology)

28.5.2 CRE/ATF Element or NF-ELAM1

Activating Transcription Factor (ATF) is a basic leucine zipper protein, whose DNA target sequence is widely distributed in cAMP response element (CRE). The CRE/ATF element of E-selectin promoter is necessary for full cytokine responsiveness. It differs from a consensus CRE by 1 nucleotide (G—>A conversion) and does not mediate transcriptional activation in response to cAMP. The cAMP actually decreases E-selectin synthesis induced by TNF-α in aortic endothelial cells; the cAMP-mediated inhibition maps to the CRE/ATF element. Study suggests that a change in the composition of the proteins binding to the CRE/ATF promoter element contributes to the competing effects of TNF-α and cAMP on E-selectin gene expression (De Luca et al. 1994). The abnormalities in activating transcription factor-2 (ATF-2) mutant mice demonstrate its absolute requirement for skeletal and CNS development, and for maximal induction of select genes with CRE sites, such as E-selectin (Reimold et al. 1996).

The cAMP-independent members of the ATF family bind specifically to the NF-ELAM1 promoter element (Fig. 28.2). This sequence operates in a cAMP-independent manner to induce transcription and thus it was defined as a

non-cAMP-responsive element (NCRE). It was found that ATFα is a component of the NF-ELAM1 complex and its over-expression activates the E-selectin promoter. Furthermore, the ability of over-expressed NF-kB to transactivate the E-selectin promoter in vivo was dependent on the NF-ELAM1 complex (Kaszubska et al. 1993). Hooft van Huijsduijnen et al. (1992) identified two proximal E-selectin promoter elements and their DNA-binding factors that are, in addition to NF-kB, essential for E-selectin transcription. This site, ACATCAT, is recognized by NF-ELAM-1. The site corresponds to NF-ELAM-1s preferential binding sequence (A/T)CA(G/T)CA(G/T). This element is identical to the T-cell δ A enhancer found in the T-cell receptor-α, -β, and CD3δ genes. Studies suggest that δ A/NF-ELAM-element can function as a modulator of NF-kB in endothelial cells both as well as a T-cell enhancer (Hooft van Huijsduijnen et al. 1992). It was shown that a novel protein-protein interaction occurs between the p50 and p65 subunits of NF-kB and certain ATFs. Furthermore, results demonstrate that NF-kB is dependent on NFELAM1 for strong induction of E-selectin expression. These studies demonstrate a novel mechanism operating to specifically control E-selectin gene transcription in response to cytokines.

28.5.3 HMG-I(Y) Mediates Binding of NF-kB Complex

The high-mobility-group protein I (Y) [HMG-I(Y)] also binds specifically to the E-selectin promoter and thereby enhances the binding of both ATF-2 and NF-kB to the E-selectin promoter in vitro. In addition to interaction of NF-kB and ATF with promotor elements, analysis of the E-selectin promoter revealed an additional region (−140 to −105 nt referred as −140/−105) which is essential in controlling promoter activation by cytokines. The HMG-I(Y) interacts specifically at two sites within this region. One of the HMG-I(Y)-binding sites overlaps a sequence element (−127/−118) diverging at only one position from the NF-kB consensus binding sequence. Lewis et al. (1994) searched whether −127/−118 element represents a second functional NF-kB-binding site within the E-selectin promoter. It was shown that p50, p65, and RelB are components of the complex interacting at this site. Mutations that interfere with HMG-I(Y) binding decrease the level of cytokine-induced E-selectin expression. Mutations at −127/−118 NF-kB site indicated that both NF-kB and HMG-I(Y) binding at this site are essential for IL-1 induction of the promoter. The binding affinity of the p50 subunit of NF-kB to both NF-kB sites within the E-selectin promoter was significantly enhanced by HMG-I(Y). Thus, HMG-I(Y) mediates binding of a distinct NF-kB complex at two sites within the E-selectin promoter (Fig. 28.2). Furthermore, a unique cooperativity between these NF-kB complexes is essential for induced E-selectin expression. These results suggest mechanisms by which NF-kB complexes are involved in specific gene activation (Lewis et al. 1994). Studies suggest that the organization of the TNF-α-inducible element of the E-selectin promoter is remarkably similar to that of the virus-inducible promoter of human β interferon gene in that both promoters require NF-kB, ATF-2, and HMG-I(Y). Thus, HMG-I(Y) functions as a key architectural component in the assembly of inducible transcription activation complexes on both promoters (Whitley et al. 1994). HMG-I(Y) binds to the A/T-rich core found at the centre of these binding sites. Distamycin, an antibiotic that also binds A/T-rich DNA, inhibits HMG-I(Y) DNA binding. The distamycin effect on transcription was mediated through one of the three NF-kB-HMG-I(Y) binding sites (NF-kBII) within the promoter. This suggests that the NF-kB-HMG-I(Y) complex interacting at the NF-kBII site plays a role not only in cytokine induction of E-selectin expression, but also in its down-modulation (Ghersa et al. 1997).

28.5.4 Phased-Bending of E-Selectin Promoter

As suggested, DNA elements required for IL-1 inducibility are located in the proximal promoter: an NF-ELAM site, two NF-kB sites (I and II), the NF-E-selectin 2 element and a TATA box. IL-1 induced promoter activity is exquisitely sensitive to the spatial arrangements of these elements. Phasing of the ATF and NF-kB II elements indicates that their relative helix orientation is more important than distance per se. This sensitivity is partly due to a requirement for correctly oriented, transcription factor-induced DNA-bending. (1) Band shift analyses with permuted ATF- and NF-kB elements showed that their associated factors all bend DNA. (2) One can functionally replace the NF-E-selectin/ATF element by a subset of a panel of DNA fragments that contain defined bends in various planes. It appears that the main role of the factors binding at the NF-E-selectin/ATF element is to alter the conformation of the E-selectin promoter, presumably looping distant enhancer elements into each other's proximity (Meacock et al. 1994).

28.6 E-Selectin Ligands

28.6.1 Carbohydrate Ligands (Lewis Antigens)

Various Lewis Antigens, Lewis A (Le^a), Lewis B (Le^b), and Sialyl Lewis A (sLe^a) have been studied in different biological contexts, for example in microbial adhesion and cancer. Adhesion molecules recognize carbohydrate

moieties sLe^x, s-diLe^x, or sLe^a, though with different affinity. The potency of oligosaccharides sLe^x, sLe^a, HSO_3Le^x, and HSO_3Le^a, their conjugates with polyacrylamide (40 kDa), and other monomeric and polymeric selectin inhibitors was compared with that of the polysaccharide fucoidan. The monomeric sLe^x is more effective as a selectin blocker in vivo than in vitro, whereas the opposite is true for fucoidan and the bi-ligand neoglycoconjugate HSO_3LeA-PAA-sTyr (Ushakova et al. 2005). Melanoma cell lines show differential binding to different Lewis antigens. For example melanoma cell line NKI-4 binds to E-selectin, but not to P-selectin. Cell line NKI-4 does not express sLe^x, but was positive for sdiLe^x and sLe^a. In contrast, melanoma cell lines, MeWo and SK-MEL-28, expressing either sdiLe^x or sLe^a on the cell surface, bound neither E-selectin nor P-selectin (Kunzendorf et al. 1994). High metastatic colonic carcinoma cells express relatively more Lamp molecules and sLe^x structures on the cell surface than their corresponding low metastatic counterparts (Saitoh et al. 1992). High and low metastatic colonic carcinoma cells differ in their adhesion efficiency to E-selectin-expressing cells. The high metastatic cells, as compared to their low metastatic counterparts, bind more efficiently to activated human endothelial cells that express E-selectin. In addition, the high metastatic cells also adhere more efficiently to mouse endothelioma cells after activation with IL-1β. It was also shown that the adhesion can be inhibited by soluble Lamp-1 or soluble leukosialin that contain sLe^x termini (Sawada et al. 1994). Among O-glycans released from an ovarian cystadenoma glycoprotein using ethylamine, three variants of the sulfated $Le^{a/x}$ sequences were identified as ligands for E-selectin, one of which is based on the unusual backbone Gal-3/4GlcNAc-3Gal-3Gal (Chai et al. 1997).

28.6.1.1 Sialyl-Lewis^x

The sLe^x determinant (Neu5Ac α2 → 3Gal β1 → 4[Fuc α-1 → 3]GlcNAc) has been identified as a major ligand in the selectin-mediated adhesion of neutrophils and monocytes to activated endothelium or platelets. This carbohydrate epitope is formed by the sequential action of α3-sialyltransferase and α3-fucosyltransferase on N-acetyllactosamine (Gal β1 → 4 GlcNAc) disaccharide termini of glycoconjugates. In addition, the α3 fucosyltransferase of the cells can use sialylated acceptors. Characterization of the product obtained with a sialylated oligosaccharide indicated that the enzyme can catalyze the formation of the sLe^x structure. The enzyme studied appeared to be a myeloid-type and was involved in the synthesis of sLe^x in leukocytes provided that its expression is at a sufficiently high level (Easton et al. 1993). A single amino acid can determine the ligand specificity of E-selectin. Kogan et al. (1995) developed a model of E-selectin binding to the sLe^x tetrasaccharide, (Neu5Ac α2 → 3Gal β1 → 4(Fuc α 1 → 3)GlcNAc), using the E-selectin-bound solution conformation of sLe^x (Cooke et al.1994) and the crystallographic structures of E-selectin (Graves et al. 1994) (ligand unbound) and the MBP (Weis et al. 1992) (ligand bound). Analysis of this model indicated that the alteration of one E-selectin amino acid, alanine 77, to a lysine residue might shift binding specificity from sLe^x to mannose. The E-selectin mutant protein possessing this change displays preferential binding to mannose containing oligosaccharides and that further mutagenesis of this mannose-binding selectin confers galactose recognition in a predictable manner. These mutagenesis data support the presented model of the detailed interactions between E-selectin and the sLe^x oligosaccharide.

Evidences suggest for density-dependent binding of the membrane-associated E-selectin not only to 3′-sialyl-lacto-N-fucopentaose II (3′-S-LNFP-II) and 3′-sialyl-lacto-N-fucopentaose III (3′-S-LNFP-III), which express the sialyl Le^a and sLe^x antigens, respectively, but also to the nonsialylated analogue LNFP-II; there is a threshold density of E-selectin required for binding to these sialylated sequences, and binding to the nonsialylated analogue is a property only of cells with the highest density of E-selectin expression. The presence of fucose linked to subterminal rather than to an internal N-acetylglucosamine is a requirement for E-selectin binding. Moreover, the presence of sialic acid 3-linked to the terminal galactose of the LNFP-II or LNFP-III sequences substantially enhances E-selectin binding where as the presence of 6-linked sialic acid abolishes E-selectin binding (Larkin et al. 1992).

28.6.1.2 Sulfated Polysaccharide Ligands

A new class of oligosaccharide ligand—sulfate-containing—for the human E-selectin molecule from among oligosaccharides was identified on ovarian cystadenoma glycoprotein. Several components with strong E-selectin binding activity were revealed among acidic oligosaccharides. The smallest among these preparations showed equimolar mixture of the Le^a- and Le^x/SSEA-1-type fucotetrasaccharides sulfated at position 3 of outer galactose. The binding activity was substantially greater than those of lipid-linked Le^a and Le^x/SSEA-1 sequences and is at least equal to that of the 3′- sLe^x/SSEA-1 glycolipid analogue (Yuen et al. 1992). The sulfo derivative of sLe^x, GM 1998-016, which blocks the P- and E-selectins interaction with a ligand, showed a significant decrease in bacterial translocation, both local (MLN) and systemic, in association with the decrease in the neutrophil infiltration, the oxygen free radicals production and the cytokines. Thus, sulfo derivative of sLe^x shows a protective effect in experimental model of bacterial translocation, downregulating the inflammatory response and the leukocyte-endothelium interactions (Garcia-Criado et al. 2005).

Interactions with Fuco-Oligosaccharides: Sweeney et al. (2000) found that sulfated fucans, whether branched and linear, are capable of increasing mature white cells in the periphery and mobilizing stem/progenitor cells of all classes. Though the presence of sulfate groups was necessary, yet it was not sufficient. Significant mobilization of stem/progenitor cells and leukocytosis, elicited in selectin-deficient mice ($L^{-/-}$, $PE^{-/-}$ or $LPE^{-/-}$) similar to that of controls, suggested that the mode of action of sulfated fucans is not through blockade of known selectins. The influence of the location of fucose residue(s) was investigated using 14 structurally defined and variously fucosylated oligosaccharides. Results showed that the recognition motifs for E-selectin include 4-fucosyl-lacto (Le^a) and 3-fucosyl-neo-lacto (Le^x) sequences strictly at capping positions and not Le^x at an internal position as a part of VIM-2 antigen sequence. Additional fucose residues α1-2-linked to neighboring galactoses or α1-3-linked to inner N-acetyglucosamines or to reducing-terminal glucose residues of the tetrasaccharide backbone had little or no effect on the selectin binding (Martin et al. 2002).

Selectin/MBP Chimeras Bind Selectin Ligand sLe^x Through Ca^{2+}Dependent Subsite: Selectin/MBP chimeras created by transfer of key sequences from E-selectin into MBP bind the selectin ligand sLe^x through a Ca^{2+} dependent subsite, present in many C-type lectins, and an accessory site containing positively charged amino acid residues. These chimeras demonstrate selectin-like interaction with sLe^x and can be faithfully reproduced even though structural evidence indicates that the mechanisms of binding to E-selectin and the chimeras are different. Selectin-like binding to the nonfucosylated sulfatide and sulfoglucuronyl glycolipids can also be reproduced with selectin/MBP chimeras that contain the two subsites involved in sLe^x binding. Results indicate that binding of structurally distinct anionic glycans to C-type carbohydrate-recognition domains can be mediated by the Ca^2-dependent subsite in combination with a positively charged region that forms an ionic strength-sensitive subsite (Blanck et al. 1996; Bouyain et al. 2001).

E-Selectin Receptors on Human l Neutrophils: The human neutrophil receptor for E-selectin has not been established. Sialylated glycosphingolipids with 5N-acetyllactosamine (LacNAc, Galβ1-4GlcNAcβ1-3) repeats and 2–3 fucose residues are major functional E-selectin receptors on human neutrophils. E-selectin-expressing cells tethered and rolled on selected glycolipids, whereas P-selectin-expressing cells failed to interact. Results support that the glycosphingolipid NeuAcα2-3Galβ1-4GlcNAcβ1-3[Galβ1-4(Fucα1-3)GlcNAcβ1-3](2)[Galβ1-4GlcNAcβ1-3](2)Galβ1-4GlcβCer (and closely related structures) are functional E-selectin receptors on human neutrophils (Nimrichter et al. 2008; Gege et al. 2008).

Enzymes Involved in the Synthesis of Lewis Antigens: The enzymes involved in the synthesis of Lewis antigens have been identified. FucT-VI is an enzyme involved in the biosynthesis of sLe^x, in myeloid cells (Koszdin and Bowen 1992). Their biosynthesis is complex and involves β1 → 3-galactosyltransferases (β3Gal-Ts) and a combined action of α2- and/or α4-fucosyltransferases (Fuc-Ts). O-glycans with different core structures have been identified, and the ability of β3Gal-Ts and Fuc-Ts to use them as substrates has been tested (Holgersson and Lofling 2006). This knowledge enables us to engineer recombinant glycoproteins with glycan- and core chain-specific Lewis antigen substitution. Such tools may be important for investigations on the fine carbohydrate specificity of Le B-binding lectins, such as Helicobacter pylori adhesins and DC-SIGN, and may also prove useful as therapeutics (Holgersson and Lofling 2006).

28.6.1.3 Transcriptional Control of Expression of Carbohydrate Ligands

Cell adhesion mediated by selectins and their carbohydrate ligands is involved in the adhesion of cancer cells to endothelial cells during the course of hematogenous metastasis of cancer. Extravasation and tissue infiltration of malignant cells in patients with adult T-cell leukemia is mediated by the interaction of selectins and their carbohydrate ligand sialyl-Le^x, which is strongly and constitutively expressed on the leukemic cells. Constitutive expression of Lewis X in these cells is due to the transcriptional activation of Fuc-T VII, the rate-limiting enzyme in the sLe^x synthesis, induced by the Tax protein encoded by the human T-cell leukemia virus-1, the etiological virus for this leukemia. This transactivation is in clear contrast to the regulation of typical CRE-element found in various cellular genes in that it is independent of phosphorylation-dependent regulation. This must be the reason for the strong and constitutive expression of sialyl-Le^x, which exacerbates the tissue infiltration of leukemic cells. This is a good example corroborating the proposition that the abnormal expression of carbohydrate determinant at the surface of malignant cells is intimately associated with the genetic mechanism of malignant transformation of cells (Kannagi 2001).

28.6.2 E-Selectin Ligand-1

E-Selectin-Ligand-1 Is a Variant of FGF-R: A 150 kDa glycoprotein is the major ligand for E-selectin on plasma membranes of myeloid cells. Mouse c-DNA for this E-selectin ligand (ESL-1) predicted amino-acid sequence, which is 94% identical (over 1,078 amino acids) to the

chicken cysteine-rich FGF-R, except for a unique 70-amino-acid aminoterminal domain of mature ESL-1. Fucosylation of ESL-1 is imperative for affinity isolation with E-selectin-IgG. ESL-1, with a structure essentially identical to that of a receptor functions as a cell adhesion ligand of E-selectin (Steegmaier et al. 1995). E-selectin binds to ESL-1 with a fast dissociation rate constant of 4.6 s^{-1} and a calculated association rate constant of 7.4×10^4 m^{-1} s^{-1}. A K_D of 62 μM resembles the affinity of L-selectin binding to glycosylation-dependent cell adhesion molecule-1. The affinity of the E-selectin-ESL-1 interaction did not change significantly from 5°C to 37°C, indicating that the enthalpic contribution to the binding is small at physiological temperatures, and that, in contrast to typical protein-carbohydrate interactions, binding is driven primarily by favorable entropic changes (Wild et al. 2001). MG160 is a conserved membrane sialoglycoprotein of the Golgi apparatus displaying over 90% amino acid sequence identities with two apparently unrelated molecules, namely, a chicken fibroblast growth factor receptor (CFR), and ESL-1 (Steegmaier et al. 1995). MG160 from rat brain membranes binds to b-FGF. The gene for MG160 has been assigned to human chromosome 16q22-23 (Mourelatos et al. 1996).

28.6.3 PSGL-1 and Relating Ligands

The selectin family of adhesion molecules mediates tethering and rolling of leukocytes to the vessel wall in the microcirculation. Selectins promote these interactions by binding to glycoconjugate ligands expressed on apposing cells. Selectin-mediated rolling is a prerequisite for firm adhesion and subsequent transendothelial migration of leukocytes into tissues. PSGL-1 interacts with selectins to support leukocyte rolling along vascular wall. PSGL-1 (CD162 P) is a dimeric mucin-like 120-kDa glycoprotein on leukocyte surfaces that binds to P-, L- and E- selectin and promotes cell adhesion in the inflammatory response (see Chap. 26 and 27). Present understanding on selectin functions reveals that selectins and their ligands have a complex role during inflammatory diseases. Among selectin ligands, mucin PSGL-1 has a well-documented role in organ targeting during inflammation in animal models. Although inhibition of selectins and their ligands in animal models of inflammatory diseases has proven the validity of this approach in vivo, only a limited number of anti-selectin drugs have been tested in humans. Results in clinical trials for asthma and psoriasis show that, although very challenging, the development of selectin antagonists holds concrete promise for the therapy of inflammatory diseases (Rossi and Constantin 2008).

PSGL-1 requires sialylated, fucosylated O-linked glycans and tyrosine sulfate to bind P-selectin. Less is known about the determinants that PSGL-1 requires to bind E-selectin. Tyrosine residues on PSGL-1 expressed in CHO cells were shown to be sulfated. Phenylalanine replacement of three tyrosines within a consensus sequence for tyrosine sulfation abolished binding to P-selectin but not to E-selectin. Results suggest that PSGL-1 requires core 2 O-linked glycans that are sialylated and fucosylated to bind P- and E-selectin. PSGL-1 also requires tyrosine sulfate to bind P-selectin but not E-selectin (Li et al. 1996). Studies using CHO-E and -P monolayers demonstrated that the first 19 amino acids of PSGL-1 are sufficient for attachment and rolling on both E- and P-selectin and that a sialyl Le^x-containing glycan at Threonine-16 was critical for this sequence of amino acids to mediate attachment to E- and P-selectin. However, a sulfated-anionic polypeptide segment within the amino terminus of PSGL-1 is necessary for PSGL-1-mediated attachment to P- but not to E-selectin. In addition, PSGL-1 had more than one binding site for E-selectin: one site located within the first 19 amino acids of PSGL-1 and one or more sites located between amino acids 19 through 148 (Goetz et al. 1997).

28.6.3.1 PSGL-1-Dependent and -Independent E-Selectin Rolling

PSGL-1 supports P-selectin-dependent rolling in vivo and in vitro. However, controversy exists regarding the importance of PSGL-1-dependent and -independent E-selectin rolling. Using antibodies against PSGL-1 and PSGL-$1^{-/-}$ mice, Zanardo et al. (2004) demonstrated abolition of P-selectin-dependent rolling but only partial inhibition of E-selectin-mediated rolling in the cremaster microcirculation following local administration of TNF-α. Data support an E-selectin ligand present on PSGL-$1^{-/-}$ neutrophils that is down-regulatable upon systemic but not local activation. In P- and E-selectin-dependent cutaneous contact hypersensitivity model, binding studies showed no E-selectin ligand down-regulation. In conclusion, it was suggested that E-selectin mediates PSGL-1-dependent and independent rolling and the latter can be down-regulated by systemic activation and can replace PSGL-1 to support the development of inflammation (Zanardo et al. 2004; Shigeta et al. 2008).

28.6.3.2 PSGL-1 Participates in E-Selectin-Mediated Progenitor Homing

The nature and exact function of selectin ligands involved in hematopoietic progenitor cell (HPC) homing to the bone marrow (BM) are not clear. Using murine progenitor homing assays, it was found that PSGL-1plays a partial role in HPC homing to BM. Homing studies with PSGL-1-deficient HPCs pretreated with anti-α4 integrin antibody revealed that PSGL-1 contributes to approximately 60% of E-selectin ligand-mediated homing activity. Results thus underscore a major difference between mature myeloid cells and immature stem/progenitor cells in that E-selectin ligands

cooperate with α4 integrin rather than P-selectin ligands (Katayama et al. 2003). PSGL-1, expressed by primitive human bone marrow $CD34^+$ cells, mediates their adhesion to P-selectin and inhibits their proliferation. Adhesion to E-selectin also inhibits the proliferation of human and mouse $CD34^+$ cells. Furthermore, a subpopulation, which does not contain the most primitive hematopoietic progenitor cells, undergoes apoptosis following E-selectin-mediated adhesion. This shows that PSGL-1 is not the ligand involved in E-selectin-mediated growth inhibition and apoptosis, but an E-selectin ligand(s) other than PSGL-1 transduces growth inhibitory and proapoptotic signals and suggests the requirement of posttranslational fucosylation (Winkler et al. 2004).

28.6.3.3 PSGL-1 Decameric Repeats Regulate Selectin-Dependent Rolling Under Flow Conditions

PSGL-1 regulates leukocyte rolling by binding P-selectin, and also by binding E- and L-selectins with lower affinity. L- and P-selectin bind to N-terminal tyrosine sulfate residues and to core-2 O-glycans attached to Thr-57, whereas tyrosine sulfation is not required for E-selectin binding. PSGL-1 extracellular domain contains decameric repeats, which extend L- and P-selectin binding sites far above the plasma membrane. It was suggested that decamers may play a role in regulating PSGL-1 interactions with selectins. Deletion of decamers abrogated sL-selectin binding and cell rolling on L-selectin, whereas their substitution partially reversed these diminutions. P-selectin-dependent interactions with PSGL-1 were less affected by decamer deletion. Analysis showed that decamers are required to stabilize L-selectin-dependent rolling. However, adhesion assays performed on recombinant decamers demonstrated that they directly bind to E-selectin and promote slow rolling. Therefore, the role of decamers is to extend PSGL-1N terminus far above the cell surface to support and stabilize leukocyte rolling on L- or P-selectin. In addition, they function as a cell adhesion receptor, which supports approximately 80% of E-selectin-dependent rolling (Tauxe et al. 2008). Tomita et al. (2009) investigated cutaneous wound healing in $PSGL\text{-}1^{-/-}$ mice in comparison with $E\text{-selectin}^{-/-}$, $P\text{-selectin}^{-/-}$, and $P\text{-selectin}^{-/-}$ mice treated with an anti-E-selectin antibody. PSGL-1 contributes to wound healing predominantly as a P-selectin ligand and partly as an E-selectin ligand by mediating infiltration of inflammatory cells.

E-selectin binding to PSGL-1 can activate β2 integrin lymphocyte function-associated antigen-1 by signaling through spleen tyrosine kinase (Syk). This signaling is independent of G αi-protein-coupled receptors, results in slow rolling, and promotes neutrophil recruitment to sites of inflammation. An ITAM-dependent pathway involving Src-family kinase Fgr and the ITAM-containing adaptor proteins DAP12 and FcRγ is involved in the initial signaling events downstream of PSGL-1 that are required to initiate neutrophil slow rolling (Zarbock et al. 2008).

28.6.3.4 Hematopoietic Cell E-/L-selectin Ligand (HCELL) (CD44) in Stem Cell Homing

Though a glycoform of PSGL-1 functions as the principal E-selectin ligand on human T lymphocytes, the E-selectin ligand(s) of human hematopoietic progenitor cells (HPCs) is not well defined. While the PSGL-1 expressed on human HPCs is an E-selectin ligand, HSCs express a novel glycoform of CD44 known as hematopoietic cell E-/L-selectin ligand (HCELL) that acts as ligand. Current understanding of the molecular basis of HSC homing describes the fundamental "roll" of HCELL in opening the avenues for efficient HSC trafficking to the bone marrow, the skin and other extramedullary sites (Sackstein 2004). The E-selectin ligand activity of CD44 is expressed on primitive $CD34^+$ human HPCs, but not on more mature hematopoietic cells. Under physiologic flow conditions, this molecule mediates E-selectin-dependent rolling interactions over a wider shear range than that of PSGL-1, and promotes human HPC rolling interactions on E-selectin expressed on human BM endothelial cells (Dimitroff et al. 2001). The LS174T colon carcinoma cell line expresses the CD44 glycoform known as hematopoietic cell E-/L-selectin ligand (HCELL), which functions as a high affinity E- and L-selectin ligand on these cells. Burdick et al. (2006) measured the binding of LS174T cells transduced with CD44 short interfering RNA (siRNA). It indicated that expression of HCELL confers robust and predominant tumor cell binding to E- and L-selectin, highlighting a central role for HCELL in promoting shear-resistant adhesive interactions essential for hematogenous cancer dissemination.

28.6.3.5 CD43 as a Ligand for E-Selectin on CLA^+ Human T Cells

Human memory T cells that infiltrate skin express the carbohydrate epitope cutaneous lymphocyte-associated antigen (CLA). Expression of the CLA epitope on T cells has been described on PSGL-1 and associated with the acquisition of both E-selectin and P-selectin ligand functions. The CD43, a sialomucin expressed constitutively on T cells, can also be decorated with the CLA epitope and serves as an E-selectin ligand. CLA expressed on CD43 is found exclusively on the 125 kDa glycoform bearing core-2-branched O-linked glycans. CLA^+ CD43 from human T cells supported tethering and rolling in shear flow via E-selectin but did not support binding of P-selectin. The identification and characterization of CD43 as a T-cell E-selectin ligand distinct from PSGL-1 expands the role of CD43 in the regulation of T-cell trafficking and provides new targets for the modulation of immune functions in skin (Fuhlbrigge et al. 2006). It has been reported that CD43 on activated T cells functions as an

E-selectin ligand and thereby mediates T cell migration to inflamed sites, in collaboration with PSGL-1, a major P- and E-selectin ligand. CD43 on neutrophils also functions as an E-selectin ligand. Observations suggest that CD43 generally serves as an antiadhesive molecule to attenuate neutrophil-endothelial interactions, but when E-selectin is expressed on endothelial cells, it also plays a proadhesive role as an E-selectin ligand (Matsumoto et al. 2008).

28.6.4 Endoglycan, a Ligand for Vascular Selectins

While several ligands have been characterized on human T cells, monocytes and neutrophils, there is limited information concerning ligands on B cells. Endoglycan (EG) together with CD34 and podocalyxin comprise the CD34 family of sialomucins. EG was previously implicated as an L-selectin ligand on endothelial cells. EG is present on human B cells, T cells and peripheral blood monocytes. Upon activation of B cells, EG increased with a concurrent decrease in PSGL-1. Expression of EG on T cells remained constant under the same conditions. Native EG from several sources (a B cell line, a monocyte line and human tonsils) was reactive with HECA-452, a mAb that recognizes sialyl Lewis X and related structures. Finally, an EG construct supported slow rolling of E- and P-selectin bearing cells in a sialic acid and fucose dependent manner, and the introduction of intact EG into a B cell line facilitated rolling interactions on a P-selectin substratum. These findings indicate that endoglycan can function as a ligand for vascular selectins (Kerr et al. 2008). Basigin (Bsg)/CD147 is a ligand for E-selectin that promotes renal inflammation in ischemia/reperfusion (I/R). Bsg is a physiologic ligand for E-selectin that plays a critical role in renal damage induced by I/R (Kato et al. 2009).

28.6.5 L-Selectin as E-Selectin Ligand

L-Selectin from Human, but Not from Mouse Neutrophils Binds E-Selectin: L-selectin has been suggested as a carbohydrate presenting ligand for E- and P-selectin. However, affinity experiments with an E-selectin-Ig failed to detect L-selectin in the isolated E-selectin ligands from mouse neutrophils. In contrast to mouse neutrophils, L-selectin from human neutrophils could be affinity-isolated as a major ligand from cell extracts using E-selectin-Ig as affinity probe. It was suggested that L-selectin on human neutrophils is a major glycoprotein ligand among very few glycoproteins that could be isolated by an E-selectin affinity matrix. The difference between human and mouse L-selectin suggests that E-selectin-binding carbohydrate moieties are attached to different protein scaffolds in different species (Zollner et al. 1997). L-selectin acquires E-selectin-binding activity following phorbol ester (PMA) treatment of the Jurkat T cell line and anti-CD3/IL-2-driven proliferation of human T lymphocytes in vitro. It appears that L-selectin on human T lymphoblasts is one of the several glycoproteins that interacts directly with E-selectin and contributes to rolling under flow (Jutila et al. 2002).

In addition to endogenous ligands for E-selectin, several proteins are found in cancer cell lines or solid tumors that act as ligands for E, L, and P selectins. E-selectin ligands present on cancer cells are: (1) Glycodelin A (GdA) is primarily produced in endometrial and decidual tissue and secreted to amniotic fluid Glycodelins is expressed in in ovarian cancer can act as an inhibitor of lymphocyte activation and/or adhesion (Jeschke et al. 2009); (2) The cysteine-rich fibroblast growth factor receptor (FGF-R) represents the main E-selectin ligand (ESL-1) on granulocytes. Hepatic stellate cells (HSC) are pericytes of liver sinusoidal endothelial cells, which are involved in the repair of liver tissue injury and angiogenesis of liver metastases. HSC express FGF-R together with FucT7 and exhibit a functional E-selectin binding activity on their cell surface (Antoine et al. 2009). (3) Although B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cell lines do not express the ligand PSGL-1, a major proportion of carbohydrate selectin ligand was carried by another sialomucin, CD43, in NALL-1 cells. CD43 plays an important role in extravascular infiltration of NALL-1 cells and the degree of tissue engraftment of BCP-ALL cells may be controlled by manipulating CD43 expression (Nonomura et al. 2008). (4) Thomas et al. (2009a) identified podocalyxin-like protein (PCLP) as an alternative selectin ligand. PCLP on LS174T colon carcinoma cells possesses E-/L-, but not P-, selectin binding activity. PCLP functions as an alternative acceptor for selectin-binding glycans. The finding that PCLP is an E-/L-selectin ligand on carcinoma cells offers a unifying perspective on the apparent enhanced metastatic potential associated with tumor cell PCLP overexpression and the role of selectins in metastasis (Thomas et al. 2009b). (5) E-selectin has been shown to play a pivotal role in mediating cell-cell interactions between breast cancer cells and endothelial monolayers during tumor cell metastasis. The counterreceptor for E-selectin was found as CD44v4. However, CD44 variant (CD44v) isoforms was functional P-, but not E-/L- selectin ligands on colon carcinoma cells. Furthermore, a ~180-kDa sialofucosylated glycoprotein(s) mediated selectin binding in CD44-knockdown cells. This glycoprotein was identified as carcinoembryonic antigen (CEA). CEA serves as an auxiliary L-selectin ligand, which stabilizes L-selectin-dependent cell rolling against fluid shear (Thomas et al. 2009b). Zen et al. (2008) identified a ~170 kDa human

CD44 variant 4 (CD44v4) as E-selectin ligand. Purified CD44v4 has a high affinity for E-selectin via sLe^x moieties.

28.7 Structural Properties of E-Selectin

28.7.1 Soluble E-Selectin: An Asymmetric Monomer

The gene coding for a soluble form of human E-selectin (sE-selectin) has been expressed in CHO cells. The sE-selectin showed a broad band of M_r 75 kDa on nonreducing SDS-PAGE and eluted with M_r 310 kDa from size exclusion chromatography. Matrix-assisted laser-desorption MS gave a molecular weight of 80 kDa, while the minimum monomer molecular weight from the gene sequence should be 58.571 kDa, demonstrating that the monomeric molecule thus expressed had 27% carbohydrate. Equilibrium ultracentrifugation gave an average solution molecular weight of 81.6 kDa. Velocity ultracentrifugation gave a sedimentation coefficient of 4.3S and, from this, an apparent axial ratio of 10.5:1, assuming a prolate ellipsoid of revolution. An analysis of the NMR NOESY spectra of sE-selectin, sialyl-$Lewis^x$, and sE-selectin with sialyl-$Lewis^x$ demonstrated that the recombinant protein binds sialyl-$Lewis^x$ productively. Hence, in solution, sE-selectin is a functional elongated monomer (Hensley et al. 1994). E-selectin-expressed in COS cells bind the promyelocytic cell line HL-60 by a Ca^{2+} dependent mechanism. Although E-selectin is homologous to mammalian lectins, its interaction with HL-60 cells is not inhibited by simple carbohydrate structures. E-selectin-expressing COS cells also bind human neutrophils and the human colon carcinoma cell line HT-29, but not the B-cell line Ramos (Hession et al. 1990).

The three-dimensional structure of the ligand-binding region of human E-selectin reveals limited contact between the two domains and a coordination of Ca^{2+} not predicted from other C-type lectins. Structure/function analysis indicates a defined region and specific amino-acid side chains that might be involved in ligand binding. The features of E-selectin/ligand interaction have important implications for understanding the recruitment of leukocytes to sites of inflammation. The selectins bind weakly to sialyl Lewis-X (sLe^X)-like glycans, but with high-affinity to specific glycoprotein counterreceptors, including PSGL-1. The crystal structure of human E-selectin constructs containing the lectin and EGF (LE) domains co-complexed with sLe^X has been elucidated (Fig. 28.3) (Somers et al. 2000). Somers et al. (2000) also solved crystal structure of P-selectin-lectin and EGF domains co-complexed with the N-terminal domain of human PSGL-1 modified by both tyrosine sulfation and sLe^X. These structures reveal differences in binding of E- and P-selectin to sLe^X and the molecular basis of interaction between P-selectin and PSGL-1.

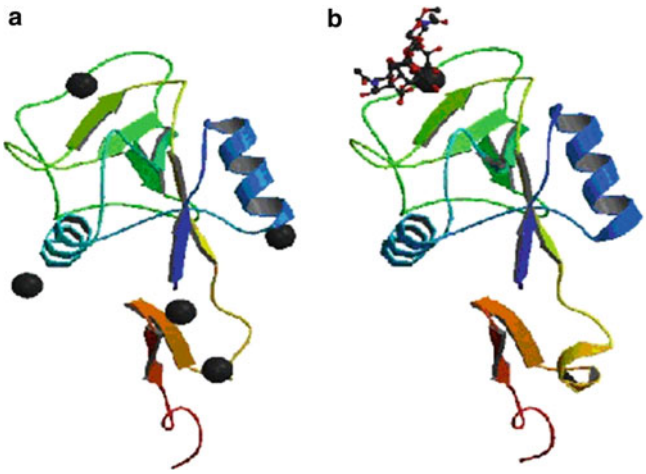

Fig. 28.3 (**a**) Crystal structure of E-selectin lectin-EGF domains (PDB ID: 1ESL) (Graves et al. 1994); (**b**) crystal structure of E-selectin lectin-EGF domains complexed with s-Le^x (PDB ID: 1G1T; Somers et al. 2000). Serum sE-selectin ranges from 0.13 to 2.8 ng/ml, suggesting that even in the absence of overt inflammatory processes E-selectin is being synthesized and released into the bloodstream. In addition, bacteremic patients with hypotension, but not those without, showed markedly elevated sE-selectin values. The blood-derived form of E-selectin is biologically active

28.7.2 Complement Regulatory Domains

Studies have shown that different animal species express E-selectin mRNAs that encode different numbers of complement regulatory (CR) domains. The isolation of these two rat E-selectin cDNA fragments, which differ only for the presence of CR5, represents the first direct evidence for the existence of E-selectin CR-variant mRNAs in the same species. Moreover, the sequence of the $CR5^-$ cDNA is consistent with its origin from an mRNA splice variant of a $CR5^+$ mRNA. Billups et al. (1995) demonstrated the presence of the two predicted mRNA species in rat heart tissue and investigated their expression in response to LPS. Although both mRNA variants were greatly induced by LPS, the $CR5^-$ form was more abundant in both treated and control tissues. This difference in mRNA abundance indicates different levels of CR5 variant proteins that perform functionally distinct tasks in E-selectin dependent inflammatory processes (Billups et al. 1995).

Consensus Repeat Domains: To study the structural characteristics of E-selectin necessary for mediating cell

adhesion, Li et al. (1994) examined the role of consensus repeat domains in E-selectin function. E-selectin containing all six consensus repeat domains (Lec-EGF-CR6) at its COOH terminus was the most potent in blocking neutrophil or HL-60 cell adhesion to either immobilized E-selectin or cytokine-stimulated HUVE cells. Therefore, although the lectin and EGF domains are necessary and sufficient for mediating cell adhesion, the additional six consensus repeat domains, present in native E-selectin, contribute to the enhanced binding of E-selectin to its ligand (Li et al. 1994).

Modulation by Metal Ions: Since E-selectin recognizes carbohydrate ligands in a Ca^{2+}-dependent manner, Anostario and Huang (1995) examined the E-selectin structure by limited proteolysis. Apo-Lec-EGF-CR6, a Ca^{2+}-free form of soluble E-selectin containing the entire extracellular domain, was sensitive to limited proteolysis by Glu-C endoproteinase. Amino-terminal sequencing analysis of the proteolytic fragments revealed that the major cleavage site is at Glu98, which is in the loop (residues 94–103) adjacent to the Ca^{2+} binding region of the lectin domain. Upon Ca^{2+} binding, Lec-EGF-CR6 was protected from proteolysis. This Ca^{2+}-dependent protection was augmented upon sialyl Lewis x (sLex) binding. This implied that Ca^{2+} binding to E-selectin induces a conformational change and perhaps facilitates ligand binding. Ba^{2+} was a potent antagonist in blocking Lec-EGF-CR6-mediated HL-60 cell adhesion. Sr2 + also bound to apo-Lec-EGF-CR6 tighter than Ca^{2+} Thus, E-selectin function can be modulated by different metal ions (Anostario and Huang 1995).

28.7.3 Three-Dimensional Structure

The three-dimensional structure of the ligand-binding region of human E-selectin has been determined at 2.0 A resolution. The structure reveals limited contact between the two domains and a coordination of Ca^{2+} not predicted from other C-type lectins. Structure/function analysis indicates a defined region and specific amino-acid side chains that may be involved in ligand binding. These features of the E-selectin/ligand interaction have important implications for understanding the recruitment of leukocytes to sites of inflammation (Graves et al. 1994). Surface presentation of adhesion receptors influences cell adhesion, although the mechanisms underlying these effects are not well understood. The orientation and length of an adhesion receptor influences its rate of encountering and binding a surface ligand but does not subsequently affect the stability of binding (Huang et al. 2004).

28.8 Functions of E-Selectin

28.8.1 Functions in Cell Trafficking

In antigen-challenged mice a significant increase in leukocyte rolling and adhesion, and a very dramatic increase in emigration have been observed for 24 h. Although rolling and adhesion was dramatically blunted in P-selectin- or P selectin/ICAM-1-deficient mice, emigrated cell number was similar to that observed in wild-type mice. Leukocyte rolling, adhesion and emigration were almost totally abrogated over in E/P-selectin-deficient mice, demonstrating that antigen-induced leukocyte recruitment can be entirely disrupted in the absence of both endothelial selectins (McCafferty et al. 2000). The absence of P-selectin, and not E-selectin, resulted in an altered adhesion environment with subsequent expansion of megakaryocyte progenitors and immature megakaryoblasts, enhanced secretion of TGF-β1, and apparent increased responsiveness to inflammatory cytokines (Banu et al. 2002; Khew-Goodall et al. 1993).

In vitro assays suggest that both E-selectin and chemokines can trigger arrest of rolling neutrophils, but E-selectin$^{-/-}$ mice have normal levels of adherent neutrophils in inflamed venules (Asaduzzaman et al. 2009). Smith et al. (2004) showed that E-selectin- and chemokine-mediated arrest mechanisms are overlapping and identified CXCR2 as an important neutrophil arrest chemokine in vivo.

28.8.1.1 Trafficking of Th1 Cells to Lung Depends on Selectins

Expression of CD62E and CD62P is induced in the lungs of mice primed and then challenged with intratracheal (i.t.) SRBC. In addition, endogenous lymphocytes accumulate in the lungs of E- and P-selectin-deficient ($E^{-/-}P^{-/-}$) mice after i.t. SRBC challenge. $E^{-/-}P^{-/-}$ mice showed a 85–95% decrease in $CD8^+$ T cells and B cells in the lungs at both early and late time points (Curtis et al. 2002). Both P- and E-selectin play an important role in Th1 lymphocyte migration to lung (Clark et al. 2004). Moreover, Yiming et al. (2005) suggested that interactions of circulating inflammatory cells with P-selectin critically determine proinflammatory endothelial activation during high tidal volume ventilation.

Changes in P-Selectin/ICAM-1 in Mutant Mice Are Organ-Specific: Mice homozygous for an E-selectin null mutation were viable and exhibited no obvious developmental alterations. Neutrophil emigration during an inflammatory response is mediated through interactions between adhesion molecules on endothelial cells and neutrophils.

P-Selectin mediates rolling or slowing of neutrophils, while ICAM-1 contributes to the firm adhesion and emigration of neutrophils. Removing the function of either molecule partially prevents neutrophil emigration. While mice with either mutation alone showed a 60–70% reduction in acute neutrophil emigration into the peritoneum during *Streptococcus pneumoniae*-induced peritonitis, double mutant mice showed a complete loss of neutrophil emigration. In contrast, neutrophil emigration into the alveolar spaces during acute *S. pneumoniae*-induced pneumonia was normal in double mutant mice. These results demonstrated organ-specific differences, since emigration into the peritoneum requires both adhesion molecules while emigration into the lung requires neither (Bullard et al. 1996).

28.8.1.2 Cooperation Between E-Selectin and CD18 Integrin

CD18-deficient mice ($CD18^{-/-}$ mice) suffer from a severe leukocyte recruitment defect in some organs, with no detectable defect in other models. Mice lacking E-selectin ($CD62E^{-/-}$ mice) have either no defect or a mild defect of neutrophil infiltration, depending on the model. The $CD18^{-/-}CD62E^{-/-}$, but not $CD18^{-/-}CD62P^{-/-}$, mice generated by cross-breeding failed to thrive, reaching a maximum body weight of 10–15 g. To explore the mechanisms underlying reduced viability, Forlow et al. (2000) suggested that the greatly reduced viability of $CD18^{-/-}CD62E^{-/-}$ mice appeared to result from an inability to mount an adequate inflammatory response. This study showed that cooperation between E-selectin and CD18 integrins is necessary for neutrophil recruitment and that alternative adhesion pathways cannot compensate for the loss of these molecules (Forlow et al. 2000).

28.8.1.3 Selectin-Independent Leukocyte Rolling and Adhesion

To study selectin-independent leukocyte recruitment and the role of ICAM-1, Forlow and Ley (2001) generated mice lacking all three selectins and ICAM-1 ($E/P/L/I^{-/-}$) by bone marrow transplantation. A striking similarity of leukocyte adhesion efficiency in $E/P/L^{-/-}$ and $E/P/I^{-/-}$ mice suggests a pathway in which leukocyte rolling through L-selectin required ICAM-1 for adhesion and recruitment. Comparison of data with mice lacking individual or other combinations of adhesion molecules revealed that elimination of more adhesion molecules further reduces leukocyte recruitment but the effect is less than additive.

28.8.2 E-Selectin in Neutrophil Activation

Neutrophil adherence to activated endothelium mediated by E-selectin is reversibly inhibited by hypothermia. The protective effect of hypothermia (e.g., in cardiopulmonary bypass) may, in part, be mediated by transiently inhibiting the expression of an endothelial cell activation phenotype (Haddix et al. 1996). In transgenic mice, the number of blood neutrophils was reduced, without any other obvious phenotype or tissue damage. These neutrophils, however, displayed two significant changes: first, an alteration in the levels of expression of two membrane receptors involved in neutrophil adhesion to endothelial cells, namely a marked increased in the Mac-1 antigen (CD11b/CD18) and a decrease in L-selectin; second, an increased oxidative activity when compared to blood neutrophils of non-transgenic mice. These observations indicated that the binding of E-selection with neutrophils bearing its ligands promotes neutrophil activation in vivo (Araki et al. 1996). Mac-1 (CD11b/CD18) is the major neutrophil glycoprotein decorated with sLe^x and ligation of these carbohydrate moieties significantly impairs neutrophil functions. Protein-binding assays indicate that sLe^x moieties on Mac-1 are critical for binding interaction of Mac-1 to E-selectin. Ligation of Mac-1 sLe^x by anti-sLe^x antibody induces a significant degranulation of neutrophil secondary granules at the absence of chemoattractant stimulation. This "dysregulated" degranulation induced by anti-sLe^x antibody strongly inhibits neutrophil transmigration in response to formyl-Met-Leu-Phe (fMLP). Thus, Mac-1 sLe^x moieties play a critical role in regulating β2 integrin functions during neutrophil transmigration and degranulation (Zen et al. 2007).

However, E-selectin-deficient mice displayed no significant change in the trafficking of neutrophils in several models of inflammation, although blocking both endothelial selectins by treatment of the E-selectin-deficient animals with an anti-murine P-selectin antibody significantly inhibited neutrophil emigration in two distinct models of inflammation. Labow et al. (1994) demonstrated that the majority of neutrophil migration in both models requires an endothelial selectin but that E-selectin and P-selectin are functionally redundant. Studies on the functional effects of mAbs against murine E-selectin on neutrophil recruitment in vivo, leukocyte rolling and circulating leukocyte concentrations in vivo, and adhesion of myeloid cells to E-selectin transfectants and recombinant E-selectin-IgG fusion protein in vitro indicated that E-selectin serves a function, other than rolling, that appears to be critically important for neutrophil recruitment to inflammatory sites in Balb/c mice (Ramos et al. 1997).

28.8.3 P- and E-Selectin in Differentiation of Hematopoietic Cells

The P- and E-selectins are critically important for adhesion and homing of hematopoietic progenitor cells (HPC) into the

bone marrow. The most primitive HPC capable of long-term in vivo repopulation express PSGL-1, a receptor common to both P- and E-selectin. In addition, P-selectin delays the differentiation of HPC whereas E-selectin enhances their differentiation along the monocyte/granulocyte pathway, describing different roles for these selectins in the regulation of hematopoiesis. Thus, the two endothelial selectins, E-selectin and P-selectin, have very different effects on HPC. E-selectin accelerates the differentiation of maturing HPC towards granulocyte and monocyte lineages while maintaining the production of more immature CFU-S(12) in ex vivo liquid suspension culture. In marked contrast, P-selectin delays the differentiation of Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ cells, allowing enhanced ex vivo expansion of CFC and CFU-S(12) but not HSCs (Eto et al. 2005; Schweitzer et al. 1996).

Role in Loose Adhesion of Allogeneic Lymphocytes and in Structural and Functional Lung Alterations: The interaction between host lymphocytes and graft endothelial cells plays an important role in graft rejection. Isogeneic perfusion induced nonspecific endothelial cell activation, which was characterized by up-regulation of E-selectin, ICAM-1, and of TNF-α. It was revealed that E-selectin expression (1) is not a consequence of TNF-α triggering, (2) E-selectin expression up-regulates its own expression and expression of I-A, VCAM-1, TNF-α, and lymphotoxin-α mRNAs, and down-regulates expression of LFA-3 and ICAM-1 mRNAs. Study indicated that the E-selectin plays major role in the loose adhesion of allogeneic lymphocytes and in structural and functional lung alterations (Joucher et al. 2004).

Memory B Lymphocytes from Secondary Lymphoid Organs Interact with E-Selectin: A subset of human tonsillar B cells that interact with E-selectin but not with P-selectin have a phenotype of non-germinal center ($CD10^-$, $CD38^-$, $CD44^+$), memory (IgD^-) cells. Furthermore, FucT-VII is expressed selectively in $CD44^+$ E-selectin-adherent B lymphocytes. These results assigned resident memory B lymphocytes a adhesion function, the rolling on E-selectin that provides insights on the adhesion pathways involved in homing of memory B cells to tertiary sites (Montoya et al. 1999).

28.8.4 Transmembrane Signaling in Endothelial Cells

In addition to supporting rolling and stable arrest of leukocytes, there is increasing evidence that E-selectin functions in transmembrane signaling into endothelial cells during these adhesive interactions. Adhesion of HL-60 cells (which express ligands for E-selectin), or antibody-mediated cross-linking of E-selectin, results in the formation of a Ras/Raf-1/phospho-MEK macrocomplex, extracellular signal-regulated protein kinase (ERK1/2) activation, and c-fos up-regulation. These downstream events require an intact cytoplasmic domain of E-selectin. Tyrosine-603 plays an important role in mediating the association of E-selectin with SHP2, and the catalytic domain of SHP2, in turn, is critical for E-selectin-dependent ERK1/2 activation. Events suggest that cross-linking of E-selectin by engagement of ligands on adherent leukocytes can initiate a multifunctional signaling pathway in the activated endothelial cell at the sites of inflammation (Hu et al. 2001).

Src-Family Kinases Mediate an Outside-In Signal: E-selectin-ligand engagement differs between lymphocytes and PMN, and that these differences may be accentuated by the CR1 and CR2 domains in the E-selectin (Hammel et al. 2001). In cell suspensions subjected to high-shear rotatory motion, human PMN adhered to E-selectin-expressing CHO (CHO-E), and formed homotypic aggregates when challenged by E-selectin-IgG fusion protein, by a mechanism that involved β2 integrins. It appeared that Src-family kinases, and perhaps Pyk2, mediate a signal necessary for β2 integrin function in PMN tethered by E-selectin (Totani et al. 2006).

E-Selectin Is Unique in Clustering Sialylated Ligands and Transducing Signals: Two adhesive events critical to efficient recruitment of neutrophils at vascular sites of inflammation are up-regulation of endothelial selectins that bind sLe^x ligands and activation of β2-integrins that support neutrophil arrest by binding ICAM-1. Neutrophils rolling on E-selectin is sufficient for signaling cell arrest through β2-integrins binding of ICAM-1 in a process dependent upon ligation of L-selectin and PSGL-1. Spatial and temporal events showed that binding of E-selectin to sLe^x on L-selectin and PSGL-1 drives their co-localization into membrane caps at the trailing edge of neutrophils rolling on HUVECs and on an L-cell monolayer co-expressing E-selectin and ICAM-1. Inhibition of p38 and p42/44 mitogen-activated protein kinase blocked the cocapping of L-selectin and PSGL-1 and the subsequent clustering of high-affinity β2-integrins. Results suggest that E-selectin is unique among selectins in its capacity for clustering sialylated ligands and transducing signals leading to neutrophil arrest in shear flow (Green et al. 2004).

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Part X

C-Type Lectins: Lectin Receptors on NK Cells

KLRB Receptor Family and Human Early Activation Antigen (CD69)

29

Rajesh K. Gupta and G.S. Gupta

29.1 Lectin Receptors on NK Cell

29.1.1 NK Cell Receptors

Natural killer cells are important component of the innate immune system, providing protection against intracellular infection particularly viruses and also neoplasia through direct cytotoxic mechanisms and the secretion of cytokines. They mediate their effects through direct cytolysis, release of cytokines and regulation of subsequent adaptive immune responses. They are called 'natural' killers because, unlike cytotoxic T cells, they do not require a previous challenge and preactivation to become active. NK cells can be activated by a range of soluble factors, including type I interferons, IL-2, IL-12, IL-15 and IL-18, but also by direct cell to cell contact between NK cell receptors and target cell ligands. NK cells possess an elaborate array of receptors, which regulate NK cytotoxic and secretory functions upon interaction with target cell MHC class I proteins. Determination of structures of NK cell receptors and their ligand complexes has led to a fast growth in our understanding of the activation and ligand recognition by these receptors as well as their function in innate immunity. B and T cells significantly and differentially influence the homeostasis and the phenotype of NK cells. The function of NK cell is tightly regulated by a fine balance of inhibitory and activating signals that are delivered by a diverse array of cell surface receptors. A prerequisite for a NK cell attack is the presence on target cells of ligands for activating receptors and low level or absence of ligands for inhibitory receptors. It was believed that NK self-tolerance was achieved by expression on each NK cell of at least one self-MHC specific inhibitory receptor. However, this dogma has been challenged after identification of a NK cell population in normal mice that lack inhibitory receptors specific for self-MHC class I molecules (Kumar and McNerney 2005; Fernandez et al. 2005). Therefore, it was made clear that some additional surface receptors contribute to NK self-tolerance and to the modulation of NK cell responses. The characterization and the identification of their physiological ligands allow us a comprehensive understanding of NK cell function.

NK cell receptors (NKR) include: (1) non-HLA class I–specific receptors, such as CD56, CD57, or CD161 (Lee et al. 2003), (2) as well as receptors that recognize HLA class I molecules or their structural relatives. Later group comprises killer receptors belonging to the immunoglobulin family (KIR), such as KIR2DL1/S1 and KIR2DL2/3/S2 (Moretta and Moretta 2004) or immunoglobulin-like transcripts (ILTs), which are expressed mainly on B, T, and myeloid cells, although some members are also expressed on NK cells. The immunoglobulin-like transcripts (ILTs) include CD94/NKG2 heterodimers (also called KLRD/KLRC), rodent Ly49 (KLRA), NKG2D (KLRK), NKR-P1 (KLRB), and KLRG1 belonging to the C-type lectin receptor family, called killer cell lectin-like receptors (KLR approved symbol) (Borrego et al. 2001; Vetter et al. 2000). The superfamily of KLRs was originally discovered in NK cells in rats. Functionally, NK cell surface receptors are divided into two groups, the inhibitory and the activating receptors. Classically, inhibitory signals are mediated by receptors that recognize MHC class I molecules or their structural relatives such as MICA, ULBP, RAE-1, and H-60. The inhibitory form of NK receptors provides the protective immunity through recognizing class I MHC molecules with self-peptides on healthy host cells (Raulet et al. 2001; Yokoyama and Plougastel 2003).

Recent studies also suggest that MHC class I-independent inhibitory signals can also result in inhibition of cytotoxic cells and some NK cell specificities may be conveyed by orphan receptors expressed on NK cells (Lebbink and Meyaard 2007; Kumar and McNerney 2005). The recent identification of non-MHC class I ligands for inhibitory immune receptors, such as KLRG1, KLRB1 and LAIR-1, indicates that MHC class I-independent inhibitory immune receptors play crucial roles in inducing peripheral tolerance.

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The presence of these receptors on many other immune cell types besides effector cells suggests that tight regulation of cell activation is necessary in all facets of the immune response in both normal and diseased tissue. Lebbink and Meyaard (2007) gave an overview of the known ligand-receptor pairs by grouping the ligands according to their properties and discussed implications of these interactions for the maintenance of immune balance and for the defense against tumors and pathogens (Lebbink and Meyaard 2007). Different mechanisms, through which NK cells can be activated, are shown in Fig. 29.1 (also see Fig. 31.1 in Chap. 31).

29.1.2 NKC Gene Locus

The human NK cell gene locus (NKC) is located on the short arm of chromosome 12 and contains a number of genes encoding C-type lectin receptors important for NK cell function. A part of human chromosome 12p12.3-p13.2 containing NKC has been sequenced. Genes among them are: CD94 and the five NKG2 genes. A detailed analysis shows that all six genes are found within a region of 100–200 kb proximal of the marker D12S77. The gene order established is D12S77—CD94—NKG2D—NKG2F—NKG2E—NKG2C—NKG2A. The NKG2 genes are of identical transcriptional orientation, whereas the CD94 gene is placed in opposite orientation. The tight genomic linkage of these genes and the identical orientation of the NKG2 genes suggest coordinate regulation of expression during differentiation of NK cells (Renedo et al. 2000; Sobanov et al. 1999). Renedo et al. (2000) localized 17 genes, 5 expressed sequence tags, and 49 STSs within this contig and established the order of the genes as tel-M6PR-MAFAL (HGMW-approved symbol KLRG1)-A2M-PZP-A2MP-NKRP1A (HGMW-approved symbol KLRB1)-CD69-AICL (HGMW-approved symbol CLECSF2)-KLRF1-OLR1-CD94 (HGMW-approved symbol KLRD1)-NKG2D (HGMW-approved symbol D12S2489E)-PGFL-NKG2F (HGMW-approved symbol KLRC4)-NKG2E (HGMW-approved symbol KLRC3)-NKG2A (HGMW-approved symbol KLRC1)-LY49L (HGMW-approved symbol KLRA1)-cen (Renedo et al. 2000).

In marmoset monkey, NKC is 1.5 times smaller than its human counterpart, but the genes are colinear and orthologous. One exception is the activating NKG2CE gene, which is probably an ancestral form of the NKG2C- and NKG2E-activating receptor genes of humans and great apes. Analyses of NKC genes in nine additional marmoset individuals revealed a moderate degree of polymorphism of the CD94, NKG2A, NKG2CE, and NKG2D genes. Furthermore, expression analyses identified several alternatively spliced transcripts, particularly of the CD94 gene. Several products of alternative splicing of NKC genes are highly conserved among primates (Averdam et al. 2007).

29.2 The Ever-Expanding Ly49 Receptor Gene Family

29.2.1 Activating and Inhibitory Receptors

The molecular mechanism that explains why NK cells do not kill indiscriminately has been elucidated. It is due to several specialized receptors that recognize MHC class I molecules expressed on normal cells. The lack of expression of one or more HLA class I alleles leads to NK-mediated target cell lysis. Different types of receptors specific for groups of HLA-C, HLA-B, and, later HLA-A alleles had been identified. While in most instances, they function as inhibitory receptors, an activatory form of the HLA-C-specific receptors has been identified in some donors. The mouse lectin-related Ly49 family and the human KIR family represent structurally distinct, yet functionally analogous, class I MHC receptors that are expressed on NK cells and some T cells. The functional similarity of these two families has been borne out by the demonstration of identical signal transduction pathways associated with each receptor family. The Ly49 family therefore provides a useful model system to study the role of this class of receptors in the regulation of the immune system. Reports relating to the Ly49 repertoire in several mouse strains have revealed an additional evolutionary parallel between KIR and Ly49 receptor families. There is now an appreciation of the variation in the number and type of Ly49s expressed in different mouse strains, similar to the previously demonstrated differences in the number of KIR genes found in humans. Different reviews have appeared describing properties of members of the Ly49 gene family, their MHC class I recognition and associated signal transduction pathways. The Ly49 receptor repertoire may be initially generated by a stochastic process that distributes receptors randomly to different cells and treats the two alleles of a given Ly49 gene independently. However, class I-MHC-dependent “education” processes shape the functional repertoire. The education processes silence potentially auto-aggressive NK cells, probably by ensuring that each NK cell expresses at least one self-specific Ly49 receptor. In addition, NK cell clones that express multiple self-specific Ly49 receptors are disfavored by the education processes, perhaps to confer greater discrimination on to individual NK cells (Raulet et al. 2001; Anderson et al. 2001).

29.2.1.1 Two Structurally Dissimilar NK Cell Receptor Families, Ly49 and KIR

In mice, C-type lectin receptors are represented by the Ly49 family of receptors, whereas in humans, NK cells express the distantly related CD94, which forms MHC class I-specific heterodimers with NKG2 family members. The Ly49 family of receptors is encoded by a highly polymorphic multigene

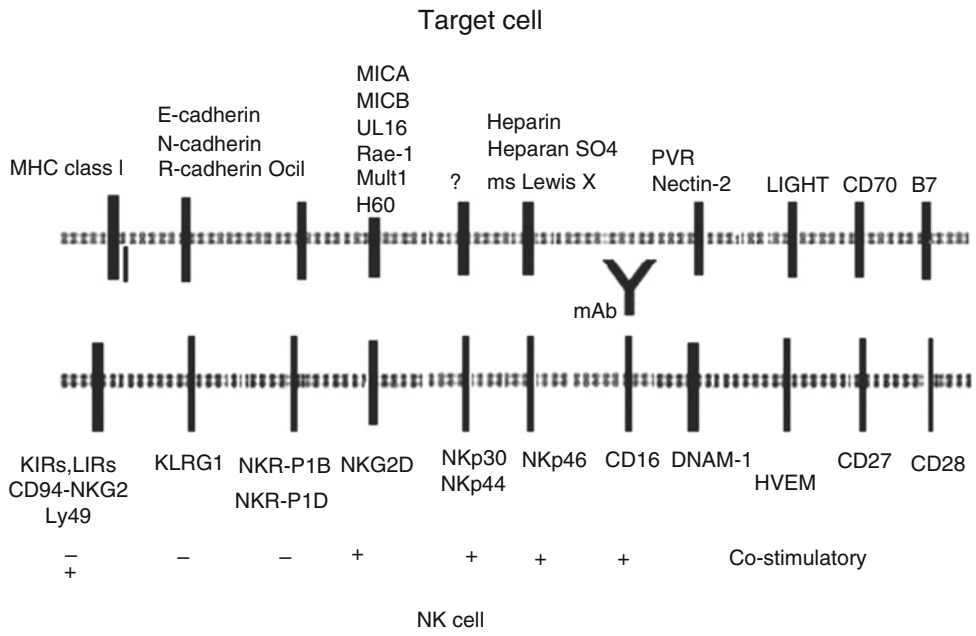

Fig. 29.1 The activating or the non-inhibitory NK receptors mediate the killing of tumor or virally infected cells through their specific ligand recognition. During target cell recognition, these cells receive both activating and inhibitory signals. Integration of opposing signals from two types of receptors determines the appropriate response: activation or inhibition of NK cells

family in mouse and is also present in multiple copies in rat. However, this gene exists as a single copy in primates and is mutated to non-function in humans. Humans also express MHC class I-specific p50/p58/p70 family of Ig-like receptors, and have been identified in mice. One Ly49-like gene, Ly49L, exists in humans but is incorrectly spliced and assumed to be nonfunctional. Mouse KIR-like genes have not been found, and the evidence suggests that the primate KIRs amplified after rodents and primates diverged. Thus, two structurally dissimilar families, Ly49 and KIR, had evolved to play similar roles in mouse and human NK cells respectively. It is not known, however, when the Ly49L gene became nonfunctional and if this event affected the functional evolution of the KIRs. The distribution of these gene families in different mammals has not been well studied, nor it is known if any species uses both types of receptors. However, the Ly49L gene shows evidence of conservation in other mammals and that the human gene likely became nonfunctional 6–10 million years ago. Furthermore, baboon lymphocytes express both full-length Ly49L transcripts and multiple KIR genes (Mager et al. 2001).

Interestingly, many activating and inhibitory receptors come in pairs, with a strong homology of the extracellular domains and partially overlapping ligand specificity but opposite signaling capacities. Examples for these paired receptors are KIRs, which comprise inhibitory and activating receptors, both recognizing the same major MHC class I ligand. Also in the family of KLRs can be found antithetic pairs that are specific for the same ligand but transmit opposing signals. For example, the NKG2A-CD94 heterodimer transmits an inhibitory signal and NKG2C-CD94 signals in an activating fashion while both receptors recognize the same ligand, HLA-E. The structures of activating and inhibitory NK cell surface receptors and their complexes with ligands and complexes with class I MHC homologs have been reviewed (Dimasi et al. 2004; Natarajan et al. 2002; Radaev and Sun 2003). Although CD94/NKG2 is expressed in both humans and rodents, KIRs are only expressed in humans and Ly49s only in rodents. Examples of other C-type lectin-like NK cell receptors that occur as individual genes are CD69, and activation-induced C-type lectin (AICL).

29.2.1.2 Ly49 Gene Expression in Different Inbred Mouse Strains

Mouse NK cells family of Ly49 contains at least 23 expressed members (A-w) and is further subdivided into activating and inhibitory subfamilies based on intracellular and transmembrane characteristics. The level of sequence identity between different members varies dramatically. However, comparison of the extracellular domain has revealed that several of the Ly49 molecules also form "pairs," where one member is activating and the other is inhibitory. Until recently, most Ly49 molecules described have come from the C57B1/6 strain of inbred mice. Using molecular cloning and immunochemical analysis it has been revealed that different mouse strains express novel Ly49

molecules. Comparison of the allelic forms of some Ly49 molecules has shown that the dividing line between different genes and different alleles is blurred (Deng and Mariuzza 2006; Deng et al. 2008).

29.2.1.3 Mouse Ly49 NK Receptors: Balancing Activation and Inhibition

In addition to lysis, a major consequence of triggering the murine activating NK receptor Ly49D is the expression of cytokines and chemokines. The activating Ly49D murine NK cell receptor can potently synergize during co-stimulation with IL-12 and IL-18 for selective production of IFN-γ. Activation both in vitro and in vivo and synergistic production of IFN-γ by Ly49D expressing NK cells results from cytokine stimulation combined with co-receptor ligation. Costimulation of the activating Ly49D murine NK cell receptor with IL-12 or IL-18 is capable of over-riding the inhibitory Ly49G2 receptor blockade for cytokine production both in vitro and in vivo. This synergy is mediated by and dependent upon Ly49D-expressing NK cells and results in significant systemic expression of IFN-γ. This would place NK cells and their activating Ly-49 receptors as important initiators of microbial, antiviral, and antitumor immunity and provide a mechanism for the release of activating Ly49 receptors from inhibitory receptor blockade (Ortaldo and Young 2003, 2005) (Fig. 29.2).

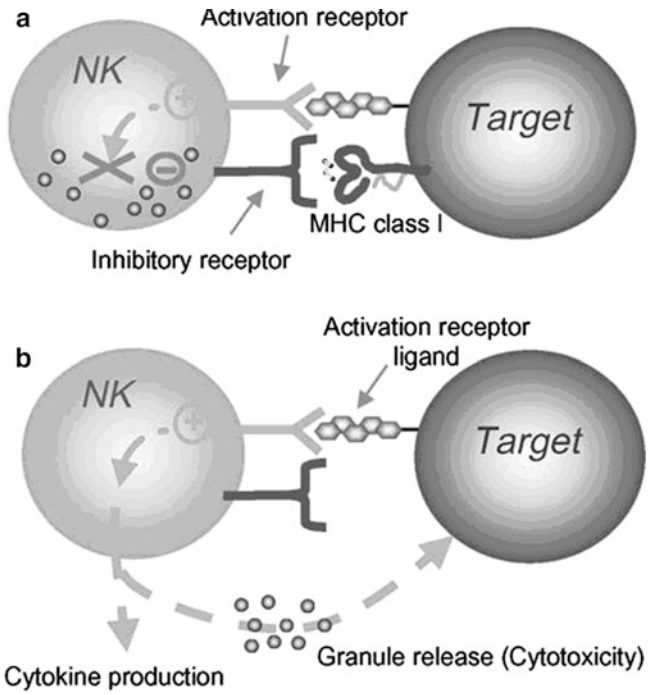

Fig. 29.2 NK cell activation is controlled by the integration of signals from activation and inhibitory receptors. (**a**) Inhibitory NK cell receptors recognize self MHC class I and restrain NK cell activation. (**b**) When unimpeded by the inhibitory receptors, binding of NK cell activation receptors to their ligands on target cells results in NK cell stimulation. In the absence or downregulation of self MHC class I on the target cells, these stimulatory signals are no longer suppressed, resulting in NK cell responses including cytokine production and granule release leading to cytotoxicity. Note that this model indicates that NK cells do not kill by default; that is, when MHC class I inhibition is absent, the NK cell must still be stimulated through activation receptors. Moreover, whether or not an individual NK cell is activated by a target is determined by this complex balance of receptors with opposing function and expression of the corresponding ligands. In general, however, inhibition dominates over activation. Finally, NK cells can be directly stimulated by cytokines such as interleukin-12 that trigger the production of other cytokines by NK cells (not shown). These direct cytokine-mediated responses are not affected by MHC class I expression (Reproduced from French and Yokoyama 2004)

29.2.2 Crystal Analysis of CTLD of Ly49I and comparison with Ly29A, NKG2D and MBP-A

Dimasi and Biassoni (2005) reviewed the functions and X-ray crystal structure of the Ly49 NK cell receptors (Ly49A and Ly49I) (Fig. 29.3a, b), and the structural features of the Ly49/MHC class I interaction as revealed by the X-ray crystal structures of Ly49A/H-2D^d and the Ly49C/H-2K^b. The Ly49 monomer consists of two α-helices (α1 and α2), and two anti-parallel β-sheets formed by β-strands β0, β1, β5; β2, β2′, β3 and β4 (Fig. 29.3). To form the Ly49 homodimer, the monomers associate through strand β0, creating an extended anti-parallel β-sheet. In Ly49A, the C-terminal ends of the α2 helices pack against one another, creating a 'closed' dimer (Dimasi and Biassoni 2005). In Ly49C and Ly49I, however, the α2 helices are not juxtaposed in the interface, opening these dimers by ~20° compared with Ly49A. An important consequence of this variability in Ly49 dimerization geometry is to modulate the way these NK receptors bind MHC, as revealed by the Ly49A/H-2D^d and Ly49C/H-2K^b structures (Tormo et al. 1999; Dam et al. 2003, 2006). The complex structures provide a framework for understanding MHC-I recognition by NK receptors from both families and reveal striking differences in the nature of this recognition, despite the receptors' functional similarity (Sawicki et al. 2001). The crystal structure of inhibitory NK receptor Ly49I monomer, at 3.0 Å adopts a fold similar to that of other C-type lectin-like NK receptors, including Ly49A, NKG2D (Fig. 29.3) and CD69.

However, the Ly49I monomers associate in a manner distinct from that of these other NK receptors, forming a more open dimer. As a result, the putative MHC-binding surfaces of the Ly49I dimer are spatially more distant than the corresponding surfaces of Ly49A or NKG2D. These structural differences probably reflect the fundamentally different ways in which Ly49 and NKG2D receptors recognize their respective ligands. For example the single MICA binding site of NKG2D is formed by the precise

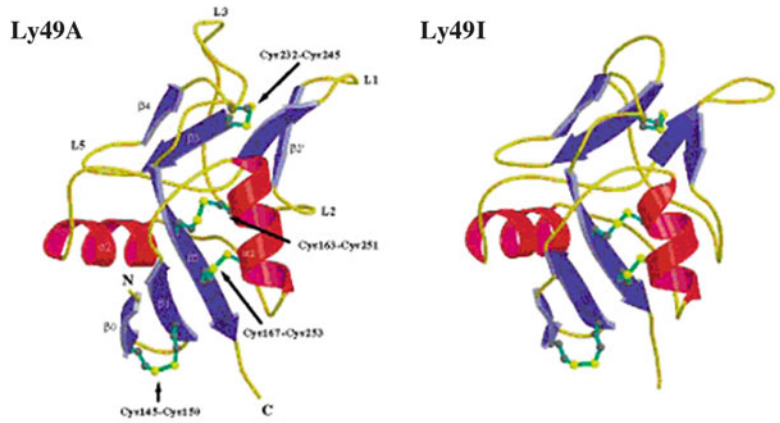

Fig. 29.3 Ribbon diagrams of C-type lectin-like domains of NK receptor Ly49A (PDB entry code 1QO3) (Tormo et al. 1999) and Ly49I (1JA3) (Dimasi et al. 2002). The secondary structural elements are colored as follows: β- strands *blue*, ®-helices *red*, and loop regions *gold*. The disulphide bonds are shown in green as ball-and-stick representation

juxtaposition of two monomers, each Ly49 monomer contains an independent binding site for MHC-I. Hence, the structural constraints on dimerization geometry may be relatively relaxed within the Ly49 family. Such variability may enable certain Ly49 receptors, like Ly49I, to bind MHC-I molecules bivalently, thereby stabilizing receptor-ligand interactions and enhancing signal transmission to the NK cell (Dimasi et al. 2002).

29.3 NK Cell Receptor Protein 1 (NKR-P1) or KLRB1

29.3.1 *Ly49* and *Nkrp1 (Klrb1)* Recognition Systems

Three classes of multigene family-encoded receptors enable NK cells to discriminate between polymorphic MHC class I molecules: Ly-49 homodimers, CD94/NKG2 heterodimers and the killer cell inhibitory receptors (KIR). Of these, CD94/NKG2 has been characterized in both rodents and humans (Chap. 31) and does not show lectin activity. In contrast, Ly-49 family members have hitherto been found only in rodents, and KIR molecules only in the human. Like *Ly49* (*Klra*), *Nkrp1(Klrb1)* locus encodes structurally and functionally related cell surface protein that regulates NK cell-mediated cytotoxicity and cytokine production. The Ly49 receptors are encoded in NKC that contains clusters of genes for other lectin-like receptors on NK cells and other hematopoietic cells. Though the NKR-P1 molecules were the first family of NK cell receptors identified, yet they remained enigmatic in their contribution to self-nonself discrimination until recently. The *Ly49* and *Nkrp1* genetically linked loci for receptor-ligand pairs suggest a genetic strategy to preserve this interaction and show several other contrasts with Ly49-MHC interactions. Despite their close relatedness and genetic linkage within NKC, these two multi-gene families have adopted dissimilar evolutionary strategies. While families, like *Ly49* are polygenic in rodents (Dissen et al. 1996), there is only a single family member (Ly49L) in humans (Westgaard et al. 1998). Ly49 genes are extremely polymorphic, with distinct gene numbers, remarkable allelic diversity, and varying MHC-I-ligand specificities and affinities among different murine haplotypes. In contrast, the *Nkrp1* genes have opted for overall conservation of genomic organization, sequences, and ligand specificities, with only limited allelic polymorphism (Carlyle et al. 2008).

29.3.2 NKRP1

The NKR-P1family of receptors has been of considerable interest due to its conservation in rats, mice, and humans (Lanier 2005; Brissette-Storkus et al. 1994). Rodents have several *Klrb1* genes encoding either activating or inhibitory NK receptors of C-type lectin superfamily, including Nkr-p1c, the NK1.1 Ag defining mouse NK cells (Plougastel et al. 2001a). In rats, the KLRs are encoded by the NK gene complex located on chromosome 4, and syntenic chromosomal regions have been identified in many other species. Several KLR gene families are clustered together in specific sub-regions of NKC, and there is considerable species-to-species variation in the number, expression patterns, and putative functions of the genes encoded by these different KLR families.

Initially thought as markers for NK cells, expression of NKR-P1 has now been documented in other cell types, including subsets of T cells as well as on granulocytes and spleen DCs. In mice, six NKR-P1 genes have been identified (Plougastel et al. 2001a), whereas in humans there appears to be only one gene, CD161 (NKR-P1A), expressed on most NK cells and subsets of thymocytes and fetal liver T cells

(Lanier 2005). Subsets of mature $CD3^+$ T cells, including $CD4^+$ and $CD8^+$ cells also express this receptor, particularly CD1d reactive NKT cells (Exley et al. 1998). This latter population is thought to be involved in the regulation of immune responses associated with a broad range of diseases, including autoimmunity, infectious diseases, and cancer (Godfrey et al. 2004). Rat NKR-P1A was shown to confer reactivity of an NK cell line to certain mouse tumor cell lines (Ryan et al. 1995), and some reports suggest a role for NKR-P1 receptors in the lysis of MHC allogeneic or semiallogeneic target cells (Dissen et al. 1996). Khalturin et al. (2003) identified a gene in the tunicate *Botryllus schlosseri* that encodes a lectin-like protein that is more similar in its extracellular domain to NKR-P1 than to other lectin-like genes (Mesci et al. 2006; Plougastel and Yokoyama 2006).

29.3.2.1 Rat NKR-P1

NKR-P1 is a 60-kDa homodimer expressed on rat NK cells. NKR-P1 may play a role in NK cell activation because antibody to NKR-P1 stimulates the release of granules from NK cells, and anti-NKR-P1 antibody causes redirected lysis by activated NK cells against targets that express FcR. NKR-P1 molecules in rodents may activate cytotoxicity by transducing biochemical signals. Structures of these molecules have not been elucidated. The murine NKR-P1 and Ly-49 molecules are encoded by members of polymorphic gene families that reside in the NK gene complex on the distal region of mouse chromosome 6. The rat NKR-P1 Ag shares several features with the mouse Ly-49 Ag, including selective cell surface expression on NK cells, homology to the C-type lectins, expression as a type II integral membrane protein, and disulfide-linked homodimeric structure. The mouse and rat NKR-P1-deduced polypeptide sequences are highly conserved, suggesting a similar tertiary structure. Although the deduced amino acid sequences of mNKR-P1 and Ly-49 reveal that these proteins are structurally similar, they are only 24% identical at the amino acid level and the cDNA sequences do not demonstrate significant nucleotide homology (Yokoyama and Plougastel 2003). NKR-P1 expresses at low levels on a small subset of rat T cells with an $NKR\text{-}P1_{dim}/TCR\text{-}\alpha\beta^+$ phenotype and on a small subset of cells with an $NKR\text{-}P1_{dim}/TCR\text{-}\alpha\beta^-$ phenotype ($\gamma\delta^+$ T cells?). The expression of functional NKR-P1 (i.e., ability to signal rADCC) correlates with and potentially contributes to MHC-unrestricted cytotoxicity (Brissette-Storkus et al. 1994; Chambers et al. 1992).

Peripheral blood neutrophils in normal animals express very low or undetectable levels of NKR-P1. Detectable level of NKR-P1 was induced as early as day 1 following small bowel transplantation in all allografted animals, whereas expression was only rarely detected in isografted animals (Webster et al. 1994). A major subset of non-alloreactive NK cells in PVG strain rats is generally low in Ly49 receptors, but expresses rat NKR-P1B(PVG) receptor (previously termed NKR-P1C). The $NKR\text{-}P1B^+$ NK subset is inhibited by a non-polymorphic target cell ligand, which is a C-type lectin-related molecule (Clr). Rat Clr molecules appear to be constitutively expressed by hematopoietic cells; expression in tumor cell lines is more variable (Kveberg et al. 2009).

Rat NK Activating and Inhibitory Receptors: While NKR-P1A is an activating receptor on rat NK cells (Giorda et al. 1992), the NKR-P1B receptor (Dissen et al. 1996), in contrast, has inhibitory structural features though it was not possible to prove NKR-P1B-specific inhibitory functions on primary cells since available anti-NKR-P1 mAb failed to distinguish NKR-P1A from NKR-P1B (Kveberg et al. 2006; Li et al. 2003). NKR-P1B functions as an inhibitory receptor because of the presence of an ITIM. Xu et al. (2005) showed that the frequency of $CD161^+$ (human NKR-P1A) NK cells in the epithelia of DA rats was greater than that of WKAH and F344 rats. DA rats have far stronger resistance in the colon to preneoplastic lesion than do other strains. Perhaps $CD161^+$ NK cells play an important role in immune-surveillance at the bottom of the crypt. These observations suggest some allelic variations among NKR-P1 molecules.

A Ly49-negative NK subset is known to selectively express an inhibitory rat NKR-P1 molecule termed NKR-P1C with unique functional characteristics. $NKR\text{-}P1C^+$ NK cells efficiently lyse certain tumor target cells, secrete cytokines upon stimulation. However, they specifically fail to kill MHC-mismatched lymphoblast target cells. The $NKR\text{-}P1C^+$ NK cell subset al.so appears earlier during development and shows a tissue distribution distinct from its complementary $Ly49s3^+$ subset, which expresses a wide range of Ly49 receptors (Kveberg et al. 2006). The NKR-P1C can act as an autonomous activation structure for NK cell cytotoxicity and cytokine secretion (Ryan et al. 1995). Thus, functionally distinct populations of rat NK cells possess different killer cell lectin-like receptor repertoires (Kveberg et al. 2006).

29.3.2.2 Mouse NKR-P1: Six Distinct Murine *Nkr-p1* Genes

An initial report described three distinct murine *Nkr-p1* genes: *Nkr-p1a, Nkr-p1b*, and *Nkr-p1c*. A fourth *Nkr-p1* gene, *Nkr-p1d*, was identified subsequently (Carlyle et al. 1999; Giorda et al. 1992). Later, genomic sequencing identified a fifth functional gene, *Nkr-p1f* (Plougastel et al. 2001a). Analysis of NK cells from different mouse strains revealed that the NK1.1 alloantigen epitope is shared by products of two distinct genes, *Nkr-p1b* and *Nkr-p1c* (Carlyle et al. 1999; Kung et al. 1999). This discovery led to the first demonstration of the inhibitory function of mouse NKR-P1B (Carlyle et al. 1999; Kung et al. 1999) and to the cDNA cloning of a related inhibitory receptor, the B6-derived NKR-P1D (Carlyle et al. 1999; Kung et al. 1999).

Since then, there have been a total of five NKR-P1 proteins identified to date: three stimulatory receptors, NKR-P1A, NKR-P1C, and NKR-P1F, which possess a charged transmembrane arginine (R) residue thought to be important for association with the FcRγ adaptor protein (Arase et al. 1997; Carlyle et al. 1999; Plougastel et al. 2001a; Ryan and Seaman 1997); and two inhibitory receptors, NKR-P1B and NKR-P1D (Carlyle et al. 2004; Iizuka et al. 2003; Plougastel et al. 2001b), which possess a consensus cytoplasmic ITIM (L/VxYxxL/I/V). The *Nkr-p1* gene cluster is located on NKC on chromosome 6 in mice, syntenic to a similar region on chromosome 4 in rats and chromosome 12 in humans (Yokoyama and Plougastel 2003; Ryan and Seaman 1997).

29.3.2.3 NK1.1 (NKR-P1C)

NK1.1 (mouse NKR-P1C) is a member of a family of NKR NKR-P1 family of disulfide-linked homodimeric type II transmembrane C-type lectin-like receptors initially defined in rats (Ryan and Seaman 1997). First described in 1977 (Glimcher et al. 1977), the original NK1.1 alloantigen has been widely used as a marker to identify NK cells and NKT cells in C57BL/6 (B6) mice. In mice, NK1.1 is expressed on almost all NK cells and on a subset of T cells, NK T cells. Almost all NK1.1$^+$ T cells recognize their TCR ligands in association with the Ag-presenting molecule CD1d, and the majority of NK1.1$^+$ T cells in the thymus, liver, and secondary lymphoid tissues express the invariant Vα14Jα18 TCR (Brigl and Brenner 2004; Godfrey et al. 2004; Kronenberg and Gapin 2002; Taniguchi et al. 2003). Subsets of NK1.1$^+$ T cells are CD4$^+$, CD8$^+$, or CD4$^-$CD8$^-$. In the thymus, blood, and secondary lymphoid tissues, NK1.1$^+$ T cells represent 1–2% of all T cells; in the liver and bone marrow, they represent 20–40% of all T cells (Hammond et al. 2001). Mouse NKR-P1C (NK1.1) is expressed on CD117$^+$ progenitor thymocytes capable of giving rise to cells of the T and NK lineages. NKR-P1C engagement with a mAb leads to IFN-γ production and the directed release of cytotoxic granules from NK cells. The NK2.1 antigen is likely to be anchored in the plasma membrane by a peptide moiety. In addition to be present on a splenic NK cell subset, the NK2.1 antigen was shown to be expressed by a small number of CD^4-CD^8-thymocytes and by a subset of CD^4-CD^8-IgG$^-$ lymph node cells. Finally, unlike NKR-P1, the rat homologue of the murine NK1.1 antigen, neither the NK2.1 nor the NK1.1 antigen is expressed by polymorphonuclear leukocytes.

In contrast to the NKR-P1C$^+$ T cells in mouse liver, the majority of NKR-P1A$^+$ T cells in the human liver are CD8$^+$. Almost all of the NKR-P1A$^+$ T cells in the human liver expressed CD69, suggesting that they were activated. Furthermore, the NKR-P1A$^+$ T cells in the human liver exhibited strong cytotoxicity against a variety of tumor cell lines including K562, Molt4 and some colonic adenocarcinoma cell lines (Ishihara et al. 1999).

Strain-Dependent NK1.1 Alloreactivity of Mouse NK Cells: Historically, NK cells from selected mouse strains have been phenotypically defined using NK-1 alloantigen-specific antisera (Glimcher et al. 1977) or the anti-NK1.1 mAb, PK136 (Koo and Peppard 1984). The NK1.1 alloantigen is now known to identify NK cells from CE, B6, NZB, C58, Ma/My, ST, SJL, FVB, and Swiss outbred mice, but not BALB/c, AKR, CBA, C3H, DBA, or 129 mice (Carlyle et al. 2006). All of *Nkr-p1* genes were reportedly identified in B6 mice, but it is now clear that *Nkr-p1b* is not expressed by B6 mice but is expressed in other strains (Iizuka et al. 2003). In B6 mice, the PK136 mAb binds only NKR-P1C, which can be considered the NK1.1 antigen (Glimcher et al. 1977), but the Ab also binds NKR-P1B in mouse strains that express it (Iizuka et al. 2003).

The underlying molecular basis for the lack of NK1.1 reactivity of NK cells from BALB/c and other mouse strains remains an enigma. The lack of NK1.1 reactivity could be due to deletion of *Nkrp1* genes, defective gene expression, or allelic polymorphism in BALB/c mice. Extreme variation in gene content between the BALB/c and B6 haplotypes has been observed previously for the related *Ly49* gene family (Anderson et al. 2005). This suggests that other NK gene complex regions, including the *Nkrp1-Ocil/Clr* region, also may be subject to rapid evolutionary divergence and/or polymorphism. Since cognate ligands for NKR-P1 have been identified (Carlyle et al. 2004; Iizuka et al. 2003; Kumar and McNerney 2005), a BALB/c defect in NKR-P1 expression could be functionally significant for NK cell function and innate immunity. Determination of the gene content of the BALB/c *Nkrp1-Ocil/Clr* region and the basis of the BALB/c defect in NK1.1 expression could have implications for the importance of the NKR-P1–Ocil/Clr system in self-nonself discrimination in mice and other species (Kumar and McNerney 2005).

To investigate the NK1.1$^-$ phenotype of BALB/c NK cells, Carlyle et al. (2006) studied NK1.1 epitope mapping and genomic analysis of the BALB/c *Nkrp1* region. Analysis reveals that, unlike the *Ly49* region, the *Nkrp1-Ocil/Clr* region displays limited genetic divergence between B6 and BALB/c mice. In fact, significant divergence is confined to the *Nkrp1b* and *Nkrp1c* genes. Strikingly, the B6 *Nkrp1d* gene appears to represent a divergent allele of the *Nkrp1b* gene in BALB/c mice and other strains. Allelic divergence of the *Nkrp1b/c* gene products and limited divergence of the BALB/c *Nkrp1-Ocil/Clr* region explain the strain-specific NK1.1 alloantigen reactivity of mouse NK cells (Carlyle et al. 2006).

Mouse NKR-P1B, a NK1.1 Antigen with Inhibitory Function: The mouse NK1.1 Ag originally defined as NKR-P1C (CD161) mediates NK cell activation. Another member of the mouse CD161 family, NKR-P1B, represents a novel NK1.1 Ag. In contrast to NKR-P1C, which functions

as an activating receptor, NKR-P1B inhibits NK cell activation. Association of NKR-P1B with Src homology 2-containing protein tyrosine phosphatase-1 provides a molecular mechanism for this inhibition. The existence of these two NK1.1 Ags with opposite functions suggests a potential role for NKR-P1 molecules, such as those of the Ly-49 gene family, in regulating NK cell function (Carlyle et al. 1999).

29.4 Human NKR-P1A (CD161)

29.4.1 Cellular Localization

CD161 (human NKR-P1A) is a major phenotypic marker of NK and NKT cell types and is thought to be involved in the regulation of functions of these cells. As against six NKR-P1 genes in mice, there appears to be only one gene in humans. Human NKR-P1A is found on a subset of peripheral T cells, including $CD4^+$ and $CD8^+$ T cells, invariant NKT cells, and $\gamma\delta$-TCR^+ T cells, fetal liver T cells and on a subset of $CD3^+$ thymocytes. It is predominantly expressed on T cells with the memory phenotype ($CD45RO^+$) (Lanier 2005; Werwitzke et al. 2003). Comparison of the predicted amino acid of human NKR-P1A with rat and mouse NKR-P1 indicates 46% homology. NKR-P1A is located on human chromosome 12, the syntenic of mouse chromosome 6, where murine NKR-P1 genes are located.

CD161 is type II transmembrane C-type lectin-like receptor and expressed on the cell membrane as disulphide-linked homodimer. In contrast to rodents, no clear functional role has been ascribed to CD161 in humans. Engagement of CD161 on cultured NK cells can result in inhibition of cytotoxicity (Poggi et al. 1999). The functional consequences of treating human NK cells with anti-CD161 mAb are more complex, resulting in no effect, activation, or inhibition, depending on the NK cell population studied (Lanier 2005; Poggi et al. 1997a), although mAb to CD161 has also been reported to trigger proliferation of immature thymocytes (Poggi et al. 1999). These diverse responses elicited by anti-NKR-P1 mAb suggest that additional, functionally distinct, isoforms or alleles of NKR-P1 may exist in humans. Consistent with the latter observation, human NKR-P1A, though not involved in direct activation of NKT cells or their recognition of the CD1d molecule, is an important costimulatory molecule for this population of T cells (Exley et al. 1998). The molecular basis of such widely different functional activities of human CD161 is not clear. Although both human NK cells and a subset of human T cells express the CD56 marker, only a small minority of $CD161^+$ T cells also express CD56 (Loza et al. 2002). Gene encoding the NKR-P1 from cattle showed identity to human nucleotide sequences at 90% and 75%, respectively, and all structural residues of C-type lectin carbohydrate recognition domains were conserved. The identification of two of its members allows to hypothesise the existence of a bovine NK gene complex, prospectively located on chromosome 5 (Govaerts and Goddeeris 2001).

While CD161 is expressed on the minority of human blood T cells, $CD161^+$ T cells expressing either CD4 or CD8 represent the majority of T cells from the epithelial and lamina propria layers of the human duodenum and colon (Ishihara et al. 1999; O'Keeffe et al. 2004). The latter cells contain few, if any, $V\alpha24V\beta11$ invariant NK T cells and secrete IFN-γ and TNF-α without IL-4 after in vitro stimulation. An abundance of $CD161^+$ T cells was also found in the liver and intestinal epithelial cells of the jejunum; the majority of the latter cells were $CD161^+CD8^+$ T cells (Iiai et al. 2002). CD161 is also expressed on human monocytes and dendritic cells. Bone marrow-derived DC of rat uniformly expressed low levels of CD161 and expressed OX62 in a bimodal distribution (Brissette-Storkus et al. 2002). Engagement of CD161 on the cell surface using an appropriate mAb induced the production of IL-1β and IL-12 by monocytes and dendritic cells, respectively (Azzoni et al. 1998; Poggi et al. 1997b). CD161on $CD4^+$ T cells and $\gamma\delta$-TCR^+ T cells has been implicated in transendothelial migration (Poggi et al. 1999). Unexpectedly, the $CD8^+CD161^+$ cells contained an anergic $CD8\alpha^+CD8^{\beta low/-}CD161^{high}$ T cell subset that failed to proliferate, secrete cytokines, or mediate NK lytic activity (Takahashi et al. 2006).

29.4.2 Transcriptional Regulation

Although the 5′-flanking region of NKR-P1C from C57Bl/6 mouse is TATA-less, there is an initiator region and a downstream promoter element, which together form the principal minimal functional promoter. Analyses of the 10-kb 5′-flanking region revealed potential binding sites for regulatory factors, and a single hypersensitive site (HS) of about a 9-kb upstream of the transcriptional initiation site. This site, HS1, could act as a transcriptional enhancer element in a NK cell line. The minimal upstream cis-acting elements point to a complex regulatory mechanism involved in the lineage-specific control of NKR-P1C expression in NK lymphocytes (Ljutic et al. 2003). IL-12, in contrast to IL-2, strongly up-regulates the expression of NKRP1A on human NK cells. The NKRP1A in turn, can regulate the NK cell activation induced via different triggering pathways. This would imply that NKRP1A-mediated functions may be regulatd by the cytokine microenvironment that NK cells may encounter at inflammatory sites (Poggi et al. 1999).

The stochastic expression of individual members of NK cell receptor gene families on subsets of NK cells has attracted considerable interest in the transcriptional

regulation of these genes. Each receptor gene can contain up to three separate promoters with distinct properties. The recent discovery that an upstream promoter can function as a probabilistic switch element in the Ly49 gene family has revealed a novel mechanism of variegated gene expression. An important question to be answered is whether or not the other NK cell receptor gene families contain probabilistic switches. The promoter elements currently identified in the Ly49, NKR-P1, CD94, NKG2A, and KIR gene families are described (Anderson 2006).

29.4.3 Ligands of CD161/ NKR-P1

29.4.3.1 Binding of Carbohydrates to NKR-P1

A diversity of high-affinity oligosaccharide ligands has been identified for NKR-P1 that contains an extracellular Ca^{2+}-dependent lectin domain. Dimerization of soluble recombinant rat NKR-P1 is predominantly dependent on the presence of an intact juxta-membrane stalk region and independent of N-glycosylation. Aminosugars have a good affinity for the NKR-P1A protein. NKR-P1 is a lectin with a preference order of GalNAc > GlcNAc >> Fuc >> Gal > Man. At neutral pH, Ca^{2+} is tightly associated with the protein. However, NKR-P1 can be decalcified at pH 10 with a total loss of carbohydrate binding. NKR-P1 differs from other calcium-dependent animal lectins in its pattern of monosaccharide recognition and in the tightness of Ca^{2+} binding. NK-resistant tumor cells are rendered susceptible by preincubation with liposomes expressing NKR-P1 ligands, suggesting that purging of tumor or virally infected cells in vivo may be a therapeutic possibility (Bezouska et al. 1994).

While N-acetylD-mannosamine was the best neutral monosaccharide ligand, its participation in the context of an extended oligosaccharide sequence was equally important. The IC_{50} value for the GalNAcβ1 —>ManNAc disaccharide was nearly 10^{-10} M with a further possible increase depending on the type of the glycosidic linkage and the aglycon nature (Krist et al. 2001). GlcNAc and chitooligomers were identified as strong activation ligands for NKR-P1 protein in vitro and in vivo. Their clustering brings about increase of their affinity to the NKR-P1 by 3–6 orders. In analogy with previous observations with GlcNAc clustered on protein or PAMAM backbones the synthetic chitooligomer clusters should provide considerably better ligands in the in vivo antitumor treatment (Semenuk et al. 2001). NKR-P1A also binds to the chitobiose core of uncompletely glycosylated N-linked glycans, and to linear chitooligomers (Bezouska et al. 1997).

Ligands for NKR-P1A include a fully sulphated disaccharide, sucrose octasulphate as observed by NMR spectroscopy and described for the screening of compound libraries for bioaffinities. These findings raise the possibility that NKR-P1A recognises sulphated natural ligands in common with certain other members of the C-type lectin family (Kogelberg et al. 2002). Uncharacterized receptors on human NK cells interact with ligands containing the terminal Galα(1,3)Gal xenoepitope. Among carbohydrate binding proteins from NK cells that bind αGal or N-acetyllactosamine (LacNAc), created by deletion of α1,3galactosyltransferase (GT) in animals is NKRPIA. Moreover, exposing LacNAc by removal of αGal resulted in an increase in binding. This may be relevant in later phases of xenotransplant rejection if $GT^{-/-}$ pigs, like $GT^{-/-}$ mice, display increased LacNAc expression (Christiansen et al. 2006). Structural studies suggested the preference of these receptors for either linear (NKR-P1) or branched (CD69) carbohydrate sequences (Pavlícek et al. 2004). Recombinant soluble form of rat NKR-P1 also recognized carbohydrate GalNac and GlcNac moieties. Ganglioside GM2 and heparin related-IS oligosaccharides representing the high affinity ligands for this receptor, increased the sensitivity of targets for killing by the rat effectors isolated from blood and spleen in vitro. Synthetic three mono- and bivalent LacdiNAc glycomimetics proved to be powerful ligands of NKR-P1 and CD69. A synthetic bivalent tethered di-LacdiNAc is the best currently known precipitation agent for both of these receptors and has promising potential for the development of immunoactive glycodrugs (Bojarová et al. 2009).

29.4.3.2 Ocil/Clr-b as Ligand in Rodents

While ligands for the stimulatory NKR-P1A/C receptors remain elusive, ligands for inhibitory NKR-P1B and NKR-P1D receptors (Carlyle et al. 2004) and activating NKR-P1F receptors are products of the *Ocil/Clr* family of genes (Carlyle et al. 2004; Iizuka et al. 2003; Zhou et al. 2001; Plougastel et al. 2001a), which are intermingled with the *Nkrp1* genes themselves in the NK gene complex. Ocil/Clr-b molecules have been shown to be the ligands for several members of the NKR-P1 family of receptors (Giorda et al. 1992; Ryan and Seaman 1997). The activating NKR-P1F receptor recognizes Clr-g (encoded by *Clec2i*) (Iizuka et al. 2003). The *Clr* genes are interspersed between the *Nkr-p1* genes. The cloning, expression, and function of Ocil/Clr-b as a ligand for the inhibitory NKR-P1B and NKR-P1D receptors have been reported. This suggests that specific receptor-ligand pairs are not inherited separately, but rather en bloc (Iizuka et al. 2003; Carlyle et al. 2004).

The NKR-P1 receptors have been shown to recognize other lectin-like molecules in contrast to other lectin-like NKRs, which recognize class I MHC molecules or relatives of class I MHC molecules. The inhibitory NKR-P1B/D orphan NK cell receptors functionally interact with Ocil/Clr-b ligand and can mediate missing self recognition of

tumor cells by mouse NK cells. The findings of Clr ligands have created a renewed interest in the NKR-P1 family, because these ligands and their receptors represent a form of self-nonself discrimination that is independent of MHC class I, a principle that can now be extended to other NK cell receptors (Kumar and McNerney 2005). It is speculated that the NKR-P1 proteins and their Clr ligands may be descended from a common C-type lectin-like ancestral protein that engaged in homophilic interactions. The Ocil/Clr-b is expressed broadly on normal hematopoietic cells but is frequently down-regulated on tumor cells, indicating that NKR-P1B/D receptors play a role in "missing self-recognition" of Ocil/Clr-b. Clr-b is also named as osteoclast inhibitory lectin, because it was also identified as an osteoblast-derived glycoprotein, which inhibits in vitro osteoclastogenesis (Hu et al. 2004; Zhou et al. 2001).

29.4.3.3 LLT1 as Ligand in Humans

Although mice have multiple *Clr* family genes, only one ortholog, *CLEC2D* (also named lectin-like transcript-1 or LLT1), exists in humans. Like mouse Clr-b, human LLT1 blocks osteoclast differentiation (Hu et al. 2004; Zhou et al. 2001). The LLT1 (alternative name, CLEC2D) is a physiologic ligand for NKR-P1A in humans. The human LLT1 is similar to the mouse Clr molecules and is expressed on cells of lymphocytic origin (Boles et al. 1999; Carlyle et al. 2004; Aldemir et al. 2005; Rosen et al. 2005). Human LLT1 is expressed on NK, T, and B cells and localized to the NK gene complex within 100 kb of CD69. In addition to NK and T cells, LLT1 is expressed on TLR-activated plasmacytoid dendritic, TLR-activated monocyte-derived DCs, and on B cells stimulated through TLR9, surface Ig, or CD40. Interactions between NKR-P1A on NK cells and LLT1 on target cells inhibit NK cell-mediated cytotoxicity and cytokine production and can inhibit TNF-α production by TCR-activated NKR-P1A$^+$ CD8$^+$ T cells. In contrast, NKR-P1A failed to inhibit or augment the TCR-dependent activation of NKR-P1A-bearing CD4$^+$ T cells. Expression of LLT1 on activated DCs and B cells suggests that it might regulate the cross-talk between NK cells and APCs (Rosen et al. 2008).

The cDNA encodes a predicted protein of 191 amino acid residues with a transmembrane domain near the N-terminus and an extracellular domain of 132 amino acid residues with similarity to CRD of C-type lectins. The predicted protein of LLT1 shows 59% and 56% similarity to AICL and CD69, respectively. The predicted protein does not contain any intracellular ITIM motifs, suggesting that LLT1 may be involved in mediating activation signals. The human LLT1 shows 43–48% of homology to Clr at the amino acid level and seems to be primarily expressed by monocytes and B cells in peripheral blood (Mathew et al. 2004).

Engagement of CD161 on NK cells with LLT1 expressed on target cells inhibited NK cell-mediated cytotoxicity and IFN-γ secretion. Conversely, LLT1/CD161 interaction in the presence of a TCR signal enhanced IFN-γ production by T cells (Aldemir et al. 2005). The lytic activity of NK cells is negatively regulated via CD161 expression mediated by IL-12 (Azzoni et al. 1998). However, LLT1 is induced rapidly in PMA-stimulated PBMCs (Eichler et al. 2001) and in IL-2-activated NK cells or T cells (Boles et al. 1999). Cross-linking of LLT1 with an Ab induced production of IFN-γ by NK cells (Mathew et al. 2004). LLT1-containing liposomes bind to NKR-P1A$^+$ cells, and binding is inhibited by anti-NKR-P1A mAb. Moreover, LLT1 on target cells can inhibit NK cytotoxicity via interactions with NKR-P1A (Rosen et al. 2005). This interaction inhibits NK cell-mediated cytotoxicity and IFN-γ production while enhancing CD3-triggered IFN-γ production by T cells. It appears that LLT1, expressed by gliomas, contributes to tumor-associated immunosuppression by affecting the lytic activity of NK cells (Roth et al. 2006, 2007).

Oosteoclast Inhibitory Lectin: Osteoclast inhibitory lectin (Ocil) is an inhibitor of osteoclast formation and shows promise as an antiresorptive protein. Murine, rat, human Ocils (mOcil, rOcil, and hOcil respectively) are type II membrane C-type lectins expressed by osteoblasts and other extraskeletal tissues, with the extracellular domain of each, are able to inhibit in vitro osteoclast formation. The hOcil is highly conserved with mOcil in its C-lectin domain, genomic structure, and activity to inhibit osteoclastogenesis (Hu et al. 2004). The hOcil gene predicts a 191 amino acid membrane protein, with the 112 amino acid C-type lectin region in the extracellular domain, having 53% identity with rOcil and mOcil. The hOcil gene is 25 kb in length, comprising five exons, and is a member of a superfamily of NK cell receptors encoded by the NK gene complex located on chromosome 12. Human Ocil mRNA expression is upregulated by IL-1α and prostaglandin E2 in human osteogenic sarcoma MG63 cells, but not by dexamethasone or 1,25 dihydroxyvitamin D3. In addition, Ocil can also inhibit bone resorption by mature, giant-cell tumor-derived osteoclasts (Hu et al. 2004). Ocil is notably localized in bone, skin, and other connective tissues, binds a range of physiologically important glycosaminoglycans, and this property may modulate Ocil actions upon other cells (Gange et al. 2004).

29.4.4 Signaling Pathways

The mechanisms of signaling pathways of CD161 are poorly understood. Different rodent NKR-P1 molecules use different signal transduction pathways to achieve their different functions. The cytoplasmic tail of rodent NKR-P1s has been reported to associate with *Src* family kinases such as p56^{lck}

and various heterotrimeric G proteins (Ljutic et al. 2005), whereas the activating NKR-P1C molecule associates with the γ-chain of the FcR (Arase et al. 1997) and the inhibitory isoform NKR-P1B (expressed by NK cells of SJL/J mice) recruits Src homology protein 1 on cross-linking (Carlyle et al. 1999; Kung et al. 1999).

Target cells expressing class I MHC bound to NKR-P1-expressing NK cells can generate a great variety of intracellular signals that are largely resistant to PTX treatment (Maghazachi et al. 1996). The functional homodimeric form of NKR-P1 selectively binds and activates G_z, G_s, $G_{q/11}$, and G_{i3}, as revealed in [^{35} S]GTP γS binding (Ho and Wong 2001) and to $G\alpha_o$ and $G\alpha_z$ which are also activated in the process of NK cell lysis of allogeneic and tumor target cells (Maghazachi et al. 1996). The activation of NKR-P1 induces IP_3 production, Ca^{2+} flux, interferon- γ secretion, degranulation and cytotoxicity of NK cells (Ho and Wong 2001).

Humans: The cytoplasmic tail of human CD161 (NKR-P1A) does not contain the CxCP/S/T lck-binding motif found in CD4, CD8, and rodent NKR-P1 (Exley et al. 1998; Ljutic et al. 2005). Although it has been reported that human NKR-P1A can be found in association with *Src* family kinases, including p56^{lck} (Cerný et al. 1997), others report a failure to reproduce these observations (Exley et al. 1998). To identify molecules that can interact with the cytoplasmic tail of human CD161 (NKR-P1A), Pozo et al. identified acid sphingomyelinase as a novel intracellular signaling pathway linked to CD161. The mAb-mediated cross-linking of CD161, in both transfectants and primary human NK cells, triggers the activation of acid, but not neutral sphingomyelinase. The sphingomyelinases represent the catabolic pathway for *N*-acyl-sphingosine (ceramide) generation, an emerging second messenger with key roles in the induction of apoptosis, proliferation, and differentiation and define a novel signal transduction pathway for the CD161 in NK and NKT cell biology (Pozo et al. 2006).

Rodents: Cross-linking of the stimulatory rat NKR-P1A or mouse NKR-P1C molecules stimulates phosphatidylinositol turnover and Ca^{2+} flux (Ryan and Seaman 1997), as well as NK cell-mediated cytotoxicity and cytokine production (Arase et al. 1997). Sequence analysis revealed that all murine NKR-P1 proteins possess the Cys-X-Cys-Pro (CxCP) motif also found in the cytoplasmic domains of CD4 and CD8 that mediates association with the Src-related nonreceptor protein tyrosine kinase, p56^{lck}. Campbell and Giorda (1997) demonstrated a physical association between the rat NKR-P1A cytoplasmic CxCP motif and p56^{lck}; however, no functional requirements for this association were shown. A feature of the inhibitory NKR-P1 receptors is the presence of an ITIM in the cytoplasmic domains of mouse NKR-P1B/D. Studies have shown that, like other ITIM-bearing receptors expressed by NK cells, mouse NKR-P1B binds Src homology 2 (SH2)-containing protein tyrosine phosphatase-1 (SHP-1) in a phosphorylation-dependent manner, suggesting a molecular mechanism for the inhibition of NK cell cytotoxicity through NKR-P1B (Carlyle et al. 1999; Kung et al. 1999). The requirement for SHP-1 recruitment to the cytoplasmic ITIM in mediating NKR-P1 inhibition was substantiated by Ljutic et al. (2005). During NKR-P1 signaling in mice, both NKR-P1B and NKR-P1C functionally associate with the tyrosine kinase, p56(lck) p56^{lck}. Mutation at putative Lck-recruitment CxCP motif abolished signal transduction through NKR-P1B/C. Lck appears to be involved in the initiation of NKR-P1 signaling, and SHP-1 in effector function of NKR-P1 receptor (Ljutic et al. 2005).

Ljutic et al. (2005) proposed a model for the initiation and effector signaling through the stimulatory and inhibitory mouse NKR-P1 receptors (Fig. 29.4). Model suggests that the initial phosphorylation of ITIM tyrosine in NKR-P1B is mediated by Lck, providing a docking site for the SH2 domain of the SHP-1 phosphatase, leading in turn to dephosphorylation and inhibition of proximal kinases. In contrast, association of Lck with the stimulatory NKR-P1C receptor leads, upon cross-linking, to transphosphorylation of the cytoplasmic ITAM tyrosines in the FcRγ adaptor protein, in turn leading to recruitment of Syk kinase and activation of downstream second messengers. Thus, findings support a stepwise model for the signaling requirements of the stimulatory and inhibitory NKR-P1 receptors (Fig. 29.4). The identification of cognate ligands for the NKR-P1 receptors (Carlyle et al. 2004; Iizuka et al. 2003) leads to new insight into the physiology of NKR-P1-mediated recognition of target cells by this functionally dichotomous receptor family (Ljutic et al. 2005).

29.4.5 Functions of NKR-P1

The biological function of human CD161 is still insufficiently understood; probably it is involved in regulation of the cytotoxic functions of the cells and in regulation of cytokine production. NKR-P1 molecules are involved in natural killing of certain tumor targets. The loss during differentiation of NKR-P1 and CD2, which are involved in target adhesion and triggering of NK cells, is consistent with the poor cytolytic capacity reported for these cells (Head et al. 1994). The NK1.1 (NKR-P1C) molecule is the most specific serological marker on murine NK cells in C57BL/6 mice. Studies of NKR-P1 have indicated that anti-NKR-P1 mAb induced NK cells to kill otherwise insensitive targets, NK cell phosphoinositol turnover and Ca^{2+} flux. Results demonstrate that immobilized anti-NK1.1 triggers only a subpopulation of NK cells

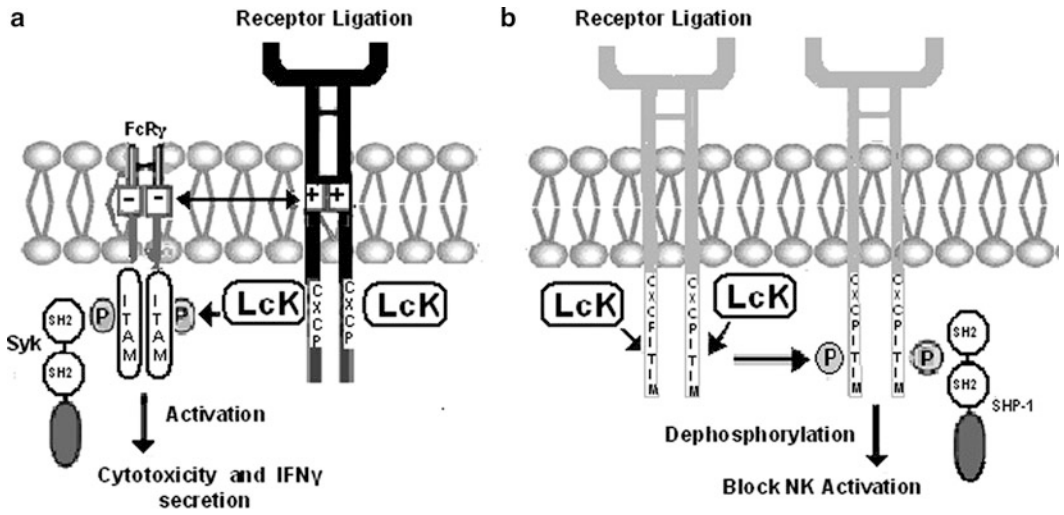

Fig. 29.4 Signaling mechanisms of stimulatory and inhibitory NKR-P1 receptors. (**a**) Upon receptor cross-linking, Lck associated with cytoplasmic tail of stimulatory NKR-P1C phosphorylates ITAM tyrosines in the FcRγ adaptor protein, in turn leading to recruitment of Syk and activation of downstream second messengers. (**b**) Phosphorylation of the cytoplasmic ITIM tyrosine in NKR-P1B is mediated by Lck, providing a docking site for the SH2 domain of SHP-1, leading to dephosphorylation and inhibition of proximal kinases

(Yokoyama and Plougastel 2003) and that NK cell responses can be greatly diminished after in vivo treatment with these mAbs (Levik et al. 2001).

The sequencing of the chicken MHC led to the identification of two ORFs, designated B-NK and B-lec that were predicted to encode C-type lectin domains. The genes for B-NK and B-lec are located next to each other in opposite orientations in the chicken MHC. The B-NK is an inhibitory receptor expressed on NK cells, whereas B-lec is an activation-induced receptor with a broader expression pattern. The B-NK and B-lec genes share greatest homology with CTLD receptors encoded by human NK gene complex, in particular NKR-P1 and lectin-like transcript 1 (LLT1), respectively. Like human NKR-P1, B-NK has a functional inhibitory signaling motif in the cytoplasmic tail and is expressed in NK cells. In contrast, B-lec contains an endocytosis motif in the cytoplasmic tail, and like LLT1, is an early activation Ag. B-NK and B-lec are potential candidate genes for the MHC-mediated resistance to MDV. Both glycosylated type II membrane proteins form disulphide-linked homodimers (Rogers and Kaufman 2008; Rogers et al. 2005).

29.5 NKR-P1A in Clinical Disorders

29.5.1 Autoimmune Reactions

Experimental Autoimmune Neuritis: NK cells are implicated in T cell-mediated autoimmune diseases. Experimental autoimmune neuritis (EAN) is a $CD4^+$ T cell-mediated animal model of the Guillain-Barré syndrome in human. Lymphoid cells co-cultured with IL-12 and IL-18 cytokines transferred aggressive clinical and histological EAN into all recipients. NKR-$P1^+$ cells (including NK and NKT cells) played an immunosuppressive function in passive transfer EAN and depletion of NKR-$P1^+$ cells by anti-NKR-P1 Ab and complement induced a more serious form of EAN. Nevertheless, lymphoid cells co-cultured with both IL-12 and IL-18 induced high levels of IFNγ and promoted Th1 differentiation partially through NKR-$P1^+$ cells and to some extent, NKR-$P1^+$ cell depletion inhibited the autoreactivity of lymphoid cells treated with IL-12 and IL-18 (Sun et al. 2007; Yu et al. 2002).

Autoimmune Uveoretinitis: Kitaichi et al. (2002) treated experimental autoimmune uveoretinitis (EAU)-susceptible mice with anti-CD161c antibodies to deplete natural NK cells. Results indicated that the severity of EAU is augmented by $NK1.1^+$ NK cells.

Rheumatic Diseases: The number of both $CD4^-CD8^-$ and $CD4^+$ NKT cells are selectively decreased in the peripheral blood of patients with rheumatic diseases. In addition, both the frequency and the absolute number of $CD161^+CD8^+$ T cells were decreased in the peripheral blood of patients suffering from SLE, MCTD, SSc and PM/DM. Thus there is also an abnormality of NKT cells in the $CD8^+$ population (Mitsuo et al. 2006).

Graves' Disease: To clarify changes in the intra-thyroidal NKT cell subset in patients with Graves' disease (GD),

Watanabe et al. (2008) examined intrathyroidal and peripheral lymphocytes patients and peripheral lymphocytes in healthy volunteers. The proportion of $CD161^+$ T cell receptor $V\alpha24^+$ $V\beta11^+$ cells was lower in the thyroid of patients than in the peripheral blood of the same patients and in the peripheral blood of healthy subjects. These results indicated that the proportion of intra-thyroidal NKT cells is decreased in patients with GD and that this decrease may contribute to incomplete regulation of autoreactive T cells in GD (Watanabe et al. 2008).

Type 1 Diabetes Mellitus: Invariant NKT cells are considered to be important in some autoimmune diseases including Type 1 diabetes mellitus (T1DM). The published reports are contradictory in regard to the role of iNKT cells in T1DM. Kis et al. (2007) studied iNKT cell frequency and the function of different iNKT cell subgroups in T1DM and suggested that the decrease in the $CD4^+$ population among the iNKT cells and their Th1 shift indicates dysfunction of these potentially important regulatory cells in T1DM.

Asthma: In humans, T cells expressing NKR-P1A constitute around 20% of the circulating $CD3^+$ cells and are potentially immunoregulatory in several diseases. González-Hernández et al. (2007) suggested that during an asthma attack, IFN-γ produced by $CD161^+$ T cells could help to reestablish the Th1/Th2 equilibrium and can be related to asthama.

Atopic Dermatitis: Atopic dermatitis is a chronic inflammatory skin disease associated with cutaneous hyperreactivity to environmental triggers and is often the first step in the atopic march that results in asthma and allergic rhinitis. Helper T cells and their cytokines, in addition to IgE and eosinophils, play a major role in the pathogenesis of atopic dermatitis. NKT cells may play a role in atopic dermatitis status. The reduction of $V\alpha24^+CD161^+$ NKT cells subtypes may be involved in the immunopathogenesis of atopic dermatitis (Ilhan et al. 2007).

29.5.2 Other Diseases

Evidence suggests that NK and NKT cells contribute to inflammation and mortality during septic shock caused by cecal ligation and puncture (CLP). Further studies indicated that NK but not CD1-restricted NKT cells contribute to acute CLP-induced inflammation. NK cells appear to mediate their proinflammatory functions during septic shock, in part, by migration into the peritoneal cavity and amplification of the proinflammatory activities of specific myeloid cell populations. This study provides a new insight into the mechanisms used by NK cells to facilitate acute inflammation during septic shock (Etogo et al. 2008). Selective reduction in the population of colonic mucosal NKR^+ T cells may contribute to the development of intestinal inflammation in ulcerative colitis (Shimamoto et al. 2007).

Hepatitis C virus (HCV) causes chronic infection accompanied by a high risk of liver failure. A subset of $CD161^+CD56^{+/-}$ NKT cells can recognize glycolipids presented by CD1d and positively or negatively regulate inflammatory responses, implicated in several models of hepatitis. CD1d is expressed at very low levels in the healthy liver, but there is a large fraction of $CD161^+CD56^+$ NKT cells. There are high levels of non-classical proinflammatory hepatic CD1d-reactive T cells in HCV infection. Durante-Mangoni et al. (2004) confirmed large numbers of hepatic $CD161^+$ T cells, lower levels of $CD56^+$ T cells, and small numbers of iNKT cells in HCV infection. However, hepatic CD1d-reactivity was not restricted to any of these populations (Durante-Mangoni et al. 2004). CD161 was significantly expressed on HCV-specific cells but not on $CD8^+$ T cells specific for human immunodeficiency virus (3.3%), cytomegalovirus (3.4%), or influenza (3.4%). Northfield et al. (2008) proposed that expression of CD161 indicates a unique pattern of T cell differentiation that might help elucidate the mechanisms of HCV immunity and pathogenesis. Functional capacities and coexpression patterns of lectin-like receptors on lymphocytes are differentially affected in HIV patients depending on the state of therapy or the cell type (NK or T cells), respectively (Jacobs et al. 2004). Reports identify a novel population of human T cells that could contribute to destructive as well as protective immune responses in the liver. It is likely that CD1d-reactive T cells may have distinct roles in different tissues (Exley et al. 2002).

Cytomegaloviruses are known to encode several gene products that function to subvert MHC-dependent immune recognition. Voigt et al. (2007) characterized a rat cytomegalovirus (RCMV) C-type lectin-like (RCTL) gene product with homology to the Clr ligands for the NKR-P1 receptors. RCMV infection rapidly extinguished host Clr-b expression, thereby sensitizing infected cells to killing by NK cells. However, the RCTL protein functioned as a decoy ligand to protect infected cells from NK killing via direct interaction with the NKR-P1B inhibitory receptor. Findings indicate a strategy adopted by cytomegaloviruses to evade MHC-independent self-nonself discrimination. The existence of lectin-like genes in several poxviruses suggests that this may be a common theme for viral evasion of innate immunity (Voigt et al. 2007). The $V\delta2^+$ $TCR\gamma\delta^+$ T lymphocyte subset, expressing the NKRP1a, is expanded in patients with relapsing-remitting multiple sclerosis and uses this molecule to migrate through endothelium. Poggi et al. (2002) suggested that subsets of γδT lymphocytes may migrate to the site of lesion in multiple sclerosis using two different signaling pathways to

extravasate. The mechanism involved in the spontaneous acceptance of liver allografts in some rat strain combinations remains unclear. Immunoregulatory NKR-P1TCRαβT (NKT) cells primarily produce IL-4 and IFN-γ, and enhance the polarization of immune responses to Th2 and Th1, respectively. The role of graft-derived NKT cells in inducing the spontaneous acceptance of rat orthotopic liver transplantation (OLTx) has suggested that graft-derived NKT cells might be responsible for spontaneous acceptance in the rat OLTx (Kiyomoto et al. 2005).

29.5.3 NKR-P1A Receptor (CD161) in Cancer Cells

NK cells were originally defined by their ability to spontaneously eliminate rare cells lacking expression of MHC-I self molecules, which is commonly referred to as "missing self" recognition. The molecular basis for missing self recognition emerges from the expression of MHC-I-specific inhibitory receptors on the NK cell surface that tolerize NK cells toward normal MHC-I-expressing cells. By lacking inhibitory receptor ligands, tumor cells or virus-infected cells that have down-modulated surface MHC-I expression become susceptible to attack by NK cells. Killer cell Ig-like receptors (KIR; CD158) and Ig-like transcripts (ILTs), CD94/NKG2 heterodimers, NKG2D, NKR-P1, and KLRG1 belonging to the C-type lectin receptor family, called killer cell lectin-like receptors (KLR approved symbol) (Borrego et al. 2001; Vetter et al. 2000). constitute a family of MHC-I binding receptors that play major roles in regulating the activation thresholds of NK cells and some T cells in humans. Although the function of NKR on T cells infiltrating tumors and their potential effect on antitumor immunity has been investigated, little is known about T cells expressing CD161 in cancer patients. $CD4^+CD161^+CD56^-$ cells represent a distinct memory T-cell population significantly increased in cancer patients. Depending on the type of signals provided by the tumor microenvironment, $CD4^+CD161^+$ cells may regulate the immune response (Iliopoulou et al. 2006).

Cutaneous malignant melanoma is one of the most immunogenic tumors. CD8 T-lymphocytes and NK cells are believed to be important effector cells involved in eliciting a protection against melanoma (Lozupone et al. 2004). The early stages of cutaneous primary melanoma are evidenced by changes in the number of $CD8^+DR^+$ or $CD8^+CD161^+$ T-cells with respect to healthy individuals. In addition, the number of CD56 NK cells was also increased at early disease stages. Campillo et al. (2006) provided evidence of immune activation in early stages of cutaneous melanoma, together with an increase of cells expressing CD158a in patients bearing the corresponding HLA-C ligand. Low expression of CD161 and activating NKG2D receptors is associated with a significant impairment in NK cell activity in metastatic melanoma (MM) patients (Konjević et al. 2007).

Glioblastoma is the most malignant intrinsic brain tumor. The activating NK and costimulatory T-cell ligands that are expressed by glioma cells are overridden by several inhibitory signals, including the immunosuppressive molecule TGF-ß (Friese et al. 2004). Glioma cells showing high surface expression of classic MHC class I molecules and a significant expression of the nonclassic MHC molecules HLA-E and HLA-G immune escape phenotype by interacting with different NK cell receptors (Lanier 2005). A tolerance factor is another molecule that may help immune escape of gliomas by inhibition of NK and T cells. Glioma cells express LLT1 mRNA and protein in vitro and in vivo, whereas expression levels in normal brain are low. It appears that LLT1, expressed by gliomas, contributes to tumor-associated immunosuppression by affecting the lytic activity of NK cells (Roth et al. 2006, 2007). LLT1 expression in human gliomas increases with the WHO grade of malignancy. TGF-β up-regulates the expression of LLT1 in glioma cells. Small iRNA-mediated down-regulation of LLT1 in LNT-229 and LN-428 cells promotes their lysis by NK cells. Thus, LLT1 acts as a mediator of immune escape and contributes to the immunosuppressive properties of glioma cells (Roth et al. 2007). Co-culture of NK cells and DCs results in their reciprocal co-activation, and an enhancement of lysis of tumor target cells (Yang et al. 2006).

In rat 9L gliosarcoma, a substantial number of cells express NKR-P1, a marker expressed only on rat lymphocytes capable of non-MHC-restricted cytotoxicity. Previous investigations have determined the existence of three populations of $NKR\text{-}P1^+$ lymphocytes in normal rats, including $NKR\text{-}P1_{bright}$/T-cell receptor $(TCR)^-/CD3\text{-}/CD5^-$ (~5–15%), $NKR\text{-}P1_{dim}$/$TCR\beta^+/CD3^+/CD5^+$ (1–5%), and $NKR\text{-}P1_{dim}$/TCR $\gamma\delta/CD3^+/CD5^+$ (~0.5–2%). It was suggested that there is selective localization of cells capable of mediating antitumor responses in 9L, but that tumor-associated factors may down-regulate their function and expression of NKR-P1 (Chambers et al. 1996).

Because the activity of cytotoxic cells is suppressed in most cancer patients, it was suggested that the *Klrb1* expression might be suppressed in cancerous cells. The transcription of the KLRB1 was suppressed in tumor tissues in 68% patients with nonsmall-lung-cancer and 57% patients with esophageal squamous-cell carcinoma. This parameter can be a marker of lung and esophageal cancers (Pleshkan et al. 2007).

29.6 Human Early Activation Antigen (CD69)

CD69 belongs to a subfamily of CTLDs, which are referred to as NK-cell domains (NKDs). Its gene maps in the NK gene complex, close to other genes coding for NK receptors.

CD69 is a dimeric glycoprotein of 33 and 27 kDa. The CD69 has a structural homology with other type II lectin cell surface receptors, such as T cell antigen Ly49, the low avidity IgE receptor (CD23), and the hepatic asialoglycoprotein receptors. The CD69, one of the first described members of the NKC family of receptors (López-Cabrera et al. 1993; Ziegler et al. 1994), is present at the cell surface as a disulfide linked homodimer, with subunits of 28 and 32 kDa resulting from the differential glycosylation at a single extracellular *N*-linked glycosylation site. Contrary to other NKC gene products, whose expression is restricted to NK cells, CD69 has been found on the surface of most hematopoietic lineages. It is one of the earliest markers induced upon activation in T and B lymphocytes, NK cells, macrophages, neutrophils, and eosinophils. In addition, it is constitutively expressed on monocytes, platelets, Langerhans cells, and a small percentage of resident lymphocytes in thymus and secondary lymphoid tissues (Sánchez-Mateos et al. 1989). The activation of T lymphocytes induces the expression of CD69. It functions as a signal transmitting receptor in lymphocytes, NK cells, and platelets. Although CD69 is absent from peripheral blood resting lymphocytes, it is expressed by in vivo activated lymphocytes infiltrating sites of chronic inflammation in several pathologies, as well as by lymphocytes after in vitro activation with different stimuli. The TNF-α gene expression and protein secretion could be induced in peripheral blood T cells through CD69 molecule. CD69-deficient mice revealed its modulatory role on B cell development and antibody synthesis (Lauzurica et al. 2000).

29.6.1 Organization of CD69 Gene

The CD69 gene mapped on chromosome 12 p13-p12. The cDNA coding for CD69 exhibited a single open reading frame of 597 bp coding and predicted a 199-amino acid protein of type II membrane topology, with extracellular (COOH-terminal), transmembrane, and intracellular domains. The CD69 clone hybridized to a 1.7-kb mRNA species, which was rapidly induced and degraded after lymphocyte stimulation, consistent with the presence of rapid degradation signals at the 3′ untranslated region. The CD69 protein shares functional characteristics with other members of this superfamily, which act as transmembrane signaling receptors in early phases of cellular activation (López-Cabrera et al. 1993)

The genomic structure of the human gene encoding CD69 sequence is divided into five exons separated by four introns. The first two exons corresponded to separate functional domains: cytoplasmic tail and the transmembrane region. The final three exons encoded the CRD. The major transcription initiation site has been located 30 nt downstream of a consensus TATA box (Santis et al. 1994). The conserved intron position between the exons encoding the CRD indicated that this protein is closely related to other type-II C-type lectins, such as the asialoglycoprotein receptor, the CD23, and NKR-P1 and Ly49. In contrast to the broad NKR-P1 and Ly-49 gene families, CD69 is a single-copy gene. The mouse CD69 gene has phorbol ester-inducible promoter element within the first 700 bp upstream of the start of transcription. Chromosomal mapping placed the mouse CD69 gene on the long arm of chromosome 6 near the NK gene complex that contains the related NKR-P1 and Ly-49 gene families. The human CD69 gene mapped to chromosome 12p13 near the related NKG2 gene cluster and in a region associated with rearrangements in approximately 10% of cases of childhood acute lymphocytic leukemia (Ziegler et al. 1994). Recombinant CD69 protein exists as a disulfide-linked homodimer on the cell surface and crystallizes as a symmetrical dimer, similar to those formed by the related NK cell receptors Ly49A and CD94.

The 5′-flanking region of the promoter of CD69 gene contains a potential TATA element 30 bp upstream of the major transcription initiation site and several putative binding sequences for inducible transcription factors (NF-kB, Egr-1, AP-1), which might mediate the inducible expression of this gene. The proximal promoter region spans positions −78 to +16 containing the cis-acting sequences necessary for basal and phorbol 12-myristate 13-acetate-inducible transcription of CD69 gene. Removal of the upstream sequences located between positions −78 and −38 resulted in decreased promoter strength and abolished the response to phorbol 12-myristate 13-acetate. The TNF-α is capable of inducing the surface expression of CD69 molecule as well as the promoter activity of fusion plasmids that contain 5′-flanking sequences of *CD69* gene, suggesting that TNF may regulate in vivo the expression of CD69 (López-Cabrera et al. 1995).

29.6.2 Src-Dependent Syk Activation of CD69-Mediated Signaling

CD69 engagement leads to the rapid and selective activation of the tyrosine kinase Syk, but not of the closely related member of the same family, ZAP70, in IL-2-activated human NK cells. The requirement for Src family kinases in the CD69-triggered activation of Syk suggests a role for Lck in this event. It was demonstrated that Syk and Src family tyrosine kinases control the CD69-triggered tyrosine phosphorylation and activation of phospholipase Cγ2 and the Rho family-specific exchange factor Vav1 and are responsible for CD69-triggered cytotoxicity of activated NK cells. Thus CD69 receptor functionally couples to the activation of Src family tyrosine kinases, which, by inducing Syk

activation, initiate downstream signaling pathways, induce rise in intracellular Ca^{2+}. Synthesis of different cytokines and/or proliferation regulates CD69-triggered functions on human NK cells (Pisegna et al. 2002; Llera et al. 2001). The receptor-proximal signaling pathways activated by CD69 cross-linking are involved in CD69-mediated cytotoxic activity. CD69 engagement leads to the activation of ERK enzymes belonging to the MAPK family, and that this event is required for CD69-mediated cell degranulation. The co-engagement of CD94/NKG2-A inhibitory receptor effectively suppressed both CD69-triggered cell degranulation in RBL transfectants, through the inhibition of ERK activation, and CD69-induced cytotoxicity in human NK cells. Thus, CD69-initiated signaling pathways and functional activity are negatively regulated by CD94/NKG2-A inhibitory complex (Llera et al. 2001). The wide distribution of CD69, along with its activating signal-transducing properties, suggest an important role of CD69 in the physiology of leukocyte activation (Llera et al. 2001)

29.6.3 Crystal Analysis of CD69

The structure of extracellular portion of NK cell receptors are divergent from true C-type lectins and are referred to as NK-cell domains (NKDs). CD69 NKD adopts the canonical CTLD fold but lacks the features involved in Ca^{2+} and carbohydrate binding by C-type lectins. The NKD of human CD69 at 2.27 A resolution reveals conservation of the C-type lectin-like fold, including preservation of the two α-helical regions found in Ly49A and MBP. Using comparative computer modeling, a 3-D model of the extracellular domain of CD69 based on the crystal structure of the MBL was generated. The sequence of CD69 appears to be highly compatible with the C-type lectin fold. Compared with MBL and selectins, CD69 displays significant deletions in loop regions. The conserved calcium binding sites found in the C-type lectin family are not conserved in CD69; only one of the nine residues coordinated to Ca^{2+} in MBP is conserved in CD69 and no bound Ca^{2+} is evident in the crystal structure. In this respect, CD69 departs from some of the conserved motifs seen in crystal structures of MBL and selectins. The CD69 model shows cavity-shaped hydrophobic regions surrounded by charged residues. One of these cavities is proximal to a potential low affinity calcium binding site and may be implicated in specific interactions with ligands. Surprisingly, electron density suggestive of a puckered six-membered ring was discovered at a site structurally analogous to the ligand-binding sites of MBP and Ly49A. This sugar-like density may represent, or mimic, part of the natural ligand recognized by CD69 (Bajorath and Aruffo 1994; Natarajan et al. 2000). CD69 NKD dimerizes noncovalently, both in solution and in crystalline state. The dimer interface consists of a hydrophobic, loosely packed core, surrounded by polar interactions, including an interdomain β-sheet. The intersubunit core shows certain structural plasticity that may facilitate conformational rearrangements for binding to ligands. The surface equivalent to the binding site of other members of the CTLD superfamily reveals a hydrophobic patch surrounded by conserved charged residues that probably constitutes the CD69 ligand-binding site (Llera et al. 2001).

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NKG2 Subfamily C (KLRC)

30

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30.1 NKG2 Subfamily C (KLRC)

30.1.1 NKG2 Gene Family and Structural Organization

NKG2 receptors are type II C-type, lectin-like, integral membrane glycoproteins, which are expressed on the cell surface as heterodimers with CD94, which is an invariant type II C-type, lectin-like polypeptide. CD94 lacks a cytoplasmic tail and therefore, cannot transduce signals. It is however essential for the expression of NKG2 receptors. Four distinct genes, A/B, C, E/H, and F, encode the NKG2 receptors. Of these receptors, CD94/NKG2A is an inhibitory one, as it contains a long cytoplasmic tail with two ITIMs. Others have short cytoplasmic tails, and each associates noncovalently with a homodimer of DAP-12, as in the case of activating KIRs. The NKG2 family of genes (HGMW-approved symbol KLRC) contains at least six members (NKG2-A, -B, -C, -E, -F and -H) which are localized to human chromosome 12p12.3-p13.2, in the same region where CD69 genes have been mapped. In addition, the human CD94 and NKR-P1A genes map to the short arm of chromosome 12. The physical distance spanned by NK gene complex (NKC) in humans ranges between 0.7 and 2.4 megabases (Renedo et al. 1997). The NKG2 and CD94 genes are localized in a small region (< 350 kb) and mapped in the following order: (NKG2-C/NKG2-A)/NKG2-E/NKG2-F/NKG2-D/CD94. Sequence analysis of 62 kb spanning the NKG2-A, -E, -F, and -D loci allowed the identification of two LINE elements that could have been involved in the duplication of the NKG2 genes. Presence of one MIR and one L1ME2 element at homologous positions in the NKG2-A and NKG2-F genes is consistent with the existence of rodent NKG2 gene(s). The 5′-ends of the NKG2-A transcripts were mapped into two separate regions showing the existence of two separate transcriptional control regions upstream of the NKG2-A locus and defining putative promoter elements for these genes (Plougastel and Trowsdale 1998). Restriction mapping and sequencing revealed the NKG2-C, -D, -E, and -F genes to be closely linked to one another, and of the same transcriptional orientation. The NKG2-C, -E, and -F genes, despite being highly similar, are variable at their 3′ ends. It was found that NKG2-C consists of six exons, whereas NKG2-E has seven, and the splice acceptor site for the seventh exon occurs in an Alu repeat. NKG2-F consists of only four exons and part of exon IV is in some cases spliced to the 5′ end of the NKG2-D transcript. NKG2-D has only a low similarity to the other NKG2 genes Glienke et al. (1998). The murine NKG2-D-like sequence also maps to the murine NK complex near CD94 and Ly49 family members.

30.1.1.1 Association of NKG2 Family Proteins with CD94

NKG2 family proteins have been shown to be covalently associated with CD94. Structural heterogeneity in NKG2 gene family and the formation of heterodimers with CD94 provides the creation of a diverse class of NK cell repertoire (Houchins et al. 1997; Lazetic et al. 1996). The human CD94 glycoprotein forms disulfide-bonded heterodimers with the NKG2A/B, NKG2C, and NKG2E glycoproteins. Human NKG2-F gene is localized 25 kb from NKG2-A, and related to NKG2-D cDNA. Despite the similarities with other NKG2 genes, NKG2-F encodes a putative protein which does not contain any lectin domain. However, a conserved 24-amino acid sequence, present in all members of NKG2 family, suggests that NKG2-F is also able to form heterodimers with CD94 (Plougastel and Trowsdale 1997). NK cells inhibited upon HLA recognition express the CD94/p43 dimer, whose specificity for HLA molecules partially overlaps the Ig-SF receptor system (López-Botet et al. 1997). In addition to NK cells, CD94-NKG2-A/B heterodimers are also expressed by TCR-γ/δ cells, and a subset of TCR-α/β cells. The human NKG2A-CD94 (KLRC1) and NKG2C-CD94 (KLRC2) heterodimers recognize the nonclassic MHC class I molecule, HLA-E (Qa-1 in

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mice) as ligand, which primarily displays peptides derived from the signal peptides of classic MHC class I molecules. NKG2D (KLRK1) molecules are also expressed by T cells, mediating co-stimulatory functions dependent on the availability of the adaptor protein DAP10 (Fig. 30.1).

30.1.2 Human NKG2-A, -B, and -C

The NKG2 cDNA clone is expressed in all NK cells. The original isolate, from a CD3- NK cell clone, was found to cross-hybridize with a family of transcripts that fell into four distinct groups designated NKG2-A, -B, -C, and -D. All four human transcripts encode type II membrane proteins of 215–233 amino acids. NKG2-A and -B peptides appear to be alternative splicing products of a single gene *Nkg2a*, which encodes two isoforms, NKG2A and NKG2B, with the latter lacking the stem region. NKG2-C is highly homologous with group A, having 94% homology in the external (C-terminal) domain and 56% homology throughout the internal and transmembrane regions. Cell surface expression of NKG2A is dependent on the association with CD94 since glycosylation patterns characteristic of mature proteins are found only in NKG2A that is associated with CD94. The induction of an inhibitory signal is consistent with the presence of two ITIM (V/LXYXXL) on the cytoplasmic domain of NKG2A. Similar motifs are found on Ly49 and KIR receptors, which also transmit negative signals to NK cells (Brooks et al. 1997). Brostjan et al. (2002) found differential expression of inhibitory (NKG2-A/B) versus triggering (NKG2-C and potentially -E, -F, -H) NK receptor chains. The generation of the splice variants NKG2-E and -H seemed to occur at a constant ratio. NKG2-D is distantly but significantly related (21% amino acid homology) to the first three groups. Large panel of NK clones indicated that NKG2-A$^-$ P25$^+$ NK clones express the NKG2-C transcript (Cantoni et al. 1998).

30.1.2.1 Promoter Sequences

Brostjan et al. (2000) established app. 3 kb of upstream promoter sequences of the human NKG2-C, -E and -F genes and compared with available NKG2-A sequences. Extended regions of homology contain numerous putative transcription factor binding sites conserved in the NKG2 genes. However, variation in Alu insertion among family members has led to promoter structures unique to the respective family members, which could contribute to differences in transcriptional initiation as well as gene-specific regulation.

NKG2C gene is deleted in Japanese population. The location of the break-point was determined to be 1.5–1.8 kb telomeric from the 3′ end of NKG2A. The frequency of NKG2C deletion haplotype was 30.2% in Japanese and 30.0% in Dutch populations. The frequency of homozygous deletion was 4.1% in Japanese and 3.8% in Dutch. *Nkg2c* deletion in Japanese and Dutch suggests that NKG2C is not essential for survival and reproduction, and is not associated with rheumatic diseases (Miyashita et al. 2004). Each of the NKG2 molecules is paired with CD94 for expression and recognizes HLA-E and Qa-1b as a ligand.

30.1.2.2 NKG2 Receptors on Fetal NK Cells and T Lymphocytes

Using mAb against NKG2A/C/E, Van Beneden et al. precipitated the NKG2A as a 38-kDa protein that decreased drastically from 2 week after birth. Phenotypic analysis showed that ~ 90% of fetal NK cells and ~ 50% of adult NK cells express high levels of CD94/NKG2. The remaining 50% of adult NK cells expressed low surface levels of CD94/NKG2. Expression of CD94/NKG2 was not restricted to NK cells, but was also observed on NK T and memory T cells. Functional analysis showed that CD94/NKG2$^+$ fetal NK cells could discriminate between MHC class I-positive and MHC class I-negative tumor cells. The expression levels of CD94/NKG2 were similar in wild-type compared with $\beta2m^{-/-}$ mice (Stevenaert et al. 2003). CD94/NKG2 receptors are also expressed on a subpopulation of peripheral CD8 memory TCR αβ lymphocytes and on mature TCR Vγ3$^+$ cells in the fetal thymus. Skin-located Vγ3 T cells, the progeny of fetal thymic Vγ3 cells, also expressed CD94/NKG2 and Ly49E but not the other members of the Ly49 family. This suggested that Vγ3 T cells expressing CD94/NKG2 receptors are mature and display a memory phenotype, and that CD94/NKG2 functions as an inhibitory receptor on these T lymphocytes (Van Beneden et al. 2002).

30.1.3 Murine NKG2A, -B, -C

The genes coding for *NKG2A,-B, -C* are clustered on the distal mouse chromosome 6 and on the rat chromosome 4 in a region designated the NK gene complex. The deduced amino acid sequence of mouse NKG2-A contains only one consensus cytoplasmic ITIM. NKG2-A from B6 and BALB/c mice differ by six amino acid residues in the extracellular domain. Murine NKG2B, like its human conterpart, appears to be a splice variant of NKG2-A, and lacks a large portion of the stalk region. Murine NKG2-C lacks an ITIM in its cytoplasmic domain, a feature shared by human and rat NKG2-C. However, unlike the human counterpart, the transmembrane domain of mouse NKG2-C does not contain a charged amino acid residue. Mouse NKG2-A mRNA was detected in IL-2-activated NK cells and spleen cells but not in other tissues (Lohwasser et al. 1999). The identification of a rat CD94 orthologue implied that NK cell receptors equivalent to NKG2/CD94 also exist in rat. The rat NKG2A

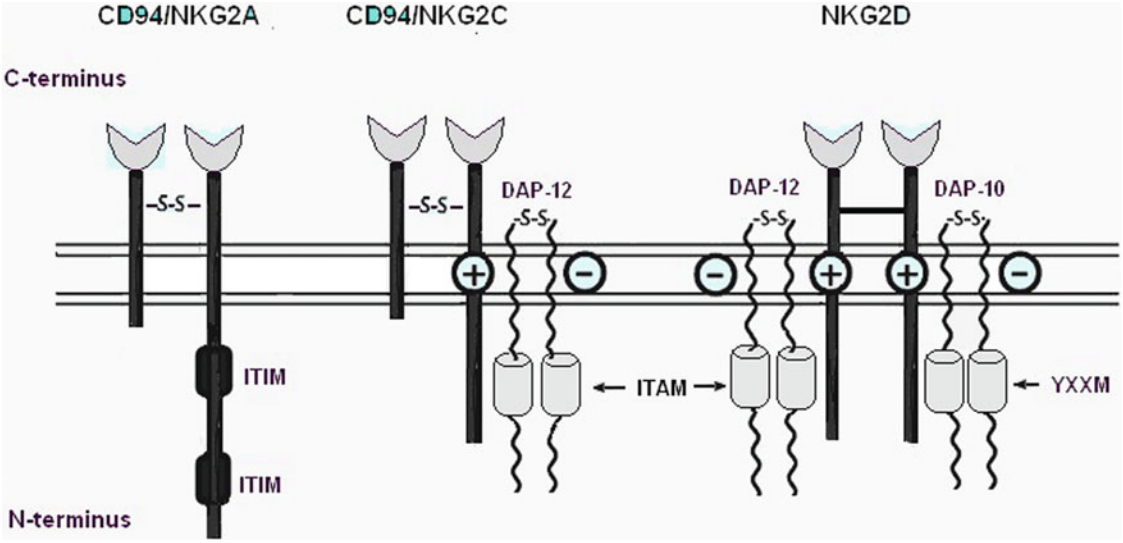

Fig. 30.1 How inhibitory and activating C-lectin receptors operate in NK cells. The C-type lectin receptors are disulfide-linked heterodimers of CD94 and NKG2 family members, either the inhibitory NKG2A or the activating NKG2C, and recognize the nonclassical MHC class I molecule HLA-E. Similar to KIRs with long cytoplasmic tails, ITIM-containing NKG2A signals through SHP-1/2 that mediate inhibitory signals. Likewise, NKG2C has a positively charged transmembrane domain that interacts with DAP-12 and transduces activating signals through Syk family members. NKG2D exists as a homodimer on the surface of NK cells. The dimmer is formed as an extension of an anti parallel β-sheet across the interface with a twofold crystallographic axis perpendicular to the extended sheet (Chap. 31) (Call et al. 2010). The NKG2D dimmer interface is primarily composed of a central hydrophobic core encased by a hydrogen bond and a salt bridge network. In contrast to NKG2C, NKG2D is only distantly related to the other NKG2 family members, does not associate with CD94, and binds to the MHC-like ligands MICA, MICB, and ULBP family. Through its positively charged transmembrane domain NKG2D associates with the adaptor molecule DAP-10 that contains an YXNK motif to bind PI-3 kinase (PI3K) and sends activating signals through this alternative pathway (see Chap. 31 for details). As the PI3K cascade is not inhibited by SHP-1/2, NKG2D may be able to mediate a dominant activation

expression is strain dependent, with high expression in DA and low in PVG NK cells, correlating with the expression of rat CD94. Presence of Ly-49 genes and the existence of rat NKG2 genes in addition to a CD94 orthologue suggest that NK cells utilize different C-type lectin receptors for MHC class I molecules in parallel.

30.1.4 NKG2 Receptors in Monkey

Labonte and Letvin (2004) demonstrated a significant variability in NKG2 mRNA expression in peripheral blood mononuclear cells (PBMCs) from rhesus monkeys. In the absence of DAP12, rhesus monkey NKG2A is preferentially expressed at the cell surface with CD94 due to a single amino acid difference in the transmembrane of NKG2A and NKG2C. In the presence of DAP12, the ability of NKG2C to compete for cell surface CD94 heterodimerization is enhanced and approaches that of NKG2A (LaBonte et al. 2004). Both *M. fascicularis* and *M. mulatta* NK cells express NKp80, NKG2D, and NKG2C molecules, which display a high degree of sequence homology with their human counterpart and reduced surface expression of selected NK cell-triggering receptors associated with a decreased NK cell function in some animals (Biassoni et al. 2005). The full-length cDNAs for CD94, NKG2A, and NKG2CE, in three unrelated squirrel monkeys showed three alternatively spliced forms of CD94 in which part of intron 4 was included in the mature transcript, suggesting evolutionary pressure for changes in the corresponding loop 3 region of the lectin domain (Andersen et al. 2004). Squirrel monkey NKG2A contains a three-nucleotide indel (insertions or deletions) that results in an additional amino acid in the predicted NKG2A protein compared to NKG2A in other species. Transmembrane-deleted forms of CD94 and NKG2CE were also expressed in the squirrel monkey (LaBonte et al. 2007). The NKC of marmoset is 1.5 times smaller than its human counterpart, but the genes are colinear and orthologous. Analyses of NKC genes in additional marmoset individuals revealed a moderate degree of polymorphism of the CD94, NKG2A, NKG2CE, and NKG2D genes. Expression analyses identified several alternatively spliced transcripts, particularly of the CD94 gene (LaBonte et al. 2007).

30.1.5 NKG2 Receptors in Other Species

In contrast to human, the cattle have multiple distinct NKG2A genes, some of which show minor allelic variation. All of the sequences designated NKG2A have two ITIM in the cytoplasmic domain and one putative gene has, in addition, a charged residue in the transmembrane domain. NKG2C appears to be essentially monomorphic in cattle. All of the NKG2A sequences are similar apart from NKG2A-01, which, in contrast, shares the majority of its carbohydrate recognition domain with NKG2-C. Most of the genes appear to generate multiple alternatively spliced forms. The CD94/NKG2A heterodimers in cattle, in contrast to other species, bind several ligands. Because NKG2C is not polymorphic, this raises questions as to the combined functional capacity of the CD94/NKG2 gene families in cattle (Birch and Ellis 2007).

The NKC is not known in chicken. Instead, NK receptor genes were found in the MHC region. In chicken, two C-type lectin-like receptor genes were identified in a region on chromosome 1 that is syntenic to mammalian NKC region. Based on 3D structure and sequence homology, one receptor is the orthologue of mammalian CD69, and the other is highly homologous to CD94 and NKG2. Like CD94/NKG2 gene found in teleostean fishes, chicken CD94/NKG2 has the features of both human CD94 and NKG2A. The arrangement of several other genes that are located outside the mammalian NKC is conserved among chicken, human, and mouse. The chicken NK C-type lectin-like receptors in the NKC syntenic region indicate that this chromosomal region existed before the divergence between mammals and apes (Chiang et al. 2007). A cDNA clone derived from the bony fish *Paralabidochromis chilotes* encodes a protein related to the CD94/NKG2 subfamily of the NK cell receptors. The gene encoding this receptor in a related species, Oreochromis niloticus, has a similar structure to the human CD94/NKG2 genes and is a member of a multigene cluster that resembles the mammalian NK cell gene complex (Sato et al. 2003).

The 1,176 bp cDNA of a C-type lectin gene, from a homozygous rainbow trout contains a 714 bp ORF predicting a 238-amino-acid (27 kDa) protein. The predicted sequence contains a 48 aa cytoplasmic domain, a 20 aa transmembrane domain (TM), a 46 aa stalk region and a 124 aa CRD. Sequence alignment and phylogenetic analysis of the CRD indicated that the protein had similarity with human dendritic cell immunoreceptor (DCIR), gp120 binding C-type lectin (gp120BCL) and mammalian hepatic lectins. The N-terminus (aa 4-183) has similarity with NKG2, important in human NK cell function (Zhang et al. 2000). This indicated that the CD94/NKG2 subfamily of NK cell receptors must have arisen before the divergence of fish and tetrapods and may have retained its function for >400 million years

30.1.6 Inhibitory and Activatory Signals

The induction of an inhibitory signal is consistent with the presence of two ITIM (V/LXYXXL) on the cytoplasmic domain of NKG2A. Similar motifs are found on Ly49 and KIR receptors, which also transmit negative signals to NK cells (Brooks et al. 1997).

Association of DAP12 with Activating CD94/NKG2C NK Cell Receptors: While inhibitory NK cell receptors for MHC class I express ITIM that recruit intracellular tyrosine phosphatases and prevent NK cell effector function, the activating NK cell receptors lack intrinsic sequences required for cellular stimulation. In contrast, the NKG2C and -E/H associate with DAP12 via a positively charged residue in their transmembrane domains and function as activation receptors. Therefore, the NKG2A/-2B contain an ITIM sequences in its cytoplasmic domain, which may be responsible for the inhibitory function of these receptors, whereas NKG2C and -E/H, lack ITIMs and associate with DAP12 via a positively charged residue in their transmembrane domains function as activation receptors and potentially transmit positive signals. Efficient expression of CD94/NKG2C on the cell surface requires the presence of DAP12. The charged residues in the transmembrane domains of DAP12 and NKG2C are necessary for this interaction (Lanier et al. 1998) (Fig. 30.1).

30.2 CD94 (KLRD1)

30.2.1 Human CD94 in Multiple Transcripts

CD94 is a receptor for human leukocyte antigen class I molecules. The CD94 is a type II membrane protein encoded by a unique gene of the C-type lectin superfamily. The primary sequences of NKG2 molecules A/B, C, and E share 27–32% identity with CD94 within the C-type lectin domain with minimal insertions or deletions, suggesting a strong structural similarity between CD94 and the NKG2 molecules. However, homodimeric CD94 has been found on the surface of certain transfected cell lines in which the expression of NKG2 is absent. Cell surface expression of NKG2A is dependent on the association with CD94 as glycosylation patterns are found only in NKG2A that is associated with CD94. Three new alternative transcripts of the *cd94* gene were identified by Lieto et al. (2006) in addition to the originally described canonical CD94Full. One of the transcripts, termed CD94-T4, lacks the portion that encodes the stem region. CD94-T4 associates with both NKG2A and NKG2B, but preferentially associates with the latter. This is probably due to the absence of a stem region in both CD94-T4 and NKG2B.

CD94 Binding with NKG2: Functionally, CD94 exists in a heterodimeric disulfide-linked complex with NKG2A/B, -C, or -E (Lazetic et al. 1996; Brooks et al. 1997; Cantoni et al. 1998) and has been shown to bind to a 26-kD NKG2A or a 43-kD protein on the surface of NK cells. The cloned CD94 molecule also covalently assembles with at least two different glycoproteins to form functional receptors. The human CD94 glycoprotein forms disulfide-bonded heterodimers with the NKG2A/B, NKG2C, and NKG2E glycoproteins. Structural heterogeneity in the NKG2 gene family and the formation of heterodimers with CD94 provides the creation of a diverse class of NK cell repertoire (Lazetic et al. 1996). Homology of CD94 with the NK cell-associated NKR-P1 and NKG2 C-type lectin genes is limited to the structural motifs conserved in the carbohydrate recognition domain. An unexpected feature of CD94 is the essential absence of a cytoplasmic domain, implying that association with other receptors may be necessary for the function of this molecule (LaBonte et al. 2001; Boyington et al. 2000).

CD94 Promoter Genes: CD94 gene expression is regulated by distal and proximal promoters that transcribe unique initial exons specific to each promoter. This results in two species of transcripts; the CD94 mRNA and a CD94C mRNA. All NK cells and $CD94^+$, $CD8^+\alpha\beta$ T cells transcribe CD94 mRNA. Stimulation of NK and $CD8^+\alpha\beta$ T cells with IL-2 or IL-15 induced the transcription of CD94C mRNA. The distal and proximal promoters both contain elements with IFN-γ-activated and Ets binding sites, known as GAS/EBS. Additionally, an unknown element, termed site A, was identified in the proximal promoter. EMSA analyses showed that constitutive factors could bind to oligonucleotide probes containing each element. After treatment of primary NK cells with IL-2 or IL-15, separate inducible complexes could be detected with oligonucleotide probes containing either the proximal or distal GAS/EBS elements. These elements are highly conserved between mice and humans, which suggests that both species regulate CD94 gene expression via mechanisms that predate their evolutionary divergence (Lieto et al. 2003).

30.2.2 Mouse CD94

Mouse CD94 is 54% identical and 66% similar to human CD94, and is a member of the C-type lectin superfamily. Mouse CD94 is expressed efficiently on the cell surface of cells transiently transfected with the corresponding cDNA, but surface CD94 was unable to mediate detectable binding to MHC class I-expressing Con-A blasts. Notably, mouse CD94, like human CD94, has a very short cytoplasmic tail, suggesting the existence of partner chains that may play a role in ligand binding and signaling. Like its human counterpart, the mouse CD94 protein associates with different NKG2 isoforms and recognizes the atypical MHC class I molecule Qa-1b.

Like many other C-type lectins expressed by NK cells, mouse *Cd94* maps to the NK complex on distal chromosome 6, synteneic to human CD94. The mouse *Cd94* is highly expressed specifically by mouse NK cells, raising the possibility that mice, like humans, express multiple families of MHC class I-specific receptors on their NK cells. The murine NKG2D-like sequence also maps to the murine NK complex near *Cd94* and Ly49 family members. The genomic organization of the mouse *Cd94* gene contains six exons separated by five introns. Exons I and II encode the 5′ untranslated region (UTR) and the transmembrane domain. Exon III encodes the stalk region and exons IV-VI encode the carbohydrate recognition domain (CRD). The *Cd94* promoter region and putative regulatory DNA elements have been identified (Lohwasser et al. 2000).

The murine *Cd94* gene has two promoters. Lymphoid cell types use these two promoters differentially. The promoter usage seen in adult cells is established during fetal development. The differential promoter usage by NK cells appears to be susceptible to perturbation, as both the murine NK cell line LNK, as well as cultured C57BL/6 NK cells showed altered promoter usage relative to fresh NK cells. Since, the promoter activity observed in transfection assays did not correlate with expression of the endogenous *Cd94* gene, it suggested the involvement of chromatin structure/methylation in transcriptional regulation (Wilhelm et al. 2003). The identification of two of NK cell receptors with residues of C-type lectin CRDs indicated the existence of a bovine NK gene complex, prospectively located on chromosome 5 (Govaerts and Goddeeris 2001). Bovine CD94, named KLRJ1, is most similar to Ly-49 (KLRA) and mapped to the bovine NK gene complex on chromosome 5. It suggests that KIR multigene families with divergent signaling motifs exist also in bovine species and that the primate and bovine KIR multigene families have evolved independently (Storset et al. 2003).

30.3 Cellular Sources of NKG2/CD94

NKG2 family proteins have been shown to be covalently associated with CD94. Structural heterogeneity in NKG2 gene family and the formation of heterodimers with CD94 provides the creation of a diverse class of NK cell repertoire (Houchins et al. 1997; Lazetic et al. 1996). The NKG2 cDNA clone is expressed on all NK cells. The proportion of $CD94^+NKG2C^-$ ($NKG2A^+$) NK cells and the level of expression of NKG2D, NKp30 and NKp46 decreases with age (Sundström et al. 2007). NK cells derived from human umbilical cord blood (CB) are the major effector cells involved in graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL). The

expression of NKG2A/CD94 was significantly higher on CB NK cells. The high expression of NKG2A/CD94 and low expression of granzyme B may be related with the reduced activity of CB NK cells (Tanaka et al. 2007; Wang et al. 2007). CD94-NKG2-A/B heterodimers are also expressed by TCR-γ/δ cells, and a subset of TCR-α/β cells. NKG2A, commonly expressed on cytotoxic T cells, has been found on activated T helper (Th) cells. In identifying markers differentiating between Th1 and Th2 lymphocytes, Freishtat et al. (2005) identified co-induction of NKG2A and CD56 on activation of Th2 cells.

As in NK cells, CD94 is expressed on Th1 cells together with members of the NKG2 family of molecules, including NKG2A, C, and E. Meyers et al. (2002) proposed that CD94/NKG2 heterodimers may costimulate effector functions of differentiated Th1 cells. Tanaka et al. (2004) found increased expression of CD94/NKG2A on $CD3^+/CD8^+$ T cells from G-CSF mobilized peripheral blood mononuclear cells (G-PBMC) after stimulation with immobilized anti-CD3 mAb with or without cytokines. A large majority of multiple myeloma (MM) $CD8^+$ cells do not express a functional CD94 receptor. It seemed that their ability to 'fine-tune' an appropriate immune response against tumor cells is impaired (Besostri et al. 2000). Activating and inhibitory CD94/NKG2 receptors regulate CTL responses by altering TCR signaling, thus modifying antigen activation thresholds set during thymic selection. Reports suggest that TCR antigenic specificity dictates NKG2A commitment, which critically regulates subsequent activation of CTL (Jabri et al. 2002). NK cells are the most abundant lymphocyte population at the maternal-fetal interface. They are considered to be important during placentation by controlling trophoblast invasion.

Bellon et al. (1999) studied the expression of CD94 heterodimers in different αβ or γ/δ T cell clones. While most of the $CD94^+NKG2A^-$ T cells have a low to intermediate expression of CD94, the cross-linking of the CD94/NKG2 heterodimer in one of these CD8αβ $CD94^+NKG2A^-$ T cell clones (K14B06) resulted in to induce the up-regulation of CD25 and the secretion of IFN-γ and to trigger the redirected cytotoxicity in a TCR-independent manner. The CD94 heterodimer showed a 39-kDa band with a similar activating heterodimer found on other NK cells and led to identification of NKG2H in K14B06 T cells. The NKG2H is an alternative spliced form of *NKG2E* gene and displayed a charged residue in the transmembrane portion and a cytoplasmic tail, which lacks ITIM. The expression of NKG2H forms part of the activating CD94/Kp39 heterodimer present on K14B06 cells.

Melanocyte differentiation antigen MART-1 specific T cells express CD94/NKG2 receptors. Detailed analysis revealed the exclusive presence of inhibitory NKG2-A/B receptors in the vitiligo-like leukoderma, whereas both the inhibitory receptors and the activating NKG2-C/E isoforms were present within the tumor (Pedersen et al. 2002).

Expression of CD94/NKG2 Receptors by Viral- and Bacterial-Specific CD8 T Cells Subsets of CD8 T cells express receptors that are critical in regulating the activity of NK cells. NKG2A/CD94 receptor expression is up-regulated by antiviral $CD8^+$ T cells during acute polyoma infection; this is responsible for down-regulating their antigen-specific cytotoxicity during both viral clearance and virus-induced oncogenesis. Miller et al. (2003) suggested that CD94/NKG2 expression is not correlated with inhibition of T cell function. This was shown following acute infection, with lymphocytic choriomeningitis virus (LCMV), of C57BL/6 and BALB/cJ mice, which expressed Ag-specific CD94/NKG2 $CD8^+$ T cells, and hence CD94/NKG2 expression is not necessarily correlated with inhibition of T cell function (c/r Miller et al. 2003). Wojtasiak et al. (2004) established that while Ag-stimulated gB-specific CD8 T cells primarily express inhibitory isoforms of CD94/NKG2 receptors, these cells remained capable of producing IFNγ upon peptide stimulation.

30.4 The Crystal Analysis of CD94

30.4.1 An Intriguing Model for CD94/NKG2 Heterodimer

Functionally, CD94 exists in a heterodimeric disulfide-linked complex with NKG2A/B, -C, or -E. A twofold related crystallographic dimer was observed in CD94 crystals in which two monomers hydrogen bond through their respective first β strands, creating an extended six-stranded antiparallel β sheet. The interface of this elongated dimer is relatively flat and contains a central hydrophobic region (residues Val66, Tyr68, Ile75, Phe107, and Met108) (Fig. 30.2a), surrounded by hydrophilic residues. A significant part of the interface observed in this dimer involves loop 3, where the helix-to-loop transformation has occurred in CD94, suggesting that this loop conformation may be partly stabilized through hydrophobic interactions across the dimer interface (Boyington et al. 1999). The extracellular ligand-binding domain of human CD94, corresponding to residues 34–179, was expressed in a bacterial expression system and refolded in vitro. The crystal structure of this fragment at 2.6 Å resolution showed that final model comprises residues Cys59 to Ile179. The molecule, with overall dimensions of approximately $42 \times 37 \times 33$ Å, consists of a three stranded antiparallel β sheet (strands 1,

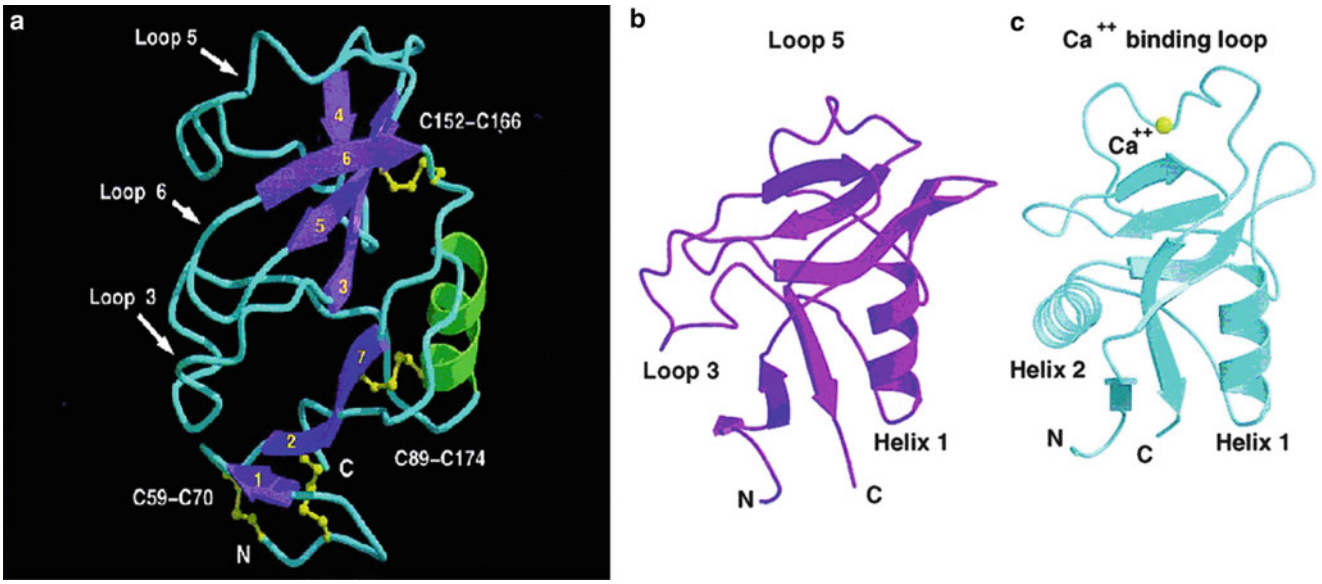

Fig. 30.2 (**a**). **Ribbon diagram of the structure of CD94**. β strands are shown in *purple* and are numbered according to their order in the sequence; the α helix is shown in *green*. The disulfide bonds are represented by *yellow ball-and-stick models* and labeled. Secondary structure assignments are as follows: β strands 1–7 corresponding to residues 66–68, 71–75, 115–122, 127–129, 151–155, 161–165, and 171–176; α helix 1 corresponding to residues 82–91. Comparison of Human CD94 (**b**) and Rat MBP-A (**c**) Superimposed and separated ribbon diagrams of C-type lectin domains from human CD94 and MBP-A (Reprinted with permission from Boyington et al. 1999 © Elsevier)

2, and 7), a four stranded antiparallel β sheet (strands 4, 3, 5, and 6) and an α helix after strand 2 (Fig. 30.2). There are four intrachain disulfide bonds in CD94, three of which are the characteristic invariant disulfides (Cys61–Cys72, Cys89–Cys174, and Cys152–Cys166) found in long-form C-type lectins. The fourth disulfide, Cys59–Cys70, which forms a looped structure with N-terminal β strands, is unique to the structure of CD94. The only extracellular cysteine not involved in intrachain disulfide pairing, Cys58, is expected to pair with the equivalent cysteine of NKG2 (e.g., Cys116 in NKG2A) to form the interchain disulfide in the CD94/NKG2 heterodimer (Boyington et al. 1999).

Unlike the canonical C-type lectin fold, CD94 lacks one of two major a helices present in other C-type lectin structures known till this date. Specifically, the region from residues 102 to 112 (within loop 3), corresponding to the second helix of the consensus C-type lectin fold, adopts a loop conformation in CD94. The putative Ca^{2+}-dependent carbohydrate-binding loop (loop 5, residues 142–152) is 2–5 residues shorter in CD94 compared to other C-type lectins of known structure and displays a markedly different conformation as well as a different sequence. Apart from the missing helix and the conformation of the Ca^{2+}-binding loop, the rest of the CD94 structure is quite similar to the classical C-type lectin fold even though the average sequence identity is only 20% between CD94 and C-type lectins of known structure.

The primary sequences of NKG2-A/-B, -C, and -E suggest a strong structural similarity between CD94 and the NKG2 molecules. The dimerization of CD94 brings the two N-terminal α carbons to within 7.4 Å of each other, which is consistent with having a disulfide bond between the two chains of the receptor. Modeling of CD94 homodimer structure reveals that of the five hydrophobic core residues at the dimer interface of CD94, three are completely conserved in the NKG2 sequences, and two are replaced with other nonpolar residues. This distinctly hydrophobic patch forms the largest contiguous nonpolar surface on both the CD94 monomer and the model of NKG2. While the CD94 homodimer has no interchain salt bridges, there are two potential regions of charge complementarity across the modeled CD94/NKG2 interface: one between Asp106 of CD94 and Lys135 of NKG2A and the other between Arg69 of CD94 and Glu122 of NKG2A. Lys135 and Glu122 are each conserved throughout NKG2A/B, -C, and -E sequences, but are replaced by Ser and Lys, respectively, in CD94, creating an unfavorable Arg69–Lys64 interaction across the CD94/CD94 interface. This helps to explain the favorable interaction between CD94 and NKG2 compared to CD94 homodimerization (Boyington et al. 1999).

The areas of greatest sequence divergence between human CD94 and NKG2 occur outside the dimer interface in the C-terminal half of the molecule, forming a contiguous surface at one end of CD94 (Fig. 30.3). These variable regions border each other to form a flat, uninterrupted surface across one face of the molecule opposite from the N- and C-termini. Based on this structure and sequence alignments, it was postulated that the putative ligand-binding site resides within this variable region including

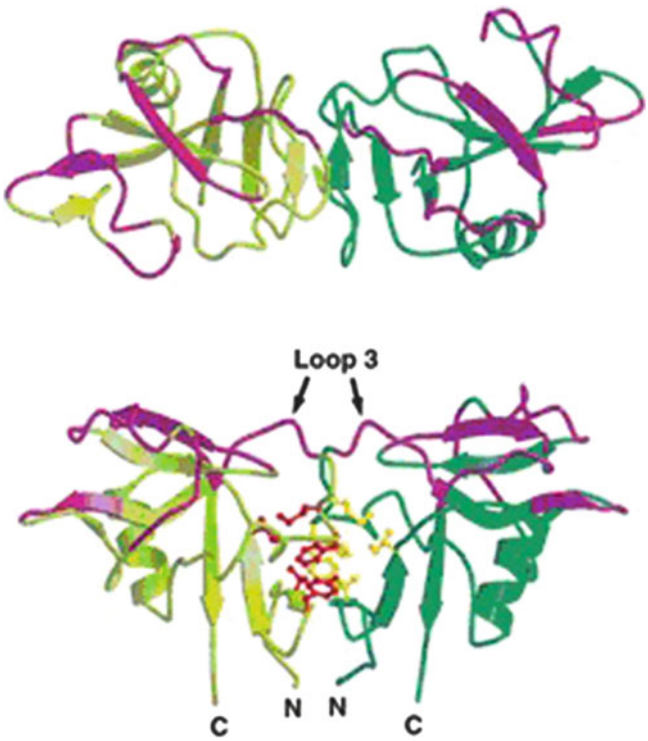

Fig. 30.3 Ribbon models showing two views of the CD94 dimer: each monomer is colored with a different *green shade*. Regions that have low sequence identity with the NKG2 sequences are colored *purple*. The *top view* is rotated 90° from the *bottom view* along the *horizontal axis*. In the *bottom view*, residues in the hydrophobic core of the dimer interface (V66, Y68, I75, F107, and M108) are represented by *ball-and-stick models*. These residues are colored *red* in one monomer and *yellow* in the other (Reprinted with permission from Boyington et al. 1999 © Elsevier)

residues 110–115, 120–124, 137–142, 144–150, 159–165, and 167–171 of human CD94 and the equivalent residues of human NKG2. The variable region of CD94 has an overall net negative charge, with seven acidic residues and only three basic residues. In contrast, the corresponding regions of the NKG2A sequence is considerably more positively charged than CD94, with six basic residues and four acidic residues (Boyington et al. 1999).

30.5 CD94/NKG2 Complex

30.5.1 CD94 and NKG2-A Form a Complex for NK Cells

The CD94/NKG2 family of receptors play an important role in regulating responses against infected and tumorigenic cells. While soluble NKG2A, -B and -C lectin domains interact with CD94 lectin domains to form complexes, NKG2D and human NKR-P1 lectin domains do not (Ding et al. (1999). The NKG2-B protein, which is an alternatively spliced product of the NKG2-A gene, can also assemble with CD94. Both NKG2-A and NKG2-B proteins contain ITIM. This provides the molecular basis of the inhibitory function mediated by the CD94/NKG2-A receptor complexes. Soluble NKG2C, -D and CD94 lectin domains bind solubilized purified HLA class I antigens independently, whereas NKG2A and -B require association of CD94 lectin domains for binding. Through differential CD94/NKG2 gene expression, human NK cells generate diverse repertoires, each cell having an inhibitory receptor for autologous HLA class I. The lytic capacity of a NK cell is regulated, in part, by the balance in cell surface expression between inhibitory CD94/NKG2A and activating CD94/NKG2C heterodimers (Lazetic et al. 1996; Borrego et al. 2006; Carretero et al. 1997).

The heterodimeric CD94/NKG2A receptor, expressed by mouse NK cells, transduces inhibitory signals upon ligation of its ligand, Qa-1b. The CD94/NKG2C and CD94/NKG2E also bind to Qa-1b. Within their extracellular CRDs, NKG2C and NKG2E share extensive homology with NKG2A (93–95% amino acid similarity). CD94/NKG2 molecules are the only Qa-1b binding receptors on NK cells. However, NKG2C/E receptors differ from NKG2A in their cytoplasmic domains (only 33% similarity) and contain features that suggest that CD94/NKG2C and CD94/NKG2E may be activating receptors (Vance et al. 1998, 1999).

30.6 Acquisition of NK Cell Receptors

Coordinated Acquisition of Inhibitory and Activating Receptors: The stages of human NK cell differentiation are not well established. Culturing $CD34^+$ progenitors, Grzywacz et al. (2006) identified 2 non-overlapping subsets of differentiating $CD56^+$ cells based on CD117 and CD94 [$CD117^{high}CD94^-$ and $CD117^{low/-}CD94^+$ cells]. Both populations expressed CD161 and NKp44, but differed with respect to NKp30, NKp46, NKG2A, NKG2C, NKG2D, CD8, CD16, and KIR. These two subsets represent distinct stages of NK cell differentiation, since purified $CD117^{high}$ $CD94^-$ cells gave rise to $CD117^{low/-}CD94^+$ cells. The identified stages of NK-cell differentiation suggested an evidence for coordinated acquisition of HLA-specific inhibitory receptors (i.e., CD94/NKG2A) and function in developing human NK cells. However, CD94/NKG2 receptor acquisition by NK cells does not require lymphotoxin-β receptor expression (Stevenaert et al. 2005).

Hierarchy of NK Cell Response Is Determined by Class and Quantity of Inhibitory Receptors for Self-HLA-B and HLA-C Ligands: Yu et al. (2007) evaluated the resting NK repertoire analyzing the responsiveness of NK subgroups expressing discrete combinations of non-KIR and KIR class I-specific receptors. CD94:NKG2A and

ILT2-expressing cells have a modest response to class I-negative target cells, but NK cells expressing inhibitory KIRs to self-MHC class I ligands, both HLA-B and HLA-C ligands, achieve significantly higher effector capacity. These findings defined how inhibitory receptor and autologous HLA interactions impact single-cell function and demonstrated how hierarchy of NK cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. These findings have important implications for the resting NK response to viral pathogens and malignancy and for donor selection in allogeneic hemopoietic cell transplantation (Yu et al. 2007; Salmon-Divon et al. 2005).

30.7 Regulation of CD94/NKG2

30.7.1 Transcriptional Regulation of NK Cell Receptors

The stochastic expression of individual members of NK cell receptor gene families on subsets of NK cells has attracted considerable interest in the transcriptional regulation of these genes. The discovery that an upstream promoter can function as a probabilistic switch element in the Ly49 gene family has revealed a mechanism of variegated gene expression. The other NK cell receptor gene families contain probabilistic switches. The promoter elements identified in the Ly49, NKR-P1, CD94, NKG2A, and KIR gene families have been reviewed. In the human population, there is a wide variation in the NK cell repertoire of KIRs and CD94:NKG2A expression. Variation is principally due to KIR gene variation and polymorphism, with a smaller effect due to MHC class I.

The human NKG2A gene of 3.9-kb genomic fragment contains a 1.65-kb region upstream of the exon 1, as well as exon 1 (untranslated), intron 1 and exon 2. A region immediately upstream from the most upstream transcriptional initiation site led to increased transcriptional activity. Within a DNase I hypersensitivity site to this region is 80-bp segment that shows two GATA (a zinc-finger transcription factor) binding sites. Mutation of GATA binding site II (−2,302 bp) but not GATA binding site I (−2,332 bp) led to decreased transcriptional activity. GATA-3 specifically binds to the NKG2A promoter in situ in NKL and primary NK cells, but not in Jurkat T cells. Thus GATA-3 is an important transcription factor for regulating NKG2A gene expression (Marusina et al. 2005; Muzzioli et al. (2007). The cAMP induced the upregulation of NKG2A at the mRNA level in human $CD8^+$ T lymphocytes. The PGE2/cAMP/PKA type I pathway appears to be involved in the expression of CD94/NKG2A on human $CD8^+$ T lymphocytes (Zeddou et al. 2005).

30.7.2 Regulation by Cytokines

Members of NKG2 family, NKG2A, -C, -D, and -E are sequentially expressed on $CD4^+$ cells. This expression was tightly regulated by cytokines, among which TGFβ1 and IL-10 are the main factors that positively contribute to the expression of CD94 and NKG2A (Romero et al. (2001). Ortega et al. (2004) found a constitutive expression of NKG2E in CD94-depleted resting human peripheral $CD4^+$ cells, whereas inductions of NKG2A (day 15) and NKG2C (day 20) after CD3-mediated stimulation required chronic cell activation and occurred after expression of CD94 (day 5). The mRNA induction of NKG2-A and NKG2-C genes was influenced by the presence of cytokines (IL-10 and TGF-β). This indicates that there are strict gene regulatory mechanisms for CD94 and NKG2 gene expression on $CD4^+$ cells, which are different from the mechanisms governing the expression of these same genes in $CD8^+$ cells (Gunturi et al. 2005). IL-12 modulates the expression of the CD94/NKG2-A inhibitory receptor by $CD8^+$ T cells in culture. Expression of the CD94/NKG2-A was also induced by IL-12 during T cell Ag stimulation. This implies the role of IL-12 in the modulation of immune responses through NKR induction (Derre et al. 2002; Gays et al. 2005).

Regulation by IL-15: The IL-15 provides an appropriate stimulus to the expression of CD94/NKG2A, but not of other class I-specific NK receptors in the process of maturation of NK cells from thymocytes (Mingari et al. 1997). Among different cytokines, only IL-2 or IL-15 induced cell proliferation when used alone. IL-15Rα-deficient (IL-15R$\alpha^{-/-}$) mice lack NK cells. However, when bone marrow (BM) progenitors from IL-15R$\alpha^{-/-}$ mice were cultured with IL-7, stem cell factor and flt3 ligand, followed by IL-15, they were able to differentiate into functional NK cells, indicating that IL-15Rα is not critical for NK cell development (Kawamura et al. 2003).

Autocrine IL-15 Mediates Intestinal Epithelial Cell Death: Intestinal intraepithelial lymphocytes (IELs), which reside between the basolateral faces of intestinal epithelial cells (IECs), provide a first-line defense against pathogens. IL-15 mediates the reciprocal interaction between IELs and IECs, an important interaction for the regulation of appropriate mucosal immunohomeostasis. These intraepithelial NK cells expressed Ly-49 antigens, NKG2 receptors, and perforin. It suggests the possibility that the apoptosis of IECs could be regulated by self-produced IL-15 through the

activation of intraepithelial NK cells. The IELs, T-cell receptor αβCD8$^+$ T cells located between epithelial cells, were thought to contribute to Fas ligand (FasL)-mediated epithelial cell death in coeliac disease, a condition characterized by excess IL-15.

IELs express CD94 in celiac disease (CD), which is characterized by IL-15 cytokine response of IELs to gluten-containing diet with concomitant increase in expression of IFN-β and down-regulatory IL-10 without increase of TNF-α or TGF-β1. CD is characterized by the presence of gliadin-specific CD4$^+$ T cells in the lamina propria and by the intraepithelial T-cell infiltration of unknown mechanism. In normal intestine, different proportions of IELs, which were mainly T cells, express NK receptors including CD94/NKG2. During active CD, the frequency of CD94$^+$ IELs, which were mostly αβT cells, conspicuously increased, while the expression of other NK markers was not modified. It appeared that the gut epithelium favors the development of T cells that express NK receptors. The specific and selective increase of IELs expressing CD94 may be related to T-cell receptor activation and/or IL-15 secretion (Jabri et al. 2000). In three major IEL-subsets γδIELs, CD4$^+$αβIELs and CD8$^+$αβELs, as well as CD94$^+$CD8$^+$αβIELs, all of which selectively expanded in active CD, CD8$^+$αβ IELs showed a significant increase in expression of both IFN-γ and IL-10. Production of IL-10 may be a common feature of IELs producing pro-inflammatory cytokines, thereby attempting to limit inflammation in an autocrine fashion (Ebert 2005; Forsberg et al. 2007; Kinoshita et al. 2002).

IL-21 Induces the Functional Maturation of Murine NK Cells: IL-21 stimulates mouse NK cell effector functions in vitro. The IL-21 is produced by CD4$^+$ T and NKT cells and mediates potent effects on a variety of immune cells including NK, -T, and -B cells. It achieves its stimulatory effect by inducing the development of mature NK cells into a large granular lymphocyte phenotype with heightened effector function. IL-21 treatment results in increased cell size and granularity and a corresponding decrease in cell viability and proliferative potential. These cells up-regulate the expression of the inhibitory CD94-NKG2A receptor complex and the activation markers CD154 and lectin-like-receptor G1. Surprisingly, IL-21 treatment also results in down-regulation of the pan-NK marker, NK1.1. These developmental changes suggested that IL-21 functions to induce the terminal differentiation of mouse NK cells, resulting in heightened NK cell-mediated cytotoxicity and immune surveillance (Brady et al. 2004).

IL-21 increases the proliferation of NKT cells in combination with IL-2 or IL-15, and particularly with the CD1d-restricted glycosphingolipid Ag α-galactosylceramide. Similar to its effects on NK cells, IL-21 enhances NKT cell granular morphology, granzyme B expression, and some inhibitory NK receptors, including Ly49C/I and CD94. This study suggests that NKT cells are potentially a major source of IL-21, and that IL-21 may be an important factor in NKT cell-mediated immune regulation (Coquet et al. 2007).

IFN-γ-Mediated Negative Feedback Regulation of NKT-Cell Function by CD94/NKG2: Activation of invariant NK T (iNKT) cells with CD1d-restricted T-cell receptor (TCR) ligands is a powerful means to modulate various immune responses. The CD94/NKG2A inhibitory receptor plays a critical role in down-regulating iNKT-cell responses. The iNKT-cell response is of limited duration and iNKT cells appear refractory to secondary stimulation. Both TCR and NK-cell receptors expressed by iNKT cells were rapidly down-modulated by priming with α-galactosylceramide (α-GalCer) or its analog OCH. TCR and CD28 were re-expressed more rapidly than the receptors CD94/NKG2A and Ly49, temporally rendering the primed iNKT cells hyperreactive to ligand restimulation. Blockade of the CD94/NKG2-Qa-1b interaction markedly augmented recall and primary responses of iNKT cells. This shows the critical role for NK-cell receptors in controlling iNKT-cell responses and provides a novel strategy to augment the therapeutic effect of iNKT cells by priming with OCH or blocking of the CD94/NKG2A inhibitory pathway in clinical applications (Kaiser et al. 2005; Ota et al. 2005).

30.8 Functions of CD94/NKG2

NK cell receptors create a delicate balance of activating and inhibitory signals. These receptors are present on different immune cells but play a major role for the innate immune system. For example, the NKG2A-CD94 heterodimer transmits an inhibitory signal and NKG2C-CD94 signals in an activating fashion while both receptors recognize the same ligand, HLA-E. CD94-NKG2-A/B heterodimers are expressed by NK cells, TCR-γ/δ cells, and a subset of TCR-α/β cells. Internalization of CD94/NKG2A is independent of ligand cross-linking or the presence of functional ITIM (Borrego et al. 2002). Thus, the mechanisms that control cell surface homeostasis of CD94/NKG2A are independent of functional signaling. Masilamani et al. (2008) indicated that CD94/NKG2A utilizes a novel endocytic mechanism coupled with an abbreviated trafficking pattern, perhaps to insure surface expression.

30.8.1 CD94/NKG2 in Innate and Adaptive Immunity

CD94/NKG2 receptor varies in function as an inhibitor or activator depending on which isoform of NKG2 is expressed. The lytic capacity of a NK cell is regulated, in part, by the balance in cell surface expression between inhibitory CD94/NKG2A and activating CD94/NKG2C heterodimers. The ligand for CD94/NKG2 is HLA-E in human and its homolog, Qa1 in mouse, which are both nonclassical class I molecules that bind leader peptides from other class I molecules. Although <5% of CD8 T cells express the receptor in a naive mouse, its expression is upregulated upon specific recognition of antigen. Similar to NK cells, most CD8 T cells that express high levels of CD94 co-express NKG2A, the inhibitory isoform. The engagement of this receptor can lead to a blocking of cytotoxicity. However, these receptors have also been implicated in the cell survival of both NK and CD8 T cells. It was found that the extent of apoptosis in $CD8^+$ T and NK cells was inversely related to the expression of CD94, with lower levels of apoptosis seen in $CD94^{high}$ cells after 1–3 days of culture. The expression of CD94/NKG2 is correlated with a lower level of apoptosis and may play an important role in the maintenance of $CD8^+$ T and NK cells. Thus, CD94/NKG2 receptors may regulate effector functions and cell survival of NK cells and CD8 T cells, thereby playing a crucial role in the innate and adaptive immune response to a pathogen (Gunturi et al. 2004, 2003; Lohwasser et al. 2001). However, NK cells whose inhibitory receptors lack any apparent self-ligand can also be found in healthy individuals. On examining these NK cells, Grau et al. (2004) detected NK cells whose sole inhibitory receptors were CD94/NKG2-A and that had no affinity for autologous HLA-C molecules. Findings demonstrated the presence of potentially autoreactive NK cells in otherwise healthy individuals.

CD94 Participates in Qa-1-Mediated Self Recognition by NK Cells: In comparison to human CD94/NKG2 heterodimer, mouse Ly-49, CD94/NKG2 homologues and CD94 have been observed on all NK and NK T cells as well as small fractions of T cells in all mouse strains tested. Two distinct populations of CD94 were identified among NK and NK T cells, $CD94^{bright}$ and $CD94^{dim}$ cells, independent of Ly-49 expression. Importantly, $CD94^{bright}$ but not $CD94^{dim}$ cells were found to be functional in the Qa-1/Qdm-mediated inhibition. Toyama-Sorimachi et al. (2001) suggested that mouse CD94 participates in the protection of self cells from NK cytotoxicity through the Qa-1 recognition, independent of inhibitory receptors for classical MHC class I such as Ly-49.

Qa-1b in Association with CD94/NKG2A on CD8 T Cells Regulates Cytotoxic T Cells: The CD94/NKG2A recognizes the non-classical MHC class I molecule Qa-1b and inhibits NK cytotoxicity. Qa-1b presents a peptide derived from the leader sequence of classical MHC class I molecules. During action of CD94/NKG2A in T cell-mediated cytotoxicity, tetrameric Qa-1b binds to almost all $CD8^+$, but not $CD4^+$ T cells. Most murine $CD8^+$ T cells constitutively express CD94 and NKG2A transcripts. Co-expression of Qa-1b and D(k) on target cells significantly inhibited cytotoxicity of D(k)-specific cytotoxic T lymphocytes generated by MLR, indicating that Qa-1b on antigen-presenting cells interacts with CD94/NKG2A on CD8 T cells and regulates classical MHC class I-restricted cytotoxic T cells (Lohwasser et al. 2001). The Qa-1-NKG2A interaction protected activated CD4+ T cells from lysis by a subset of $NKG2A^+$ NK cells and was essential for T cell expansion and development of immunologic memory. Findings suggest a new clinical strategy for elimination of antigen-activated T cells in the context of autoimmune disease and transplantation (Lu et al. 2007).

30.8.2 Modulation of Anti-Viral and Anti-Tumoral Responses of γ/δ T Cells

Viral, bacterial, protozoal, and cancer-associated Ags elicit strong responses in human γ/δ T lymphocytes. Vγ9Vδ2 T cells stimulated with nonpeptidic mycobacterial antigens produce IFN-γ and TNF-α. Signaling through the CD94/NKG2 receptor interferes with the synthesis of these cytokines. The CD94/HLA class I interaction is also involved in the cytotoxic activity of Vγ9Vδ2 T cells. The Vγ9Vδ2 T cell regulation through the CD94 receptor may be important for the potentially dual function in innate immunity, i.e., (1) NK-like and (2) TCR ligand-induced cytolytic activities (Poccia et al. 1997).

Different Mechanisms of CD94/NKG2-A Expression on γ/δ T Lymphocytes: Most adult peripheral blood γ/δ T cells express Vγ9Vδ2-encoded TCR that recognize a restricted set of nonpeptidic phosphorylated compounds, referred to as phosphoantigens. They also express MHC class I-specific inhibitory receptors, in particular CD94/NKG2-A heterodimers, which participate in the fine tuning of their TCR-mediated activation threshold. Most mature Vγ9Vδ2 T cells express surface CD94 receptors, unlike cord blood or thymus-derived Vγ9Vδ2 clones, thus suggesting a role for the microenvironment in inhibitory receptors expression. Most $CD94^-$ Vγ9Vδ2 peripheral blood lymphocytes (PBL), ex vivo, express an intracellular pool of CD94/

NKG2-A receptors that is translocated to the cell surface upon activation by phosphoantigens or IL-2. In sharp contrast, intracellular CD94/NKG2-A complexes are undetectable in $CD94^{-}$ thymus or PBL-derived mature Vδ2 T cell clones, and no surface induction is observed following phosphoantigen activation of T cell clones. These results provide the existence of distinct mechanisms controlling in vivo and in vitro induction of inhibitory receptors on Vγ9Vδ2 T cells (Boullier et al. 1998).

CD94/NKG2 Complex or KIRs Exert Inhibitory Effect on HLA-I-Mediated NK Cell Apoptosis: The $CD8^{dull}$, $CD8^{intermediate}$, and $CD8^{bright}$ NK cell clones can be identified. Triggering of CD8 with its natural ligand(s), such as soluble HLA class I (sHLA-I) leads to NK cell apoptosis. The magnitude of apoptosis directly correlated with the level of CD8 expression. The sHLA-I-induced apoptosis depends on the interaction with CD8, as it was inhibited by masking this molecule with specific mAbs. However, inhibitory receptor, such as CD94/NKG2 complex or KIRs exerted an inhibitory effect on sHLA-I-mediated apoptosis and secretion of FasL. Thus the interaction between sHLA-I and CD8 evoking an apoptotic signal is down-regulated by inhibitory receptor superfamily that functions as survival receptors in NK cells (Spaggiari et al. 2002).

30.9 NK Cells in Female Reproductive Tract and Pregnancy

NK cells are present in the various female reproductive tract (FRT) tissues; their regulation is largely dependent upon the FRT tissue where they reside. NK cells in the Fallopian tube, endometrium, cervix, and ectocervix expressed CD9 while blood NK cells did not. Unique subsets of NK cells are found in specific locations of the FRT. The NK cells in the lower reproductive tract did not express CD94, but they did express CD16. In contrast, NK cells in the upper FRT express high amounts of CD94 and CD69, but few NK cells expressed CD16. All FRT NK cells were able to produce IFN-γ upon stimulation with cytokines. These unique characteristics of the tissues may account of specific localization of different NK cell subsets (Mselle et al. 2007).

30.9.1 Decidual NK Cell Receptors

NK cells are the most abundant lymphocyte population at the maternal-fetal interface. They are considered to be important during placentation by controlling trophoblast invasion. In early human pregnancy, uterine decidual NK cells (dNK) are abundant (50–90% of lymphocytes) and considered as cytokine producers but poorly cytotoxic despite their cytolytic granule content, suggesting a negative control of this latter effector function. There is no clear evidence that dNK cells kill trophoblast cells. Instead they are able to secrete cytokines which are likely to be beneficial for the placental development, maternal uterine spiral arteries remodeling, and the antiviral immune response (Rabot et al. 2005). To investigate the basis of a negative control of effector function, El Costa et al. (2008) examined the relative contribution to the cytotoxic function of different activating receptors expressed by dNK. In fresh dNK, mAb-specific engagement of NKp46- and to a lesser extent NKG2C-, but not NKp30-activating receptors induced intracellular calcium mobilization, perforin polarization, granule exocytosis and efficient target cell lysis. It was found that in dNK, mAb-specific engagement of NKp30, but not NKp46, triggered the production of IFN-γ, TNF-α, MIP-1α MIP-1β, and GM-CSF pro-inflammatory molecules.

The balance of inhibitory and activating NK receptors on maternal dNK cells, most of which are $CD56^{bright}$, is thought to be crucial for the proper growth of trophoblasts in placenta and maintenance of pregnancy. The CD94/NKG2, a receptor for HLA-E, is expressed on trophoblasts. Women with alloimmune abortions have a limited inhibiting KIR repertoire and such miscarriages may occur because trophoblastic HLA class I molecules are recognized by dNK cells lacking the appropriate inhibitory KIRs (Varla-Leftherioti et al. 2003). dNK cells express normal surface levels of certain activating receptors, including NKp46, NKG2D, and 2B4, as well as KIRs and CD94/NKG2A inhibitory receptor. In addition, they are characterized by high levels of cytoplasmic granules despite their $CD56^{bright}$ $CD16^{-}$ surface phenotype. Moreover, in dNK cells, activating NK receptors display normal triggering capability whereas 2B4 functions as an inhibitory receptor. This might suggest that dNK cells, although potentially capable of killing, are inhibited in their function when interacting with cells expressing CD48 (Eidukaite et al. 2004; Vacca et al. 2006).

Kusumi et al. (2006) compared the expression patterns of NK receptor, CD94/NKG2A, and CD94/NKG2C, on dNK cells in early stage of normal pregnancy with those on peripheral NK cells, most of which are $CD56^{dim}$. The rate of NKG2A-positive cells was significantly higher for decidual $CD56^{bright}$ NK cells than for peripheral $CD56^{dim}$ NK cells, but the rates of NKG2C-positive cells were comparable between the two cell types. Interestingly, peripheral $CD56^{dim}$ NK cells reciprocally expressed inhibitory NKG2A and activating NKG2C, but decidual $CD56^{bright}$ NK cells that expressed activating NKG2C simultaneously expressed inhibitory NKG2A. The co-expression of inhibitory and activating NKG2 receptors may fine-tune the immunoregulatory functions of the dNK cells to control the

trophoblast invasion in constructing placenta (Kusumi et al. 2006).

Pre-eclamptic women had higher number of $CD56^+$ and $CD94^+$ cells in the decidua, indicating an altered receptor expression of dNK cells. The villous trophoblasts from women suffering from pre-eclampsia had significantly less IL-12 in placentae and significantly elevated IL-12 and IL-15 levels in serum. The altered receptor expression of dNK cells together with diminished placental IL-12 expression could implicate an altered NK cell-regulation in pre-eclampsia (Bachmayer et al. 2006).

Potentially cytotoxic $V\delta^+$ T lymphocytes recognize HLA-E on the trophoblast via their CD94/NKG2A receptors. While the percentage of viable $V\delta2^+$ T cells was higher, the percentage of $V\delta1^+$ T cells was lower in women at risk of premature pregnancy termination than in healthy pregnant women. Nonetheless, the percentage of $NKG2A^+$ $V\delta2^+$ T cells was significantly lower in pregnant women at risk of premature pregnancy termination. This indicates the involvement of γδ T lymphocytes in the pathogenesis of premature pregnancy termination (Szereday et al. 2003).

30.10 Signal Transduction by CD94/NKG2

30.10.1 Engagement of CD94/NKG2-A by HLA-E and Recruitment of Phosphatases

Many receptors share common signaling motifs to transmit their signal into the cell. Activating receptors usually signal via the immunoreceptor tyrosine-based activation motif (ITAM) containing tyrosine residues that can be phosphorylated upon receptor engagement and can recruit Syk family kinases. Inhibitory receptors often possess an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM), which can recruit phosphatases upon phosphorylation. These phosphatases, like SHP-1 or SHP-2, are able to dephosphorylate and therefore inhibit intracellular factors that otherwise would promote cellular activation. NKG2C and NKG2D, the C-type lectin-like receptors, play important roles for the activation of NK cells. These receptors have no intracellular signaling domain but instead pair with the adaptor molecules DAP10 and/or DAP12 to mediate an activating signal. The src-family tyrosine kinases include SH2-domain-containing protein tyrosine phosphatase 1 (SHP-1), SHP-2, and SH2-domain-containing inositol polyphosphate 5′ phosphatase (SHIP1). SHP-1, in particular, has been demonstrated to associate with phosphorylated ITIMs and to mediate inhibition of NK cell cytotoxicity (Fig. 30.1). A selective engagement of the CD94/NKG2A receptor with a specific mAb was sufficient to induce tyrosine phosphorylation of NKG2A subunit and SHP-1 recruitment. Furthermore, mAb cross-linking of the CD94/NKG2A receptor, segregated from other NK-associated molecules by transfection into a leukemia cell line (RBL-2H3), promoted tyrosine phosphorylation of NKG2A and co-precipitation of SHP-1, together with an inhibition of secretory events triggered via FcεRI (Carretero et al. 1998).

Specific engagement of the receptor complex expressed on the surface of an NK clone induced the phosphorylation of MAPK. It was demonstrated that the MAPK pathway participates in the CD94-dependent TNF-α production and cytotoxicity. Cross-linking of the receptor induced calcium mobilization, serotonin release and phosphorylation of MAPK (Carretero et al. 2000). Palmieri et al. (1999) indicated that CD94/NKG2-A inhibits the CD16-triggered activation of two signaling pathways involved in the cytotoxic activity of NK cells. They thus provide molecular evidence to explain the inhibitory function of CD94/NKG2-A receptor on NK effector functions.

NK Cell inhibitory receptors can interfere with tyrosine phosphorylation of 2B4 (CD244), which is an NK cell activation receptor that can provide a co-stimulatory signal to other activation receptors. Cross-linking of 2B4 on NK cells and ligation of 2B4 in the context of an NK cell-target cell interaction leads to 2B4 tyrosine phosphorylation, target cell lysis, and IFN-γ release. Coligation of 2B4 with CD94/NKG2 completely blocks NK cell activation. The rapid tyrosine phosphorylation of 2B4 after contact of NK cells with sensitive target cells is abolished when CD94/NKG2 are engaged by their cognate MHC class I ligand on resistant target cells. These results demonstrate that NK inhibitory receptors can interfere with a step as proximal as phosphorylation of an activation receptor (Watzl et al. 2000; Zingoni et al. 2000.

30.11 Ligands for CD94/NKG2

30.11.1 HLA-E as Ligand for CD94/NKG2A

The protein HLA-E is a non-classical MHC molecule of limited sequence variability. Human HLA-E, transcribed in most tissues, is the primary ligand for CD94/NKG2A-inhibitory receptors. The apparent CD94-mediated specific recognition of different HLA class Ia allotypes, transfected into the HLA-defective cell line 730.221, depends on their selective ability to concomitantly stabilize the surface expression of endogenous HLA-E molecules, which confer protection against $CD94/NKG2A^+$ effector cells. Further studies on $CD94/NKG2^+$ NK cell-mediated recognition of .221 cells transfected with different HLA class I allotypes confirmed that the inhibitory interaction was mediated by CD94/NKG2A recognizing the surface HLA-E molecule, because only antibodies directed against either HLA-E, CD94, or CD94/NKG2A specifically restored lysis.

Consistent with the prediction that the ligand for CD94/NKG2A is expressed ubiquitously, the HLA-E distribution indicated that it is detectable on the surface of a wide variety of cell types (Lee et al. 1998; Brooks et al. 1999).

The inhibitory CD94/NKG2-A receptor has a higher binding affinity for HLA-E than the activating CD94/NKG2-C receptor and, that the recognition of HLA-E by both CD94/NKG2-A and CD94/NKG2-C is peptide dependent. There appeared to be a strong, direct correlation between the binding affinity of the peptide-HLA-E complexes for the CD94/NKG2 receptors and the triggering of a response by the NK cell (Braud et al. 1998; Vales-Gomez et al. 1999; Wada et al. 2004).

HLA-E-Bound Peptides Influence Recognition by CD94/NKG2 Receptors: Reports support the notion that the primary structure of HLA-E-bound peptides influences CD94/NKG2-mediated recognition, beyond their ability to stabilize surface HLA-E. Further, CD94/NKG2A$^+$ NK clones appeared more sensitive to the interaction with most HLA-E-peptide complexes than did effector cells expressing the activating CD94/NKG2C receptor. However, a significant exception to this pattern was HLA-E loaded with the HLA-G-derived nonamer. This complex triggered cytotoxicity very efficiently over a wide range of peptide concentrations, suggesting that the HLA-E/G-nonamer complex interacts with the CD94/NKG2 triggering receptor with a significantly higher affinity. Study raises the possibility that CD94/NKG2-mediated recognition of HLA-E expressed on extravillous cytotrophoblasts plays an important role in maternal-fetal cellular interactions (Llano et al. 1998). HLA-E interacts with CD94/NKG2A receptors on NK cells and this inhibits NK cell lysis of the cell displaying HLA-E (LaBonte et al. 2001; Boyington et al. 2000).

The uterine mucosa in early pregnancy (decidua) is infiltrated by large numbers of NK cells, which are closely associated with placental trophoblast cells. Trophoblast cells express HLA-E on their cell surface in addition to the expression of HLA-G and HLA-C. The vast majority of decidual NK cells bind to HLA-E tetrameric complexes and this binding is inhibited by mAb to CD94. This shows that recognition of fetal HLA-E by decidual NK cells may play a key role in regulation of placentation. The functional consequences of decidual NK cell interaction between CD94/NKG2 and HLA-E is the inhibition of cytotoxicity by decidual NK cells. It was suggested that HLA-E interaction with CD94/NKG2 receptors may regulate other functions besides cytolysis during implantation (King et al. 2000).

A Peptide from Leader Sequences of HSP 60 Binds Also to HLA-E: HLA-E also presents a peptide derived from the leader sequence of human heat shock protein 60 (HSP60). This peptide gains access to HLA-E intracellularly, resulting in up-regulated HLA-E/HSP60 signal peptide cell-surface levels on stressed cells. Notably, HLA-E molecules in complex with the HSP60 signal peptide are no longer recognized by CD94/NKG2A inhibitory receptors. Thus, during cellular stress an increased proportion of HLA-E molecules may bind the nonprotective HSP60 signal peptide, leading to a reduced capacity to inhibit a major NK cell population. Such stress induced peptide interference would gradually uncouple CD94/NKG2A inhibitory recognition and provide a mechanism for NK cells to detect stressed cells in a peptide-dependent manner (Crew et al. 2005; Michaelsson et al. 2002; Wooden et al. 2005).

30.11.2 CD94/NKG2-A Recognises HLA-G1

There is no evidence that p58 and p70 KIRs may interact with HLA-G1. By contrast, NK recognition of cells expressing HLA-G1 involves at least two non-overlapping receptor-ligand systems: (1) the direct engagement of the ILT2 (LIR1) receptor by HLA-G1; and (2) the interaction of CD94/NKG2A and CD94/NKG2C receptors with the non-classical class I molecule HLA-E, co-expressed on the surface upon binding to a nonamer (VMAPRTLFL) from the HLA-G leader sequence (López-Botet et al. 1999 ; Navarro et al. 1999). Perez-Villar et al. (1997) supported the idea that the CD94/NKG2 receptor is involved in the recognition of cells expressing HLA-G1.

CD94/NKG2 Is Predominant Receptor in Recognition of HLA-G at Maternal-Fetal Interface: NK cells isolated from adult peripheral blood kill the HLA-A-, HLA-B-, and HLA-C-deficient B lymphoblastoid cell line 730.221, but many are unable to kill 730.221 cells transfected with HLA-G, a molecule expressed preferentially on fetal cytotrophoblasts. The nonclassical human HLA-G is selectively expressed on fetal trophoblast tissue at the maternal-fetal interface in pregnancy. It seems that HLA-G may inhibit maternal NK cells through interaction with particular NK cell receptors. Recognition of HLA-G by NK cells was prevented in the presence of anti-CD94 mAb, implicating CD94/NKG2 as the predominant inhibitory NK cell receptor for HLA-G used by dNK cells (Soderstrom et al. 1997). Allan et al. (1999) suggested that the primary role of HLA-G may be the modulation of myelomonocytic cell behavior in pregnancy.

HLA-E and HLA-G Expression on Porcine Endothelial Cells Inhibit Xenoreactive Human NK Cells: Studies showed that peptides derived from the leader sequence of HLA-G binds and up-regulates the surface expression of HLA-E molecules, which was considered to consequently

provide negative signals to human NK cells. HLA-G protects porcine cells from human NK cells through a CD94/NKG2-independent pathway. These results demonstrated that both HLA-E and HLA-G could directly inhibit human NK cells in the absence of other endogenous HLA class I molecules (Sasaki et al. 1999). These results have implications in preventing xenograft rejection mediated by human NK cells.

30.11.3 Non-Classical MHC-I Molecule Qa-1b as Ligand

Vance et al. (1998) provided evidence that mouse NK cell CD94/NKG2A heterodimer recognizes Qa-1b. The NK recognition of Qa-1b results in the inhibition of target cell lysis. Inhibition appears to depend on the presence of Qdm (Qa-1 determinant modifier), a Qa-1b-binding peptide derived from the signal sequences of some classical class I molecules. Qa-1 predominantly assembles with a single Qdm. The Qa-1/Qdm complex is the primary ligand for CD94/NKG2A inhibitory receptors expressed on a major fraction of NK cells. Cells become susceptible to killing by NK cells under conditions where surface expression of the Qa-1/Qdm inhibitory ligand is reduced (Borrego et al. 1998).

Qdm (Qa-1 Determinant Modifier): Qdm peptide, derived from the signal/leader sequence of many MHC class Ia protein molecules, has the sequence (AMAPRTLLL). This peptide binds with- and accounts for almost all of the peptides associated with this molecule. The Qa-1b was found to bind related ligands representing peptides derived from the leaders of class I molecules from several mammalian species, indicating a conservation of this "Qdm-like" epitope throughout mammalian evolution (Kurepa et al. 1998). Human HLA-E, the homologue of Qa-1b, binds similar peptides derived from human class Ia molecules and interacts with CD94/NKG2 receptors on NK cells. All of the peptides, which bind HLA-E bound readily to Qa-1b. However, the exact circumstances under which Qa1 protects cells from NK lysis and, in particular the role of Qdm, were addressed by Gays et al. (2001). Results obtained with a series of substituted Qdm peptides suggest that residues at positions 3, 4, 5, and 8 of the Qdm sequence, AMAPRTLLL, are important for recognition of Qa1-Qdm complexes by inhibitory CD94/NKG2 receptors (Jensen et al. 2004). The CD94/NKG2A receptor, expressed by mouse NK cells, transduces inhibitory signals upon recognition of its ligand, Qa-1b. The CD94/NKG2C and CD94/NKG2E also bind to Qa-1b. Within their extracellular CRDs, NKG2C and NKG2E share extensive homology with NKG2A (93–95% amino acid similarity) and may bind Qa-1b.

The Qa-1b/Qdm Tetramer Binds to CD94/NKG2 Complex Expressed on CD94^{high} Murine NK Cells: Although very few CD8$^+$ T cells from naive mice express CD94/NKG2 receptors, approximately 50% of CD8$^+$ T cells taken from mice undergoing a secondary response against *Listeria monocytogenes* (LM) are CD94^{high} and bind the Qa-1b/Qdm tetramer. Also, CD94^{int} NK cells do not bind the tetramer, where as CD94^{int} CD8$^+$ T cells do, and this binding is dependent on the CD8 co-receptor (Gunturi et al. 2003).

A Peptide from HSP60 Binds to Qa-1 in Absence of Qdm: In the light of stress induced peptide interference by HSP-60 peptide to uncouple CD94/NKG2A inhibitory recognition and to provide a mechanism for NK cells to detect stressed cells in a peptide-dependent manner (Michaelsson et al. 2002), Davies et al. (2003) reported the isolation and sequencing of a HSP-60-derived peptide (GMKFDRGYI) from Qa-1. This peptide is the dominant peptide bound to Qa-1 in the absence of Qdm. A Qa-1-restricted CTL clone recognizes this HSP60 peptide, further verifying that it binds to Qa-1 and a peptide from the homologous *Salmonella typhimurium* protein GroEL (GMQFDRGYL). These observations have implications for how Qa-1 can influence NK cell and T cell effector function via the TCR and CD94/NKG2 family members, and how this effect can change under conditions that cause the peptides bound to Qa-1 to change (Davies et al. 2003).

30.12 Structure Analysis of CD94-NKG2 Complex

30.12.1 Crystal Analysis of NKG2A/CD94: HLA-E Complex

HLA-E binds peptides derived from the leader sequences of other HLA class I molecules. NK cell recognition of HLA-E molecules, via the CD94-NKG2 NK family, represents a central innate mechanism for monitoring MHC expression levels within a cell. Kaiser et al. (2008) determined the crystal structure of the NKG2A/CD94/HLA-E complex at 4.4-Å resolution, revealing two critical aspects of this interaction. First, the C-terminal region of the peptide, which displays the most variability among class I leader sequences, interacts entirely with CD94, the invariant component of these receptors. Second, residues 167–170 of NKG2A/C account for the approximately sixfold-higher affinity of the inhibitory NKG2A/CD94 receptor compared to its activating NKG2C/CD94 counterpart. These residues do not contact HLA-E or peptide directly but instead form part of the heterodimer interface with CD94. An evolutionary analysis across primates reveals that whereas CD94 is

evolving under purifying selection, both NKG2A and NKG2C are evolving under positive selection. It seemed that the evolution of the NKG2x/CD94 family of receptors has been shaped both by the need to bind the invariant HLA-E ligand and the need to avoid subversion by pathogen-derived decoys (Kaiser et al. 2008).

Changes in Peptide Conformation Affect Recognition of HLA-E by CD94-NKG2: The leader sequence-derived peptides bound to HLA-E exhibit very limited polymorphism, yet subtle differences affect the recognition of HLA-E by the CD94-NKG2 receptors. In order to prove this point, the structure of HLA-E was determined in complex with two leader peptides, namely, HLA-Cw*07 (VMAPRALLL), which is poorly recognised by CD94-NKG2 receptors, and HLA-G*01 (VMAPRTLFL), a high-affinity ligand of CD94-NKG2 receptors. A comparison of these structures revealed that allotypic variations in the bound leader sequences do not result in conformational changes in HLA-E heavy chain, although subtle changes in the conformation of the peptide within the binding groove of HLA-E were evident. Accordingly, results indicate that the CD94-NKG2 receptors interact with HLA-E in a manner that maximises the ability of the receptors to discriminate between subtle changes in both the sequence and conformation of peptides bound to HLA-E (Hoare et al. 2008).

30.12.2 CD94-NKG2A Binding to HLA-E

Petrie et al. (2008) described the crystal structure of CD94-NKG2A in complex with HLA-E bound to a peptide derived from the leader sequence of HLA-G. The CD94 subunit dominated the interaction with HLA-E, whereas the NKG2A subunit was more peripheral to the interface. Moreover, the invariant CD94 subunit dominated the peptide-mediated contacts, albeit with poor surface and chemical complementarity. There were few conformational changes in either CD94-NKG2A or HLA-E upon ligation, and such a "lock and key" interaction is typical of many receptor-ligand interactions. The structure provided insight into how this interaction can be modulated by subtle changes in the peptide ligand or by the pairing of CD94 with other members of NKG2 family (Petrie et al. 2008) (Figs. 30.4 and 30.5).

Results showed that the CD94-NKG2A docked toward the C-terminal end of the HLA-E antigen-binding cleft, binding at an angle of ~70° (Fig. 30.4a, b) in a manner that permitted CD94-NKG2A to sit across both the α1 and α2 helices of HLA-E (Fig. 30.4). A comparison of the CD94-NKG2A–HLA-$E^{VMAPRTLFL}$ complex with the nonligated CD94-NKG2A and HLA-$E^{VMAPRTLFL}$ structures revealed no significant conformational change in either HLA-E or CD94-NKG2A upon complex formation. The one disordered loop of the nonligated CD94-NKG2A heterodimer (residues 199–204 in NKG2A) became ordered in the complex, although this observation was attributable to crystal-packing effects, as this loop did not contact HLA-$E^{VMAPRTLFL}$. Only one residue, Gln 112 of CD94, was reorientated upon ligation to maximize the complementarity at the interface. Accordingly, this "lock and key" engagement between HLA-$E^{VMAPRTLFL}$ and CD94-NKG2A exemplified the "innate characteristic" of this interaction.

Analysis of the electrostatic surfaces of HLA-E and CD94-NKG2A highlighted a role for charge complementarity at the CD94-NKG2A–HLA-E interface. Namely, a basic region on the α1 helix of HLA-E interacted with an acidic region on CD94 and, conversely, an acidic region on the HLA-E α2 helix docked with a distinct patch of basic charge on NKG2A. The CD94 footprint on HLA-E was broad, with residues within loops 2, 3, and 5, and β strands 6 and 7 from CD94 interacting with a region spanning residues 65–89 from α1 helix of HLA-E (Petrie et al. 2008). When compared with that of CD94, the footprint of NKG2A on HLA-E was markedly smaller and more focused with loop 3 and β strands 2, 5, and 6 interacting with residues 151–162 of the α2 helix of HLA-E. Nevertheless, analogous to the CD94–HLA-E interactions, the NKG2A–HLA-E contacts were dominated by polar residues. Accordingly, the large and predominantly polar network of interactions between CD94-NKG2A and HLA-E underscored the specificity of this interaction and highlighted the dominant role of the CD94 subunit with respect to the NKG2A subunit (Petrie et al. 2008).

The location of the CD94-NKG2A footprint on HLA-$E^{VMAPRTLFL}$ was analogous to the positioning of the footprint of human NKG2D on its MHC-like ligands, MICA and, to a lesser extent, ULBP3 (Fig. 30.5). Nevertheless, there were notable differences between these footprints, which are attributable to the narrower cleft between the α helices of MICA and ULBP3 compared with HLA-E, and the structural differences between CD94-NKG2A and NKG2D. CD94-NKG2A possessed a flat interacting surface with HLA-E, whereas that of NKG2D was more "saddle-like," which enabled it to clamp around both α helices of the MHC-like ligands (Fig. 30.5) (Petrie et al. 2008). Accordingly, the innate NK receptors and the αβTCR focus on a similar region of the HLA-E or MHC-like ligand, regardless of whether the ligand presents peptide or not. However, the characteristics of the footprint deviate between these receptors, thereby providing a basis for the NKG2D promiscuity versus the CD94-NKG2A specificity.

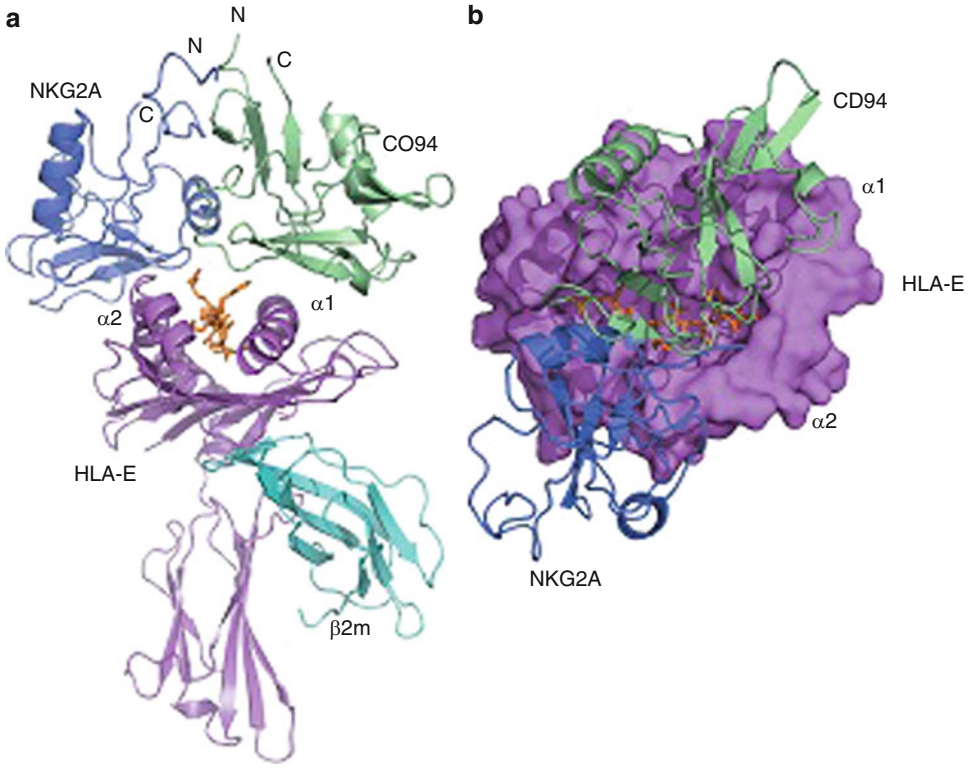

Fig. 30.4 The CD94-NKG2A – HLA-E VMAPRTLFL complex: NKG2A and CD94 are represented as *blue* and *pale green* ribbon structures, respectively. The heavy chain of HLA-E and β 2 m are shown as *violet* and *cyan ribbons*, respectively, with the VMAPRTLFL peptide in *orange sticks*. (**a**) Side view of CD94-NKG2A docking onto HLA-E VMAPRTLFL. (**b**) *Top view* of CD94-NKG2A docking onto the surface of HLA-E VMAPRTLFL (Adapted with permission from Petrie et al. 2008 © Rockefeller University Press)

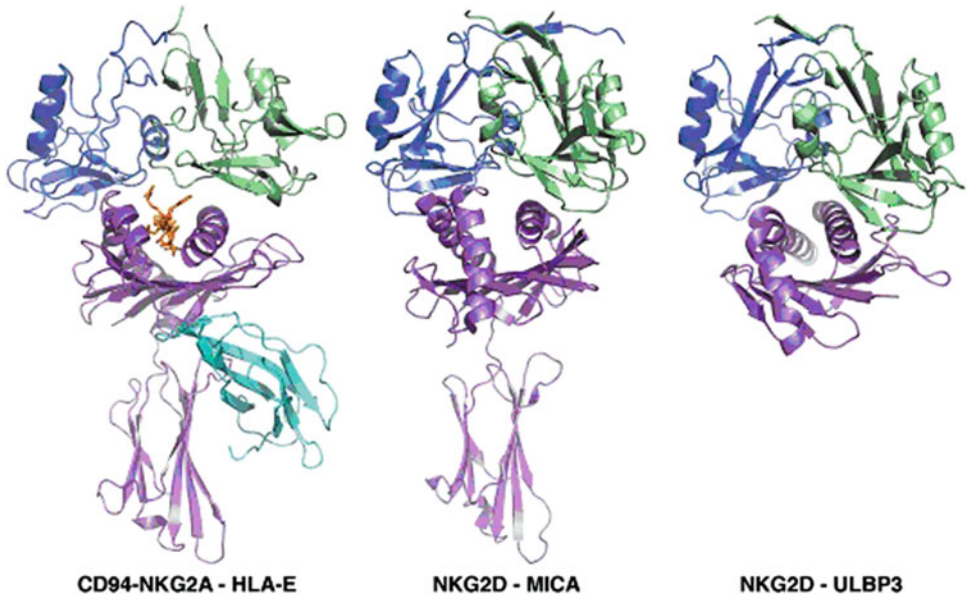

Fig. 30.5 Comparison of polar versus nonpolar interactions by CD94-NKG2A and NKG2D homodimer: Nonpolar interactions are represented in *purple*, and polar interactions are in *blue*. In situations where a residue makes both nonpolar interactions as well either a salt bridge or hydrogen bond, it was represented as making a polar interaction. Ribbon representation of CD94-NKG2A in complex with HLA-E ^{VMAPRTLFL} and NKG2D homodimers binding to MICA and ULBP3. The subunit of the NKG2D homodimer equivalent to CD94 is represented in *pale green*, and the subunit equivalent to NKG2A is in *blue*. The MHC-like molecules, MICA and ULBP3, not associated with β2m (represented in *cyan*), are represented in *violet* (Adapted with permission from Petrie et al. 2008 © The Rockefeller University Press)

30.13 Inhibitory Receptors in Viral Infection

Virus Associated Innate Immunity in Liver: The expression of inhibitory receptor CD94/NKG2A is up-regulated on NK cells in patients with chronic hepatitis C. HLA-E, a ligand for NKG2A, was expressed in all human hepatoma cell lines as well as in nontransformed hepatocytes, but not in K562 cells, a classic NK-sensitive target. Aberrant expression of CD94/NKG2A should have negative impact on innate resistance and subsequent adaptive immunity toward HCV-infected or transformed cells in chronic hepatitis C (Takehara and Hayashi 2005).

Kanto (2008) sought to clarify the role of innate immune system in the pathogenesis of HCV infection. DCs are known to sense virus infection via toll-like receptors (TLR) or retinoic acid inducible gene-I (RIG-I), resulting in the secretion of type-I IFN and inflammatory cytokines. Blood DCs consist of two subsets; myeloid DC (MDC) and plasmacytoid DC (PDC). In MDC from HCV-infected patients, the levels of TLR/RIG-I-mediated IFN-β or TNF-α induction are lower than those in uninfected donors suggesting that their signal transduction in MDC is impaired. In response to IFN-α, DC are able to express MHC class-I related chain A/B (MICA/B) and activate NK cells following ligation of NKG2D.

Interestingly, DC from HCV-infected patients are unresponsive to exogenous IFN-α to enhance MICA/B expression and fail to activate NK cells. Alternatively, NK cells from HCV-infected patients down-regulate DC functions in the presence of HLA-E-expressing hepatocytes by secreting IL-10 and TGF-β1. Such functional alteration of NK cells in HCV infection is ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts. Invariant NKT cells activated by CD1d-positive DC secrete both Th1 and Th2 cytokines, serving as immune regulators. The frequency of NKT cells in chronic HCV infection does not differ from those in healthy donors. Activated NKT cells produce higher levels of IL-13 but comparable levels of IFN-γ with those from healthy subjects, showing that NKT cells are biased to Th2-type in chronic HCV infection. It is evident that the cross-talks among DC, NK cells and NKT cells are critical in shaping subsequent adaptive immune response against HCV (Kanto and Hayashi 2007; Kanto 2008).

Qa-1b-Dependent Modulation of DC and NK Cell Cross-Talk In Vivo: DC trigger activation and IFN-γ release by NK cells in lymphoid tissues, a process important for the polarization of Th1 responses. Colmenero et al. (2007) showed that the interaction between Qa-1b expressed on DC and its CD94/NKG2A receptor on NK cells is important in the regulation of DC-induced NK cell IFN-γ synthesis. NK cells from CD94/NKG2A-deficient mice displaying higher IFN- γ production upon DC stimulation along with other experiments demonstrated that Qa-1b is critically involved in regulating IFN- γ synthesis by NK cells in vivo through its interaction with CD94/NKG2A inhibitory receptors. This receptor-ligand interaction may be essential to prevent unabated cytokine production by NK cells during an inflammatory response (Colmenero et al. 2007).

CD94/NKG2 Receptors During HIV-1 Infection: NK HIV has evolved several strategies to evade recognition by the host immune system including down-regulation of MHC class I molecules. However, reduced expression of MHC class I molecules may stimulate NK cell lysis in cells of haematopoietic lineage. Dysfunction of cytotoxic activity of T and NK lymphocytes is main feature in patients with AIDS. The augmented expression of CD94 observed in HIV-infected individuals could be related to high levels of IL-10 in HIV-1-infected individuals (Galiani et al. 1999). The functional capacities and coexpression patterns of NK receptors (CD94 and CD161) are differentially affected depending on the state of therapy [HAART or HAART naïve] or the cell type (NK or T cells), respectively (Jacobs et al. 2004). However, Mela et al. (2005) suggested that changes in the NK cell repertoire during HIV-1 infection were not the result of HIV-1 viraemia alone but resembled those associated with concomitant infections (Mela et al. 2005).

Increased NK Cell Activity in Viremic HIV-1 Infection: Abnormal upregulation of CD94/NKG2A inhibitory NKR on CTLs could be responsible for a failure of immunosurveillance in cancer or HIV infection. Despite reduced NK cell numbers in subjects with ongoing viral replication, NK cells were significantly more active in secreting both IFN-γ and TNF-α than NK cells from aviremic subjects or HIV-1-negative controls and expressed significantly higher levels of CD107a, while the numbers of $CD3^-/CD56^+/CD94^+$ and $CD3^-/CD56^+/CD161^+$ NK cells were reduced. Therefore, viremic HIV-1 infection is associated with a reduction in NK cell numbers and a perturbation of NK cell subsets, but increased overall NK cell activity (Alter et al. 2004). However, Zeddou et al. (2007) showed that the chronic stimulation with HIV antigens in viraemic patients leads to a decreased rather than increased CD94/NKG2A expression on $CD8^+$ T lymphocytes and NK cells. Ballan et al. (2007) observed an increased frequency of NK cells expressing inhibitory KIRs in infected children. Moreover, increased expression of KIR2DL3, NKG2C, and NKp46 on NK cells correlated with decreased $CD4^+$ T-lymphocyte percentage, an indicator of disease severity in HIV-1- infected children (Ballan et al. 2007; Goodier et al. 2007)

NKG2C is a Triggering Receptor in Vδ1 T Cell-Mediated Cytotoxicity Against HIV-Infected CD4 T Cells: γδT cells share with NK cells many cell-surface proteins, including the NKG2 receptor. A subset of γδT cells that express the variable Vδ1 region plays a critical role in immune regulation, tumor surveillance and viral infection. Dramatic expansion of Vδ1 T cells has been observed in HIV infection. Results raise the possibility that induction of NKG2C expression on Vδ1 T cells plays a key role in the destruction of HIV-infected CD4 T cells during HIV disease (Fausther-Bovendo et al. 2008). Nattermann et al. (2005) demonstrated that HIV-mediated up-regulation of HLA-E is an additional evasion strategy targeting the antiviral activities of NK cells, which may help the virus in establishing chronic infection (Nattermann et al. 2005; Martini et al. 2005).

SIV-Infected Rhesus Monkeys

The NK cells from simian immunodeficiency virus (SIV)-infected rhesus monkeys are significantly impaired in their ability to secrete IFN-γ, TNF-α, and IL-2, while NK cell function in SIV-infected long-term non-progressor monkeys is similar to that of normal monkeys. The activating molecules NKG2C and NKG2C2 are significantly down-regulated in peripheral blood mononuclear cells of SIV-infected rhesus monkeys, suggesting that the dysregulation of these molecules may contribute to the abnormal NK cell function observed in the setting of infection (LaBonte et al. 2006).

Regulation of Cytolytic Activity of HSV-Specific Memory CD8$^+$ T Cells: Evidences suggest that sensory neurons regulate the effector functions and phenotype of CD8$^+$ T cells during active immunosurveillance of herpes simplex virus (HSV) latency. After ocular infection, HSV-specific CD8$^+$ T cells migrate to and retained in the ophthalmic branch of the trigeminal ganglia (TG). Virus-specific CD8 + T cells maintain an activation phenotype and secrete IFN-γ in the latent TG. The activated virus-specific memory CD8$^+$ T cells, although potentially cytolytic, also express the CD94-NK cell receptor subfamily G2a inhibitory molecule and are unable to exert cytotoxicity when engaged by Qa-1b expressing targets. Suvas et al. (2006) indicated that the Qa-1b and CD94-NKG2a interaction regulate cytolytic activity of HSV-specific memory CD8$^+$ T cells in the latently infected TG and serves to protect irreplaceable neurons from destruction by the immune system.

CD94/NKG2A Expression During Polyoma Virus Infection: Memory CD8$^+$ T cells form critical component of durable immunity because of their capacity to rapidly proliferate and exert effector activity upon Ag rechallenge. In mice infected with polyoma virus, the majority of persistence-phase antiviral CD8$^+$ T cells expressed the inhibitory NK cell receptor CD94/NKG2A. The CD94/NKG2A expression was associated with Ag-specific recall of polyoma virus-specific CD8$^+$T cells. Polyoma virus-specific CD8$^+$ T cells that expressed CD94/NKG2A were found to preferentially proliferate; this proliferation was dependent on cognate Ag both in vitro and in vivo. In addition, CD94/NKG2A$^+$ polyoma-specific CD8$^+$ T cells had an enhanced capacity to produce IL-2 upon ex vivo Ag stimulation compared with CD94/NKG2A$^-$ polyoma-specific CD8$^+$ cells (Byers et al. 2006).

NK Cell Receptors in Response to HCMV/MCMV Infection: Human cytomegalovirus (HCMV) infection is a pattern of complexity shown by host-pathogen interactions. To avoid recognition by CTLs, HCMV inhibits the expression of HLA class I molecules. As a consequence, engagement of inhibitory NKRs specific for HLA class I molecules is impaired, and infected cells become vulnerable to an NK cell response driven by activating receptors. In addition to the well-defined role of the NKG2D, the involvement of other triggering receptors (i.e., activating KIR, CD94/NKG2C, NKp46, NKp44, and NKp30) in response to HCMV is under investigation. (Gumá et al. 2006a).

During HCMV infection, the CD94/NKG2C$^+$ cells express lower levels of NKp30 and NKp46 and higher proportions of KIR$^+$ and CD85j$^+$ cells than CD94/NKG2A$^+$ cells. The CD8$^+$ T cell compartment of human CMV-seropositive individuals characteristically contains a high proportion of cells that express NKRs which may contribute to the surveillance of virus-infected cells. Like other NKR, CD94/NKG2C is predominantly expressed by a CD8$^+$ T cell subset, though TCRγδ$^+$ NKG2C$^+$ and rare CD4$^+$ NKG2C$^+$ cells are also detected in some individuals. Studies support the notion that CD94/NKG2C may constitute an alternative T cell activation pathway capable of driving the expansion and triggering the effector functions of a CTL subset in response to HCMV (Guma et al. 2004, 2005).

The increase of CD94/NKG2C$^+$ NK cells in healthy individuals infected with human CMV suggested that HCMV infection may shape the NK cell receptor repertoire (Gumá et al. 2006c). The CD94/NKG2C$^+$ cells outnumbered the CD94/NKG2A$^+$ subset. Results support that the interaction of CD94/NKG2C with HCMV-infected fibroblasts, concomitant to the inhibition of HLA class I expression, promotes an outgrowth of CD94/NKG2C$^+$ NK cells (Gumá et al. 2006b). In addition to NKG2C, to escape from NK cell-mediated surveillance, HCMV interferes with the expression of NKG2D ligands in infected cells. Saez-Borderias et al. (2006) suggested that NKG2D functions as a prototypic costimulatory receptor in a subset of HCMV-specific CD4$^+$ T lymphocytes and thus might have a role in the response against infected HLA class II$^+$ cells displaying NKG2D

ligands. In the acute phase of infection, a strong induction of CD94 on $CD3^+$ T cells was observed with surface expression of activating $CD94^{dim}$ NKG2C dimers appearing before inhibitory $CD94^{bright}$ NKG2A ones (van Stijn et al. 2008).

NK Cell Recognition of MCMV-Infected Cells: Studies on mouse model have shown that NK cells deploy multiple mechanisms to deal with mouse cytomegalovirus (MCMV) infection, which involve receptors of the C-lectin type superfamily. While reviewing these attack-counterattack measures, Vidal and Lanier (2006) pointed to the central role of NK cells in host resistance to infection. Following acute infection, MCMV replicates persistently in the salivary glands, despite the vigorous response of activated CD8 T cells that infiltrate this gland. Virus-specific CD8 T lymphocytes from this organ were found to express the CD94/NKG2A receptor that confers an inhibitory response to CTLs. In response to MCMV infection, expression of the CD94/NKG2A ligand, Qa-1b, increased dramatically in the sub-mandibular gland (SMG) prior to up-regulation of $H\text{-}2D^d$. However, there was no net negative impact on virus-specific T-cell function. Thus, the expression of inhibitory CD94/NKG2A receptors does not account for the failure of MCMV-specific CTLs to clear the SMG of infection (Cavanaugh et al. 2007).

Epstein-Barr Virus Differs with HCMV: Following activation of Epstein-Barr virus (EBV)-infected B cells from latent to productive (lytic) infection, there is a concomitant reduction in the level of MHC class I molecules and an impaired antigen-presenting function that may facilitate evasion from EBV-specific $CD8^+$ cytotoxic T cells. In some other herpesviruses studied, such as HCMV, evasion of virus-specific $CD8^+$ effector responses via down-regulation of surface MHC class I molecules is supplemented with specific mechanisms for evading NK cells. Pappworth et al. (2007) reported that EBV differs from HCMV in matters such as: resistance to lysis by NK cell lines, down-regulation of HLA-A, -B, and -C molecules that bind to the KIR family of inhibitory receptors and also the down-regulation of HLA-E molecules that bind CD94/NKG2A inhibitory receptors. Conversely the ligands for NK cell-activating receptors NKG2D and DNAM-1, respectively, were elevated. These results highlight a fundamental difference between EBV and HCMV with regards to evasion of innate immunity.

Induction of CD94 and NKG2 in $CD4^+$ T Cells by Influenza A Virus (IAV) Infection: Graham et al. (2007) investigated CD94 and NKG2 gene expression in memory CD4 T-cell clones established from the spleens of C57BL/10 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice infected with IAV. The in-vivo-generated CD4 Th1 cells, but not Th2 cells, expressed full-length CD94 and NKG2A following activation with viral peptide. A truncated isoform of NKG2A was detectable in a Th2 cell clone. NKG2D, but not NKG2C or E, was also differentially expressed in Th1 cells. Graham et al. (2007) suggested that CD94 and NKG2A may exist in multiple isoforms and help to distinguish helper T-cell subsets.

30.14 Pathophysiological Role of CD94/NKG2 Complex

30.14.1 Polymorphism in NKG2 Genes

CD94 is highly conserved, while the NKG2 genes exhibit some polymorphism. For all the genes, alternative mRNA splicing variants were frequent among the human clones. Similar alternative splicing occurs in human and chimpanzee to produce the CD94B variant from the CD94 gene and the NKG2B variant from the NKG2A gene. Whereas single chimpanzee orthologs for CD94, NKG2A, NKG2E, and NKG2F were identified, two chimpanzee paralogs of the human NKG2C gene were defined. The chimpanzee Pt-NKG2CI gene encodes a protein similar to human NKG2C, whereas in the chimpanzee Pt-NKG2CII gene the translation frame changes near the beginning of the carbohydrate recognition domain, causing premature termination. Genomic DNA from 80 individuals representing six primate species was typed for the presence of CD94 and NKG2. Each species gave distinctive typing patterns, with NKG2A and CD94 being most conserved. Seven different NK complex genotypes within the panel of 48 common chimpanzees were due to differences in Pt-NKG2C and Pt-NKG2D genes (Shum et al. 2002).

30.14.2 Phenotypes Associated with Leukemia

The polymorphic nature of NK cell receptor genes generates diverse repertoires in the human population, and displays specificity in the innate immune response. This was substantiated by Verheyden et al. (2004) who suggested that an important percentage of leukemic patients express a KIR phenotype in favor of escape from NK cell immunity. Studies have revealed the existence of a distinct type of NK cell leukaemia of the juvenile type, which shows with hypersensitivity to mosquito bites (HMB) as a clinical manifestation and is infected with clonal Epstein-Barr virus (EBV). This disorder is thus called HMB-EBV-NK disease and has been reported mostly from Japan. More than 98% of NK cells from the patients with HMB-EBV-NK disease exhibited CD94 at a higher level than did normal NK cells, whereas p70 or NKAT2, belonging to Ig-like receptor, was not expressed in those NK cells. Leukaemic NK cells transcribed mRNA for CD94-associated molecule NKG2C at an abnormally high level, and upon stimulation with IL-2 and/or IL-12 they

expressed NKG2A as well. The expression of these receptors provides insights into phenotypic markers for the diagnosis of this type of NK cell leukaemia (Seo et al. 2000).

B-Cell Chronic Lymphocytic Leukaemia: Reduced reactivity of cytotoxic T cells (CTL) towards tumor cells can result into tumor progression and loss of tumor control. In B-cell chronic lymphocytic leukaemia (B-CLL), the reactivity of tumor-reactive CTL seems to relate inversely to disease stage. Analysis of $CD8^+$ T from patients with advanced disease (Binet stage C) had a significantly greater percentage of CTL expressing CD158b, CD158e, and CD94 than patients with non-progressive disease (Binet stage A) and healthy controls. The increased expression of KIR and CD94 on CTL in advanced stage B-CLL may significantly contribute to the impaired anti-tumor immune response in these patients (Junevik et al. 2007).

Heat Shock Protein 70: A Link Between NK Cells and Tissue Response: Stress-inducible heat shock protein 70-kDa (Hsp70) provides a molecular link between inflammatory responses and tissue repair. In addition to molecular chaperoning, Hsp70 exerts modulatory effects on endothelial cells and leukocytes involved in inflammatory networks. The 14 amino acid sequence (aa-450-463) TKDNNLLGRFELSG (TKD) of Hsp70 has been identified as a tumor-selective recognition structure for NK cells. The receptor CD94 participates in the interaction of NK cells with Hsp70-protein and Hsp70-peptide TKD. Hsp70 reactivity could be associated with elevated cell surface densities of CD94 after TKD stimulation independent of MHC class I molecules (Gross et al. 2003). Incubation of peripheral blood lymphocytes with TKD plus low-dose IL-2 enhances the cytolytic activity of NK cells against Hsp70 membrane-positive tumors, in vitro and in vivo. From clinical Phase I trial, reinfusion of Hsp70-activated autologous NK cells is safe and warrant additional studies in patients with lower tumor burden (Krause et al. 2004). Further studies of Gross et al. (2008) revealed that Hsp70 peptide initiates NK cell killing of leukemic blasts after stem cell transplantation and that Hsp70 is the target structure for TKD-activated NK cells. Studies indicated that Hsp70 (as an activatory molecule) and HLA-E (as an inhibitory ligand) expression influence the susceptibility of leukemic cells to the cytolytic activities of cytokine/TKD-activated NK cells (Stangl et al. 2008).

30.14.3 CD94/NKG2A on NK Cells in T Cell Lymphomas

The NK and cytotoxic T-cell lymphomas and noncytotoxic nodal T-cell lymphoma controls are stained for variety of NKR molecules including CD94, NKG2A, and p70. The NK-cell lymphomas expressed at least the CD94/NKG2A complex. Detection of CD94/NKG2A may be a useful tool to the diagnosis of NK-cell lymphomas and to delineate the subgroup of cytotoxic T-cell lymphomas (Haedicke et al. 2000; Kamarashev et al. 2001). However, a restricted KIR repertoire without monoclonal T-cell receptor rearrangement (mTCR-R) supports a NK lineage in nasal-type extranodal NK/T-cell lymphoma (NTENL) but does not correlate with clinical findings. Developing NK cells express first CD94, then NKG2A, NKG2E, and finally NKG2C. This sequence suggests an immature $CD94^-$ and a mature $CD94^+$ subtype of NTENL (Lin et al. 2003).

30.14.4 CD94/NKG2 Subtypes on Lymphocytes in Melanoma Lesions

Vetter et al. (2000) characterized the expression of CD94/NKG2 on tumor-infiltrating lymphocytes in melanoma lesions. 5–10% of the tumor-infiltrating lymphocytes, both in primary and metastatic melanoma lesions, expressed CD94. More than 95% of these $CD94^+$ cells coexpressed CD8 and the percentage of CD94 expression within the $CD8^+$ cell population ranged from 5% to 20% with a higher expression in metastatic lesions. RT-PCR revealed the presence of NKG2-C/E in all primary and metastatic lesions. In contrast, the inhibitory NKG2-A/B was only present in 50% of primary tumors, whereas 80% of tumor-infiltrating lymphocytes in metastatic lesions expressed these transcripts. In healthy humans, the number of inhibitory NK cell receptors is higher than that of activating receptors, but the opposite was true for tumor-infiltrating lymphocytes in melanoma (Casado et al. 2005; Naumova et al. 2005).

Becker et al. (2000) demonstrated that the expression of CD94/NKG2 on T cells depends on the state of differentiation during the immune response to solid tumors. To this end Becker et al. identified clonally expanded T cells which were present both in the sentinel lymph node of primary melanoma, as well as in the tumor itself. Within the early stages of T cell activation, i.e. priming in the lymph node, T cells did not express CD94/NKG2 whereas the same T cell clones expressed high levels of CD94/NKG2 having reached the effector state at the tumor site. It is likely that NK cell receptors are involved in peripheral regulatory mechanisms avoiding overwhelming immune responses and immunopathology, particularly in situations of long-lasting immune activation. However, DBA/2J mice are naturally CD94-deficient and do not express cell-surface CD94/NKG2A receptors, even on neonatal NK cells. Thus, self-tolerance of neonatal NK cells cannot be attributed to CD94/NKG2A expression. The results of Vance et al. (2002) lead to the reconsideration of current models of NK cell development and self-tolerance.

30.14.5 Cancers of Female Reproductive Tract

Very often, up-regulation of inhibitory NKRs has been linked to the modulation of virus- and/or tumor-specific immune responses in animal models. However, in human cervical cancer, the percentage expression of Ig-like NKR^+CD8^+ T lymphocytes was similar in gated $CD8^+$-autologous tumor-infiltrating lymphocytes (TILs) and peripheral blood mononuclear cells (Sheu et al. 2005). On the contrary, cervical cancer-infiltrating $CD8^+$ T lymphocytes expressed up-regulated CD94/NKG2A compared with peripheral blood $CD8^+$ T cells and/or normal cervix-infiltrating $CD8^+$ T lymphocytes, where as CD94 and NKG2A mainly expressed on $CD56^-$ $CD161^-$ $CD8^+$ TILs within the cancer milieu. Cytotoxicity experiments showed that up-regulated expressions of CD94/NKG2A restrain $CD8^+$ T lymphocyte cytotoxicity. This study indicated that cervical cancer cells could promote the expression of inhibitory NKRs via IL-15- and possibly TGF-β-mediated mechanism and abrogate the antitumor cytotoxicity of TILs. $CD8^+$ T cells express higher ratios of CD94 and NKG2A in TILs than in peripheral blood mononuclear cells (PBMCs) in human endometrial carcinoma (EC). Increased expression of CD94/NKG2A restricted to tumor-infiltrating $CD8^+$ T cell subsets may shape the cytotoxic responses, which indicate a possible role of tumor escape from host immunity in human EC (Chang et al. 2005).

30.14.6 Disorders of Immune System

CD94 in Pathogenenesis of Behcet's Disease: Behcet's disease (BD) is a multisystemic disorder with a possible underlying pathology of immune-mediated vasculitis. An imbalance in cytotoxic activity and cytokine production and genetic susceptibility associated with HLA-B*51 and B*2702 has been implicated in its pathogenesis. The BD patients show increased $CD3^+$ T cells, with no change in NK cells. Increased expression of CD94 in BD was observed on $CD16^+CD56^+$ and on $CD3^+$ and $CD3^+CD56^+$ T cells. Considering the defined regulatory mechanisms of NK cells through HLA class I binding receptors, the interactions of NK and T cells through the NK receptors may be important in the pathogenesis of BD. Although the effects of SNPs remain unclear, results indicate that the SNPs of CD94/NKG2A and its haplotypes, as well as its ligand HLA-E, are associated with BD immune systems (Seo et al. 2007).

Behcet's Uveitis: Behçet's uveitis, characterized by chronic recurrent uveitis and obliterating retinal vasculitis, frequently causes bilateral blindness. Intraocular infiltration of $TCR\alpha\beta^+$ $CD8^{bright}$ $CD56^+$ cells is a distinct feature in Behçet's uveitis. Interestingly, $CD45RA^{dim}$ $CD45RO^-$ phenotypes were expanded, and CD94 expression was markedly up-regulated in contrast to the down-regulation of NKG2D. Furthermore, these subsets were polarized to produce IFN-γ and contained high amounts of preformed intracellular perforin while exclusively expressing surface FasL upon PI stimulation. Hence, $CD8^{bright}$ $CD56^+$ T cells in Behçet's uveitis are characterized by cytotoxic effector phenotypes with functional NK receptors and function as strong cytotoxic effectors through both FasL-dependent and perforin-dependent pathways. The combined low dose cyclosporine and prednisone treatments in active Behçet uveitis may down-regulate the NK-like effector functions of $CD8^{bright}$ $CD56^+$ T cells (Ahn et al. 2005).

$CD8^+$ Regulatory T Cells in Multiple Sclerosis: To investigate $CD8^+$ regulatory T cell influence on multiple sclerosis development, peripheral blood and cerebrospinal fluid (CSF) $CD8^+$ T cell clones (TCCs) recognizing MBP(83–102) and MOG(63–87)-specific $CD4^+$ T cells were analysed. During exacerbations, CD94/NKG2A expression was significantly higher in $CD8^+$ TCCs, limiting their cytotoxic activity. Moreover, IL-15 and IFN-γ significantly increased CD94 and NKG2A expression. Evidence suggests that CD94/NKG2A receptors play an important role in regulating T cell activity during the course of MS (Correale and Villa 2008).

Negative Regulation of CD8+ TNF-α by CD94/NKG2A: Lung injury due to influenza pneumonia is mediated after Ag recognition by $CD8^+$ T cell in the distal airways and alveoli. TNF-α produced by Ag-specific $CD8^+$ T cells appears primarily responsible for this immunopathology. Zhou et al. (2008) suggested negative regulation of $CD8^+$ TNF-α by CD94/NKG2A when engaged with its receptor, Qa-1b. TNF-α production by $CD8^+$ T cells was enhanced by NKG2A blockade in vitro, and mice deficient in Qa-1b manifested greater pulmonary pathology upon $CD8^+$ T cell-mediated clearance in influenza pneumonia. It appears that CD94/NKG2A transduces a biologically important signal in vivo to activated $CD8^+$ T cells that limits immunopathology in severe influenza infection.

Susceptibility for Rheumatoid Arthritis: To examine a possible association between variations in CD94 and NKG2 genes and genetic susceptibility to rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), Hikami et al. (2003) carried out a systematic polymorphism screening of NKG2A, NKG2C and CD94 genes on a population basis. Although human NKG2-A, -C and CD94 are generally conserved with respect to amino acid sequences, NKG2A is

polymorphic in the noncoding region, and that the number of genes encoded in the human NKC is variable among individuals, as shown for leukocyte receptor complex (LRC), HLA and Fcγ receptor (FCGR) regions (Hikami et al. 2003). Park et al. (2008) revealed that the major NKG2A c.338-90*A/*A, NKG2C102*Ser/*Ser, and NKG2D72*Ala/*Ala genotypes in RA were significantly associated compared with controls. The minor NKG2A c.338-90*G/*G, NKG2C102*Phe/*Phe, and NKG2D72*Thr/*Thr genotypes showed a risk of RA compared with controls.

Synovial NK cells were similar to the well-characterized $CD56^{bright}$ peripheral blood (PB) NK-cell subset present in healthy individuals. However, compared to peripheral blood subset the synovial NK cells expressed a higher degree of activation receptors including CD69 and NKp44, the latter being up-regulated also on CD56(bright) NK cells in the peripheral blood of patients. Activated synovial NK cells produced IFN-γ and TNF, which were further up-regulated by antibody masking CD94/NKG2A, and down-regulated by target cells expressing HLA-E in complex with peptides known to engage CD94/NKG2A. It suggested that synovial NK cells have an activated phenotype and that CD94/NKG2A is the key regulator of synovial NK-cell cytokine synthesis. Human eosinophils express several inhibitory receptors IRp60, LIR3/ILT5, FcγRIIB, and p75/AIRM but not LIR1/ILT2, p58.1, p58.2, p70, or NKG2A/CD94 (Munitz et al. 2006; de Matos et al. 2007).

$CD94/NKG2A^+$ T Cells in Patients with Psoriasis: Psoriasis is a common inflammatory cutaneous disorder characterized by activated T-cell infiltration. T lymphocytes bearing NKRs have been suggested to play an important role in the pathogenesis of psoriasis. Liao et al. (2006) demonstrated increased proportions of particular subsets of inhibitory $CD158b^+$ and/or $CD94/NKG2A^+$ T cells in patients with psoriasis. The elevation of these inhibitory NKR-expressing T cells was correlated with disease severity, which may signify the unregulated cytokine production in the pathogenesis of psoriasis.

CD94/NKG2A in Sarcoidosis Patients: The majority of the lung NK cell subpopulation expressed CD56(bright). In contrast, there was a predominant CD56(dim) subset in the blood of both patients and healthy controls. Most lung NK cells expressed HLA-E-specific inhibitory receptor (i.e. CD94/NKG2A), but only a few lung NK cells expressed KIRs specific for HLA-A, -B or -C molecules. After in vitro stimulation, both lung NK and $CD56^+$ T-cells produced considerable amounts of IFN-γ and TNF-α. Thus, patients with pulmonary sarcoidosisproduce a distinct phenotype of NK cells with the capacity to produce cytokines (Katchar et al. 2005).

Frequency of CD94/NKG2A in Peritoneal NK Cells in Patients with Endometriosis: In women with endometriosis, the percentage of CD94/NKG2A-positive peritoneal NK cells was significantly higher than in the control group. It was associated with high levels mRNA transcripts of HLA-E, the ligand of CD94/NKG2A. The increased expression of CD94/NKG2A in peritoneal NK cells may mediate the resistance of endometriotic tissue to NK cell-mediated lysis leading to the progression of the disease (Galandrini et al. 2008).

Lymphoproliferative Disease of Granular Lymphocytes: Mitsui et al. (2004) analysed the chemokine receptors and NKRs in addition to NK-cell markers in patients with lymphoproliferative disease of granular lymphocytes (LDGL). There were no marked differences in the expression patterns of chemokine receptors between NK- and T-LDGL patients. Although restricted NKR subsets were expressed on both NK- and T-large granular lymphocytes (LGLs), CD94 was the most widely expressed marker.

CD94 and KIRs After Transplantation: The effect of NK cell alloreactivity on the outcome of haploidentical hematopoietic stem cell transplantation (HSCT), with or without in vitro T cell depletion, remains controversial. KIRs recognize HLA-C and -B epitopes on target cells, thereby regulating NK cell activity. The occurrence of a GVHD or the receipt of high doses of T cells in the allograft altered KIR reconstitution. The high levels of CD94 expression in donors or in recipients by day 60 might be a good predictor for poor prognosis (Zhao et al. 2007). While hepatic $CD56^+$ T cells are not expanded in malignancy, downregulation of KIR and CD94 expression may be a mechanism by which the hepatic immune system can be activated to facilitate tumor rejection (Norris et al. 2003).

NK cells mediate bone marrow allograft rejection. It was shown that murine NK cells recognize allogeneic target cells through Ly-49s and CD94/NKG2 heterodimers. NKG2I is the activating receptor mediating recognition and rejection of allogeneic target cells (Koike et al. 2004). NKG2I was composed of 226 amino acids, showed ~40% homology to the murine NKG2D and CD94 The expression of NKG2I was largely confined to NK and NKT cells. The cross-linking of NKG2I enhanced interleukin 2– and interleukin 12–dependent interferon- production (Koike et al. 2004).

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NKG2D Activating Receptor

31

Rajesh K. Gupta and G.S. Gupta

31.1 NKG2D Activating Receptor (CD314, Synonyms KLRK1)

Information on receptor ligand systems used by NK cells to specifically detect transformed cells has been accumulating rapidly. Killer cell lectin-like receptor subfamily K, member 1, also known as KLRK1, is the product of human gene. The KLRK1 has been designated as CD314 and contains a C-type lectin-like domain (CTLD). KLRK1 is also known as: KLR; NKG2D; NKG2-D; FLJ17759; FLJ75772; D12S2489E. Human NKG2D was originally identified in 1991 as an orphan receptor on NK cells (Houchins et al. 1991). Although genetically mapping near the C-type lectin receptors CD94 and NKG2A-E, the NKG2D activating NK cell receptor has little sequence homology with these receptors and is expressed as a homodimer that signals through DAP10 rather than CD94 (Chap. 30). NKG2D binds to two distinct families of ligands, the MHC class I chain-related peptides (MICA and MICB) and the UL-16 binding proteins (ULBP). These ligands are upregulated in cells that have undergone neoplastic transformation, and NK cytotoxicity on tumor cells correlates with tumor expression of MICA and ULBP. The NKG2D differs from other members of the NKG2 family in significant ways. They do not form heterodimers with CD94 on the cell surface. Instead, they are expressed as homodimers, and each homodimer associates noncovalently with a homodimer of the adaptor protein DAP-10. The cytoplasmic tail of DAP-10 carries a YxxM motif, which can recruit the regulatory subunit p85 of phosphatidylinositol-3 kinase and Grb2 (see also Chap. 30).

The NKG2D (KLRK1) is also widely expressed on T cells and other immune system cells, providing stimulatory or co-stimulatory signals. NKG2D drives target cell killing following engagement of diverse, conditionally expressed MHC class I-like protein ligands whose expression can signal cellular distress due to infection or transformation. The NKG2D ligand-binding site recognition is highly degenerate that demonstrates its ability to simultaneously accommodate multiple non-conservative allelic or isoform substitutions in the ligands. The NKG2D degeneracy is achieved using distinct interaction mechanisms at each rigid interface: recognition degeneracy by "rigid adaptation." While forming similar complexes with their ligand (HLA-E), other NKG2x NKR family members do not require such recognition degeneracy. However, NK cells are known to efficiently kill target cells that do not express HLA class I molecules, thus implying the existence of triggering receptors for non-HLA ligands (Zhang et al. 2005).

31.2 Characteristics of NKG2D

31.2.1 The Protein

NKG2D is encoded by the *KLRK1* (killer cell lectin-like receptor subfamily member 1) gene. NKG2D is a type II homodimeric transmembrane protein with an extracellular C-type (i.e. Ca^{2+}-binding) lectin-like domain. The NKG2D gene exists within the "NK complex" on human chromosome 12 and mouse chromosome 6 (Ho et al. 1998). The physical location of the *Cd94* (centromeric) and *Nkg2d* (telomeric) genes were mapped between *Cd69* and the *Ly49* cluster in the NK complex. Genes within this complex encode structurally similar type II lectin-like receptors, and many of these genes are expressed primarily in NK cells. In mice, alternative DNA splicing generates two isoforms of NKG2D that differ in the length of their cytoplasmic domains (NKG2D-S (short) and NKG2D-L (long)). Their ability to induce cellular activation is mediated via association with two membrane-bound, signaling adaptor molecules, DAP10 and DAP12. Two NKG2D isoforms, NKG2D-S and NKG2D-L, are known to associate differentially with DAP10 and DAP12 adaptor proteins. The mouse (Ho et al. 1998), rat (Berg et al. 1998), and porcine (Yim et al. 2001) homologues of NKG2D

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have been identified. Interspecies amino acid sequence identities range from 52% to 78% for the entire protein (mouse and rat are the most closely related sequences) and from 72% to 90% within the lectin domain. Function of NKG2D was first described in 1999 by two separate groups investigating MICA/MICB ligands (Bauer et al. 1999) or signal transduction through the DAP10 adapter protein (Wu et al. 1999). Ho et al. (1998) cloned C57BL/6-derived cDNAs homologs to human NKG2-D and CD94. Among normal tissues, murine NKG2-D transcripts are highly expressed only in activated NK cells, including both $Ly49A^+$ and $Ly49A^-$ subpopulations. Additionally, mNKG2-D is expressed in murine NK cell clones KY-1 and KY-2. NKG2D is present on the surface of natural killer (NK) cells, some NKT cells, $CD8^+$ cytotoxic T cells, subsets of γδ T cells, and under certain conditions $CD4^+$ T cells. Present in both humans and mice, NKG2D binds to a surprisingly diverse family of ligands that are distant relatives of MHC-class-I molecules. There is increasing evidence that ligand expression can result in both immune activation (tumor clearance, viral immunity, autoimmunity, and transplantation) and immune silencing (tumor evasion).

Tolerance of NK cells toward normal cells is mediated through their expression of inhibitory receptors that detect the normal expression of self in the form of MHC-I molecules on target cells. These MHC-I-binding inhibitory receptors recruit tyrosine phosphatases, which are believed to counteract activating receptor-stimulated tyrosine kinases. The perpetual balance between signals derived from inhibitory and activating receptors controls NK cell responsiveness and provides an interesting paradigm of signaling cross talk. MacFarlane and Campbell (2006) and Champsaur and Lanier (2010) reviewed the knowledge of the intracellular mechanisms by which cell surface receptors influence biological responses by NK cells with a special emphasis on the dynamic signaling events at the NK immune synapse and the unique signaling characteristics of specific receptors, such as NKG2D.

31.2.2 Orthologues to Human NKG2D

The CLEC12B is a lectin-like NK cell receptor on myeloid cells. The extracellular domain of CLEC12B shows a considerable homology to activating NKG2D, but unlike NKG2D, CLEC12B contains an ITIM in its intracellular domain. Despite the homology, CLEC12B does not appear to bind NKG2D ligands and therefore does not represent the inhibitory counterpart of NKG2D. However, CLEC12B has the ability to counteract NKG2D-mediated signaling, and this function is dependent on the ITIM and the recruitment of the phosphatases SHP-1 and SHP-2. This receptor seems to play a role in myeloid cell function (Hoffmann et al. 2007).

The rat orthologue to human NKG2D (hNKG2D) represents a separate, single gene NKLLR family, named NKR-P2. Rat NKR-P2 is expressed in NK cells and strongly expressed in resting CD81 and CD41 T cells. Sequence analysis showed that the two molecules form a distinct NKLLR family, not related to NKG2A/B, -C or -E than to other NKLLR families. *Nkrp2* is a single-copy gene containing seven introns, mapping to the rat NK gene complex. Rat NKR-P2 differs from the human orthologue in that its cytoplasmic tail contains 13 additional amino acids, encoded by a separate exon (Berg et al. 1998). NKR-P2 is a potent activation receptor on rat DCs (Srivastava et al. 2008)

31.3 NKG2D Ligands

31.3.1 The Diversity of NKG2D Ligands

The NKG2D drives target cell killing following engagement of diverse, conditionally expressed MHC class I-like protein ligands whose expression can signal cellular distress due to infection or transformation. NKG2D recognizes some self ligands such as MICA/B in human and RAE-1 in mice. The interaction of NKG2D with its ligands plays an important role in immunosurveillance of tumors and infectious pathogens, but dysregulation of this system may lead to autoimmunity. Several human NKG2D ligands (NKG2DLs), UL16-binding proteins (ULBP) 1, 2, 3 and 4, retinoic acid early transcript 1G (RAET1G) and MHC class I-related chains A and B have been reported. However, the list of ligands for NKG2D, often up-regulated during cellular distress, is growing. Moreover, NKG2D ligands have an intrinsic ability to regulate tissue-associated immune compartments. Certain cytokines and specifically the IFNs regulate expression of specific NKG2D ligands on murine tumors. Why there is such diversity of NKG2D ligands is not known but one hypothesis is that they are differentially expressed in different tissues in response to different stresses. The expression of diverse NKG2D-binding molecules in different tissues and different properties is consistent with multiple modes of infection- or stress-induced activation.

Rodent homologs of MICA and MICB have not been identified, but other molecules with weak homology to MHC class I, notably RAE-I and H-60, serve as ligands for mouse NKG2D. Like MICA and MICB, RAE-1 and H-60 are frequently expressed on tumor but not normal cells, suggesting that NKG2D functions as a receptor for tumor surveillance by NK cells. In addition, HCMV glycoprotein UL16 binding proteins (ULBP) and mouse UL16-binding protein–like transcript 1 (MULT1) have been identified as ligand for human and mouse NKG2D respectively. UL16 is believed to act as a decoy receptor for NKG2D ligands, facilitating viral evasion of the immune system. Although the homodimeric NKG2D

ligands are distant structural homologs of MHC class I, including MICA, MICB, ULBP3, and RAE-1β, unlike true MHC class I molecules, NKG2D ligands neither bind peptides (or other small molecules) nor β_2m, and ULBP3 and RAE-1β even lack the heavy chain α3 domain, existing on the cell surface as glycophosphatidylinositol-linked α1/α2 platform domains. In humans, MICA and MICB are minimally expressed in normal tissues, but are upregulated in stressed cells and epithelial tumors. The interaction of mouse NKG2D with RAE-1, H-60 and MULT1 is disrupted by the HCMV-encoded MHC class I homologs m152, m155, m157 and m144. By preventing NK cell activation, this disruption promotes viral survival (Cerwenka 2009; Deng and Mariuzza 2006; Eagle et al. 2006 Eagle and Trowsdale 2007; Mans et al. 2007).

31.3.2 MHC Class I Chain Related (MIC) Proteins

31.3.2.1 A Second Lineage of Mammalian MHC Class I Genes

MHC class I genes typically encode polymorphic peptide-binding chains which are ubiquitously expressed and mediate the recognition of intracellular antigens by cytotoxic T cells. They constitute diverse gene families in different species and include the numerous so-called non-classical genes in the mouse H-2 complex, of which some have been adapted to variously modified functions. Bahram et al. (1994, 2005) identified a distinct family of five related sequences in human MHC which are distantly homologous to class I chains. These MIC genes (*M*HC class *I c*hain-related genes) evolved in parallel with the human class I genes and with those of most if not all mammalian orders. The MIC-A gene is located near HLA-B and is by far the most divergent mammalian MHC class I gene known. It is further distinguished by its unusual exon-intron organization and preferential expression in fibroblasts and epithelial cells. However, the presence of diagnostic residues in the MIC-A amino acid sequence translated from cDNA suggests that the putative MIC-A chain folds similarly to typical class I chains and may have the capacity to bind peptide or other short ligands. These results define a second lineage of evolutionarily conserved MHC class I genes. Major structural deviations of MIC-A from HLA-A2 (Bjorkman et al. 1987) include a deletion of amino acid positions 45–49 and an insertion of 6 amino acids at position 147. This implies that MIC-A and possibly other members in this family have been selected for specialized functions that are either ancient or derived from those of typical MHC class I genes, in analogy to some of the nonclassical mouse H-2 genes (Bahram et al. 1994).

The MIC-A and MIC-B are inducible by stress on cell surface and recognized by immunocytes bearing the receptor NKG2D, including intestinal epithelial Vδ1 γδ T cells, which may play a role in immunological reaction in intestinal mucosa. NKG2D ligands are expressed by infected and transformed cells. They transmit danger signals to NKG2D-expressing immune cells, leading to lysis of NKG2D ligand-expressing cells. Expression of MIC-A and MIC-B has been proposed to play an important role in the immunosurveillance of tumors Groh et al. (1998). Proteolytic shedding of NKG2D ligands from cancer cells therefore constitutes an immune escape mechanism impairing anti-tumor reactivity by NKG2D-bearing cytotoxic lymphocytes. Serum levels of sMICA have been shown to be of diagnostic significance in malignant diseases of various origins (Sutherland et al. 2006). NKG2D homodimers form stable complexes with monomeric MICA in solution and also with cell surface MICB, which has structural and functional properties similar to those of MICA. Comparison of allelic variants of MICA revealed large differences in NKG2D binding that were associated with a single amino acid substitution at position 129 in α2 domain. Varying affinities of MICA alleles for NKG2D may affect thresholds of NK-cell triggering and T-cell modulation (Steinle et al. 2001). MICA has no function in antigen presentation.

31.3.3 Retinoic Acid Early (RAE) Transcripts

31.3.3.1 A Cluster of MHC Class I Related Genes on Human Chromosome 6

In addition to human MIC-A and MIC-B, human ligands also include retinoic acid early transcript-1 proteins (RAET-1, originally called unique long 16) [UL-16-binding proteins (ULBP)]. Radosavljevic et al. (2002) identified a cluster of ten human MHC class I related genes, among which 6 encode potentially functional glycoproteins. The 180-kb cluster containing these genes is not located within the MHC on 6p44.3, but near the tip of its long arm at q24.2-q31.3, close to the human equivalent of the mouse H2-linked t-complex, a sub-chromosomal region syntenic to a segment of mouse chromosome 10. Mouse chromosome 10 harbors the orthologous MHC class I related transcript loci, *Raet1a-d*. The human identified loci were called *RAET1E-N*. Human RAET1 products are all devoid of membrane-proximal Ig-like α3 domain. Most, but not all RAET1 products, are predicted to remain membrane-anchored via GPI-linkage and are known to display an atypical pattern of polymorphism. *RAET1* transcripts are absent from hematopoietic tissues, but largely expressed in tumors.

Mouse NKG2D ligands include the family of retinoic acid inducible genes-1 (RAE-1α), the minor histocompatibility antigen H60, two H60 variants (H60b and H60c), and mouse UL16-binding protein–like transcript 1 (MULT1) (Diefenbach et al. 2003; Carayannopoulos et al. 2002). Ligands for mouse NKG2D were identified by their capacity to bind dimeric NKG2D-Fc fusion proteins or tetrameric NKG2D-streptavidin

complexes (Cerwenka et al. 2000; Diefenbach et al. 2000). Members of RAET1 family (RAE-1α) are MHC class I-related genes located within a 180-kb cluster on chromosome 6q24.2-q31.3. RAET1 proteins contain MHC class I-like α-1 and α-2 domains. RAET1E and RAET1G differ from the other RAET1 proteins (e.g., RAET1I, or ULBP1) in that they have type I membrane-spanning sequences at their C termini rather than GPI anchor sequences (Radosavljevic et al. 2002).

The mouse NKG2D ligands are expressed at appreciable levels on thymocytes but at low levels or not at all on most normal tissues (Cerwenka et al. 2000; Diefenbach et al. 2000). Expression is up-regulated, however, on many tumor cells. RAE-1 mRNA was detected throughout early embryos and in the brain/head region of day 10–14 embryos, but transcripts were not observed in day 18 embryos (Cerwenka et al. 2000; Nomura et al. 1996). Very low levels of *Rae-1* transcripts were detected in adult spleen and liver, and transcripts were absent from adult brain and kidney. *Rae-1* transcripts were present in several transformed cell lines. Expression of NKG2D ligands RAE-1, MULT1, and H60 within the CNS following JHM strain of mouse hepatitis virus (JHMV) infection demonstrated a functional role for NKG2D in host defense during acute viral encephalitis by selectively enhancing CTL activity by infiltrating virus-specific $CD8^+$T cells (Walsh et al. 2008).

The cDNAs encoding NKG2D ligands, RAE-1ß and RAE-1 (Cerwenka et al. 2000; Diefenbach et al. 2000) had been described earlier as products of retinoic acid early inducible (Rae-1) transcripts (Zou et al. 1996; Nomura et al. 1996). This family is known to contain at least four distinct loci encoding polypeptides that share 92–95% amino acid identity. All RAE-1 family members were shown to bind NKG2D (Zou et al. 1996; Savithri and Khar 2006). Savithri and Khar (2006) described the cloning of a ligand for NKR-P2, the rat ortholog of human and mouse NKG2D, termed as rat RAE-1-like transcript (RRLT). The RRLT ligand is homologous to mouse RAE-1 family of proteins, but differs from them in being transmembrane anchored. Strid et al. (2008) reported that epidermis-specific upregulation of RAE-1 induces changes in the organization of tissue-resident Vγ5Vδ1 TCR$γδ^+$ intraepithelial T cells and Langerhans cells, followed by epithelial infiltration by unconventional αβT cells. Whereas local $Vγ5Vδ1^+$ T cells attempted to limit carcinogenesis, Langerhans cells unexpectedly promoted it.

31.3.3.2 The UL16-Binding Proteins

The UL16-binding proteins (ULBPs, also termed as retinoic acid early transcripts, encoded by RAET1 genes) are a family of human, MHC class I-related, cell surface proteins. ULBP1 was identified based on its ability to bind to human CMV (HCMV) glycoprotein, UL16. ULBP2 and ULBP3 were subsequently discovered as expressed sequence tags with high homology to ULBP1. Unlike traditional MHC class I proteins, ULBPs are GPI-linked, lack an $α_3$ domain, and do not associate with $β_2$-microglobulin (Cosman et al. 2001). ULPB transcripts were found in various tissues in healthy individuals, including heart, lung, liver, and testis (Cosman et al. 2001). UL16 also binds to a member of another family of nonclassical MHC class I proteins, MIC-B (Groh et al. 1996). Presently, the human RAET1/ULBP gene family comprises of ten members (RAET1E to N) with six loci encoding for potentially functional proteins. These are ULBP1 or RAET1I, ULBP2 or RAET1H, ULBP3 or RAET1N, and RAET1L, which are glycosylinositol phospholipid (GPI)-linked glycoproteins and ULBP4 or RAET1E and ULBP5 or RAET1G, which are transmembrane glycoproteins. The RAET1 products contain α1 and α2 domains but lack α3 domain and do not associate with β2-microglobulin. RAET1/ULBPs have tissue-specific expressions, and some of them are also polymorphic. The MIC-B and the closely related MIC-A protein share some similar properties with ULBPs (Sutherland et al. 2002).

ULBPs are important activators of NK cells. Soluble recombinant forms of ULBPs bind to human NK cells and stimulate NK cytotoxicity against tumor targets (Kubin et al. 2001). Soluble ULBPs also induce production of the cytokines IFN-γ, GM-CSF, TNF-α, and TNF-β, and the chemokines macrophage-inflammatory protein (MIP)-1α, MIP-1β, and I-309. In all cases, costimulation with IL-12 has a superadditive effect on the production of these factors (Cosman et al. 2001; Kubin et al. 2001).

UL16-binding proteins are frequently expressed by malignant transformed cells, a variety of leukemias, carcinomas, melanomas, and tumor cell lines and mediate cytotoxicity of NKG2D-positive NK cells, $CD8^+$ αβ T cells and γδ T cells in vitro and in vivo to tumor cells. ULBPs 1, 2, 3 and 4 are functional ligands of the NKG2D/DAP10 receptor complex on human NK cells. ULBP1, −2, −3, −4 and RAET1L are linked to membrane through GPI. Two more members of the RAET1/ULBP gene cluster, RAET1E and RAET1G have been characterized by Bacon et al. (2004). The encoded proteins RAET1E and RAET1G were similar to the ULBP in their class I-like α1 and α2 domains, but differed in one aspect: instead of being GPI-anchored, their sequences were like type 1 membrane-spanning molecule and contain transmembrane and cytoplasmic domains. Both proteins were capable of being expressed at the cell surface. Both proteins bound the activating receptor NKG2D, and RAET1G bound the human CMV protein UL16. Tissue expression of ULBP4 differs from other members of the family, in that it is expressed predominantly in the skin (Chalupny et al. 2003). ULBP-1 can be detected on mature DCs both in situ in the T cell areas of lymph nodes as well as in vitro after artificial maturation (Schrama et al. 2006). Human gastric cancer cell lines, which expressed ULBP are susceptible to NK cells in a NKG2D-dependent manner. However, cancer cells which had no ULBP on their surface were resistant to NK cells. ULBP 1, 2, and 3 being GPI-anchored proteins are sensitive to

phosphatidylinositol-specific phospholipase C (PI-PLC). Down-regulation of NKG2D by soluble ULBP provides a potential mechanism by which gastric cancer cells escape NKG2D-mediated attack by the immune cells (Song et al. 2006). The ULBP4 binds to TCRγ9/δ2 and induces cytotoxicity to tumor cells through both TCRγδ and NKG2D (Kong et al. 2009).

In the NKG2D/ULBP3 complex, the structure of ULBP3 resembles α1 and α2 domains of classical MHC molecules without a bound peptide. The lack of α3 and β2m domains is compensated by replacing two hydrophobic patches at the underside of the class I MHC-like β sheet floor with a group of hydrophilic and charged residues in ULBP3. NKG2D binds diagonally across the ULBP3 α helices, creating a complementary interface, an asymmetrical subunit orientation, and local conformational adjustments in the receptor. The interface is stabilized primarily by hydrogen bonds and hydrophobic interactions. Unlike the KIR receptors that recognize a conserved HLA region by a lock-and-key mechanism, NKG2D recognizes diverse ligands by an induced-fit mechanism (Radaev et al. 2001).

Monocyte-derived DCs (mDCs) and NK cells are reciprocally activated via cytokines and cell-cell contact. ULBPs have been detected on human mDCs. While ULBP1 was upregulated on mDCs in response to LPS or infection with human respiratory syncytial virus (RSV), the expression of ULBP2 was induced by LPS and poly I:C, indicating that the TLR-containing adapter molecule-1, IFN inducing pathway is associated with ULBP2 (Ebihara et al. 2007).

Proteolytic cleavage of ULBP2 produces truncated and soluble forms that may counteract NKG2D-mediated tumor immune surveillance. It is suggested that RAET1E can produce a soluble, 35-kDa protein (termed as RAET1E2) lacking the transmembrane region by selective splicing in tumor cells. The expressions of both RAET1E2 transcripts and protein can be found in different tumor cells and tissues. Incubation of NK-92 cells with RAET1E2 protein decreases the surface expression of NKG2D and in marked reduction in cytotoxiccity to tumor cells. These results suggest for an immune escape mechanism of tumors via alternative splicing of ULBP RNA to generate RAET1E2 that may impair NKG2D-mediated NK cell cytotoxicity to tumors (Cao et al. 2007).

31.3.4 Role of NKG2D Ligands

As NKG2D plays an important role in the immunosurveillance of tumors, studies suggest that release of MIC-A from cancer cells constitutes an immune escape mechanism that systemically impairs antitumor immunity. The interaction of human NK cells with MIC-A-positive human cancer cells in an in vivo setting showed that MIC-A overexpression can function as NK cell-mediated immunotherapy in experimental lung cancer. Moreover, low-dose application of the proteasome inhibitor bortezomib enhances expression of human NKG2D ligands MIC-A and MIC-B on hepatocellular carcinoma cells (Armeanu et al. 2008; Busche et al. 2006).

An understanding of effects of hyperthermia on NK cell cytotoxicity is important for maximizing the clinical benefits in cancer therapy. At temperatures above 40°C, (normally achieved during fever or exercise), both enhancing and inhibitory effects on cytotoxic activity of NK cells against tumor cells have been reported. Dayanc et al. (2008) have shown that thermal stress (using a temperature of 39.5°C) enhances human NK cell cytotoxicity against tumor target cells. This effect requires function of the NKG2D receptor of NK cells, and is maximal when both NK and tumor cell targets are heated. Heat sensitive cellular targets affected by hyperthermia on tumor cells include HSPs, MIC-A and MHC Class I. The expressions of NKG2D ligands were induced by both heat shock and ionizing radiation in various cell lines with peaks at 2 h and 9 h after treatment, respectively, although inducibility of each NKG2D ligand was dependent on cell lines (Kim et al. 2006). The available studies indicate a strong potential for heat-induced enhancement of NK cell activity in mediating, at least in part, the improved clinical responses seen when hyperthermia is used in combination with other therapies (Dayanc et al. 2008).

31.3.5 Regulation of Ligands

The three main mechanisms by which NKG2D ligand transcription can be induced are: DNA damage, TLR stimulation, and cytokine exposure. The DNA damage response pathway is involved in maintaining the integrity of the genome. The PI3K-related protein kinases ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3 related) sense DNA lesions, specifically double-strand breaks and stalled DNA replication, respectively. This sensing results in cell-cycle arrest and DNA repair, or cell apoptosis if the DNA damage is too extensive to be repaired. This pathway has been shown to be constitutively active in human cancer cells (Bartkova et al. 2005; Gorgoulis et al. 2005; Stephan and David 2006). The DNA damage response, previously known to arrest the cell cycle and enhance DNA repair functions, or to trigger apoptosis, may also participate in alerting the immune system to the presence of potentially dangerous cells (Gasser et al. 2005). Gasser et al. provided evidence that this pathway actively regulates NKG2D ligand transcription (Gasser et al. 2005). Both mouse and human cells upregulated NKG2D ligands following treatment with DNA-damaging agents. This effect was dependent on ATR function, as inhibitors of ATR and ATM kinases prevented ligand upregulation in a dose-dependent fashion. These findings provide a link between the constitutive activity of

the DNA damage response in tumors (Bartkova et al. 2005; Gorgoulis et al. 2005) and the frequent upregulation of NKG2D ligands by these transformed cells. The exact mechanism linking the ATR/ATM-dependent recognition of DNA damage and the transcription of NKG2D ligands remain elusive (Champsaur and Lanier 2010). Toll-like receptor (TLR) signaling and Cytokines can also influence NKG2D ligand expression. In particular, interferons have pleiotropic effects on NKG2D ligand expression. Transforming growth factor (TGF-β) also decreases the transcription of MICA, ULBP2, and ULPB4 on human malignant gliomas. Therefore, cytokines and interferons can differentially affect NKG2D ligand expression in different cell types and environments. Other stimuli have also been reported to induce NKG2D ligand transcription.

Various mechanisms are responsible for the post-transcriptional regulation of NKG2D ligands. A group of endogenous cellular microRNAs (miRNAs) that bound to the 3′-UTR (untranslated region) of MICA and MICB, repressed their translation. Nice et al. showed that MULT1 protein undergoes ubiquitination dependent on the lysines in its cytoplasmic tail, resulting in its rapid degradation. Thomas et al. have recently described the capacity of the KSHV (Kaposi's sarcoma-associated herpesvirus)-encoded E3 ubiquitin ligase K5 to down-regulate cell surface expression of MICA and MICB. In this case, ubiquitination resulted in the redistribution of MICA to the plasma membrane, rather than its targeting to degradation as observed with MULT1 (Champsaur and Lanier 2010).

31.4 Crystal Structure of NKG2D

The basic folding pattern of murine NKG2D is similar to other C-type lectin domains such as CD94, Ly49A, rat MBP-A, and CD69 despite relatively low sequence homology (Boyington et al. 1999; Natarajan et al. 2000; Tormo et al. 1999; Weis et al. 1991). However, the precise mode of dimeric assembly varies among these natural killer receptors, as well as their surface topography and electrostatic properties.

31.4.1 Structures of NKG2D-Ligand Complexes

The NKG2D structure provides the first structural insights into the role and ligand specificity of this stimulatory receptor in the innate and adaptive immune system (Wolan et al. 2001) (Fig. 31.1). Crystal structures have been determined for mouse and human NKG2Ds in complex with MICA (Li et al. 2001). The structure of MICA - NKG2D complex reveals an NKG2D homodimer bound to a MICA monomer in an interaction that is analogous to that seen in αß-T cell receptor-MHC-I protein complexes. Similar surfaces on each NKG2D monomer interact with different surfaces on either the α1 or α2 domains of MICA. The binding interactions are large in area and highly complementary. The central section of the α2-domain helix, disordered in the structure of MICA alone, is ordered in the complex and forms part of the NKG2D interface. The extensive flexibility of the interdomain linker of MICA is shown by its altered conformation when crystallized alone or in complex with NKG2D (Li et al. 2001). Although there is little obvious sequence similarity between the human and mouse NKG2D ligands, there appears to be considerable structural similarity as evidenced by the ability of mouse NKG2D to react with human ligands, MICB, ULBP1, and ULBP2 (Kubin et al. 2001).

In NKG2D/ULBP3 complex, the structure of ULBP3 resembles the α1 and α2 domains of classical MHC molecules without a bound peptide. The lack of α3 and β2m domains is compensated by replacing two hydrophobic patches at the underside of the class I MHC-like β-sheet floor with a group of hydrophilic and charged residues in ULBP3. NKG2D binds diagonally across the ULBP3 α-helices, creating a complementary interface, an asymmetrical subunit orientation, and local conformational adjustments in the receptor. The interface is stabilized primarily by hydrogen bonds and hydrophobic interactions. Unlike the KIR receptors that recognize a conserved HLA region by a lock-and-key mechanism, NKG2D recognizes diverse ligands by an induced-fit mechanism (Radaev et al. 2001).

Rodent RAE-1 proteins (α, β, γ and δ) are distant MHC class I homologs, comprising isolated α1α2 platform domains. The crystal structure of RAE-1β was distorted from other MHC homologs and displayed noncanonical disulfide bonds. The loss of any remnant of a peptide binding groove was facilitated by the close approach of the groove-defining helices through a hydrophobic, leucine-rich interface. The RAE-1β-murine NKG2D complex structure resembled the human NKG2D-MICA receptor-ligand complex and further demonstrated the promiscuity of the NKG2D ligand binding site (Li et al. 2002).

The interaction of NKG2D receptor with several MHC I-like ligands has been analyzed thermodynamically. NKG2D ligand-binding site recognition is highly degenerate, as demonstrated by NKG2D's ability to simultaneously accommodate multiple non-conservative allelic or isoform substitutions in the ligands. In TCRs, "induced-fit" recognition explains cross-reactivity, but analyses of multiple NKG2D-ligand pairs showed that rather than classical "induced-fit" binding, NKG2D degeneracy is achieved using distinct interaction mechanisms at each rigid interface: recognition

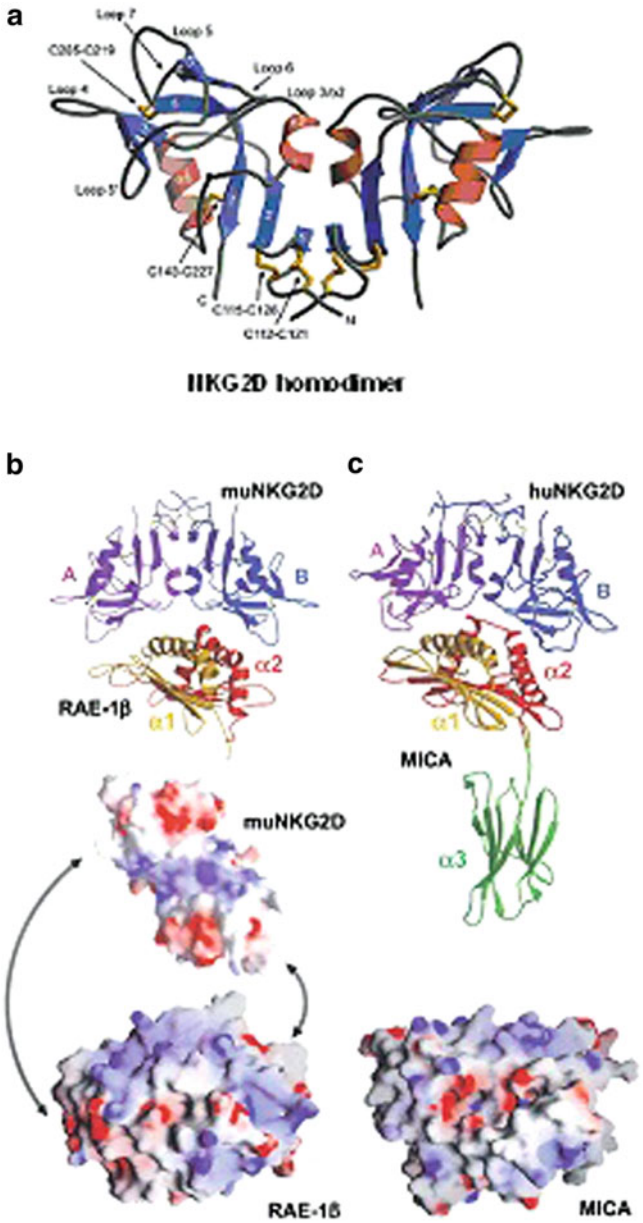

Fig. 31.1 (**a**) **Anatomy of killer cell lectin-like receptor subfamily K, member 1 (KLRK1) or NKG2D**. Structure of murine NKG2D homodimer shows side view showing the NH_2 terminus at the *bottom* of the structure that would connect to the membrane at the cell surface by a stalk comprised of 16 residues. The dimer is formed primarily of an extension of the first β-sheet (β1) across the interface. The NKG2D structure has a C-type lectin fold like the one reported in CD94 (Boyington et al. 1999), Ly49A (Tormo et al. 1999) and rat MBP-A (Weis et al. 1991). However, loop 5 in NKG2D is not a Ca^{2+}-dependent carbohydrate-binding loop like rat MBP-A. Only CD94 does not contain an α2-helix in loop 3 (Figure not shown) (Adapted with permission from Boyington et al. 1999 © Elsevier). (**b**) and (**c**) **Structures of murine NKD NK cell receptor-ligand complexes:** ribbon representations (*top*) and GRASP molecular surfaces (*bottom*) are shown for the structures of (**a**) the muNKG2D–RAE-1β, and (**b**) huNKG2D–MICA. Ribbons of the ligands are colored by domain: α1, *yellow*; α2, *red*; α3 (when present), *green*; and β_2-m (when present), *cyan*; ribbons of the receptors are colored by chain: *blue* or *purple*. Molecular surfaces of the platform domains are oriented such that the view is looking down onto the NKG2D binding surface of RAE-1 and MICA. In (**a**), the molecular surface of muNKG2D was included in an orientation looking down onto the RAE-1 binding surface, as if the receptor had been peeled away from the complex. Molecular surfaces are colored by electrostatic potential, with positively charged areas in blue and negatively charged areas in red. The RAE-1β-murine NKG2D complex structure resembles the human NKG2D-MICA receptor-ligand complex and demonstrates the promiscuity of the NKG2D ligand binding site (Adapted with permission from Li et al. 2002 © Elsevier)

Table 31.1 Activating homodimeric receptors and co-receptors with two basic TM residues

	Receptor	Signaling polypeptide	Ligand	Protein family
Human	NKG2D	DAP10	MIC-A/MIC-B; ULBPs(RAET1)	C-type lectin
	NKG2F (NKp80)	DAP12	AICL	C-type lectin
	NKp46	ξ/γ	Heparin, lewis-x	Ig-SF
	NKp30	ξ/γ	HLA-B-associated transcript 3;B7h6	Ig-SF
	NKp44	DAP12	Heparan sulfate epitope(s)	Ig-SF
Mouse	NKG2D	DAP10	MIC-A/MIC-B; ULBPs (RAET1)	C-type lectin
	Ly49D	DAP12	H2-D	C-type lectin
	Ly49H	DAP12	m157	C-type lectin
	NKRP1C	ξ/γ	unknown	C-type lectin
	NKRP1F	?	Clr-g	C-type lectin

TM transmembrane residues

degeneracy by "rigid adaptation." While likely forming similar complexes with their ligand (HLA-E), other NKG2x NKR family members do not require such recognition degeneracy (Strong and McFarland 2004).

31.5 DAP10/12 Adapter Proteins

31.5.1 DAP10

A number of receptors that are primarily expressed by human and/or murine NK cells represent homodimers with a basic residue in each transmembrane (TM) domain and may thus form a hexameric structure (Table 31.1). The human NKp46 receptor can assemble with Fcγ or ξ-ξ signaling dimers (Pessino et al. 1998), and experiments demonstrated that it forms a homodimer (Garrity et al. 2005). The human NKp80 and NKp40 and mouse Ly49H and Ly49D receptors assemble with DAP12 (Smith et al. 1998), whereas the mouse NKR-P1C receptor assembles with Fcγ (Lanier 2005). The stoichiometry described for NKG2D–DAP10 complex may be relevant for several receptors from the C-type lectin and immunoglobulin families.

In the recognition of self from non-self, activating and inhibitory receptors signal immune cells via adapter molecules, which determine the outcome of immune response. NKG2D lacks signal elements in its cytoplasmic domain. However, DAP10, the transmembrane adapter protein expressed broadly in hematopoietic cells, associates with NKG2D activating receptor forming a multisubunit complex, which recognizes self-proteins upregulated during tumorigenesis, infection, and autoimmune response. NKG2D can deliver stimulatory signals only in association with DAP10 or DAP12. Human NKG2D transduces activation signals exclusively via an associated DAP10 adaptor containing a YxNM motif, whereas murine NKG2D can signal through either DAP10 or the DAP12 adaptor, which contains an ITAM sequence. DAP10 signaling is thought to be mediated, at least in part, by PI3K and is independent of Syk/Zap-70 kinases. NKG2D couples to the non-ITAM-containing DAP10 and initiates at least two signaling branches that are both required for cytotoxicity. The NKG2D-mediated response can be modulated by factors such as cytokine milieu and possibly the particular ligand expressed. This nontraditional NKG2D-DAP10 initiated signal pathway enables lymphocytes to differentially respond to ligands based on cellular environment (Upshaw and Leibson 2006).

31.5.2 KARAP (DAP12 or TYROBP)

The signaling adaptor protein KARAP/DAP12/TYROBP (killer cell activating receptor-associated protein/DNAX activating protein of 12 kDa/tyrosine kinase binding protein) belongs to the family of transmembrane polypeptides bearing an intracytoplasmic immunoreceptor tyrosine-based activation motif (ITAM). This adaptor, initially characterized in NK cells, is associated with multiple cell-surface activating receptors expressed in both lymphoid and myeloid lineages. The main features of KARAP/DAP12 describe its involvement in a broad array of biological functions. KARAP/DAP12 is a wiring component for NK cell anti-viral function (e.g. mouse cytomegalovirus via its association with mouse Ly49H) and NK cell anti-tumoral function (e.g. via its association with mouse NKG2D or human NKp44). KARAP/DAP12 is also involved in inflammatory reactions via its coupling to myeloid receptors, such as triggering receptors expressed by myeloid cells (TREM) displayed by neutrophils, monocytes/macrophages and dendritic cells. Moreover, bone remodeling and brain function are also dependent upon the integrity of KARAP/DAP12 signals, as shown by the analysis of KARAP/DAP12-deficient mice and KARAP/DAP12-deficient Nasu-Hakola patients (Tomasello and Vivier 2005).

31.5.3 Characterization of DAP10 and DAP12

Human, mouse, pig, and the bovine DAP10 cDNA clones codes for a phosphatidyl-inositol-3 (PI-3) kinase-binding site (YxxM) in its cytoplasmic region. Similar to human, mouse, pig, the bovine DAP12 demonstrates one ITAM in its cytoplasmic domain (Fig. 31.2). The short mouse NKG2D-S and the bovine NKG2D could associate with either DAP10 or DAP12 adaptor protein for its cell surface expression. The advances in our understanding of the structural properties and signaling pathways of the inhibitory

and activating NK cell receptors, with a particular focus on the ITAM-dependent activating receptors, the NKG2D-DAP10 receptor complexes system have been reviewed (Lanier 2008, 2009).

NKG2D is required for cytolysis of tumor cells and both activated and expanded $CD8^+$ T cells and NK cells use DAP10. In addition, direct killing was partially dependent on the DAP12 signaling pathway. Silencing human NKG2D, DAP10, and DAP12 reduces cytotoxicity of activated $CD8^+$ T cells and NK cells in human effector cell function (Karimi et al. 2005), although NKG2D function was normal in patients lacking functional DAP12, indicating that DAP10 is sufficient for human NKG2D signal transduction (Rosen et al. 2004).

31.5.3.1 NKG2D Receptor Assembles in the Membrane with Two Signaling Dimers into a Hexameric Structure

The human receptor NKG2D assembles with the DAP10 signaling dimer and it was thought that one NKG2D homodimer pairs with a single DAP10 dimer by formation of two salt bridges between charged transmembrane (TM) residues. However, stoichiometry analysis demonstrated that one NKG2D homodimer assembles with four DAP10 chains giving hexameric structure (Fig. 31.2). Selective mutation of one of the basic TM residues of NKG2D resulted in loss of two DAP10 chains, indicating that each TM arginine serves as an interaction site for a DAP10 dimer. Assembly of the hexameric structure was cooperative because this mutation also significantly reduced NKG2D dimerization. A monomeric NKG2D TM peptide was sufficient for assembly with a DAP10 dimer, indicating that the interaction between these proteins occurs in the membrane environment. Formation of a three-helix interface among the TM domains involved ionizable residues from all three chains: the TM arginine of NKG2D and both TM aspartic acids of the DAP10 dimer. The organization of the TM domains thus shows similarities to the T cell antigen receptor-CD3 complex, in particular to the six-chain assembly intermediate between T cell antigen receptor and the CD3δε and CD3γε dimers. Binding of a single ligand can thus result in phosphorylation of four DAP10 chains, which may be relevant for the sensitivity of NKG2D receptor signaling, in particular in situations of low ligand density (Garrity et al. 2005) (Fig. 31.2). DAP10 signaling is involved in adjusting the activation threshold and generation of NKT cells and T_{reg} to avoid autoreactivity, but also modulates antitumor mechanisms (Hyka-Nouspikel and Phillips 2006).

31.5.3.2 Association of Adapter Proteins with NKG2D Splice Variants

It has been reported that the long form of murine NKG2D (NKG2D-L) associates exclusively with DAP10, whereas the short form of NKG2D (NKG2D-S) can pair with either DAP10 or DAP12. The short isoform was almost undetectable in naive NK cells. However, using two distinct cell types, it was observed that like the short isoform, the long variant of NKG2D also associates not only with DAP10 but also with DAP12. Cross-linking either isoform of NKG2D induces a calcium flux when associated exclusively with DAP10 or DAP12 (Rabinovich et al. 2006). The differential adapter pairing has functional consequences, as the different adapters trigger distinct signaling cascades. The cytoplasmic YINM motif of DAP10 recruits the p85 subunit of phosphoinositide kinase-3 (PI3K) and growth factor receptor-bound protein 2 (Grb2). DAP12 carries an immunoreceptor tyrosine-based activation motif (ITAM) whose phosphorylation leads to the recruitment of the zeta-chain associated protein kinase 70 (Zap70) and spleen tyrosine kinase (Syk). Thus, NKG2D engagement can result in both the PI3K and Grb2 and Syk and Zap70 signaling cascades (Fig. 31.2). It appears that signaling via mouse NKG2D isoforms is more complex than originally presented (Champsaur and Lanier 2010).

31.5.4 NKG2D Receptor Complex and Signaling

Each NKG2D homodimer associates with two homodimers of DAP10, forming a hexameric structure. Whether a single NKG2D homodimer can pair with both DAP10 and DAP12 homodimers has not yet been determined, but one could imagine this scenario to be beneficial to induce both signaling cascades upon triggering a single receptor. Crystal structures of both mouse and human NKG2D receptors in the soluble form and bound to ligands suggest that NKG2D binds to its ligands through "rigid adaptation" recognition, allowing binding to a wide variety of ligands (Champsaur and Lanier 2010).

The NK cell signaling pathway illustrates the signals produced by the activating cell surface receptors that initiate PTK (protein tyrosine kinase)-dependent pathways through their noncovalent association with transmembrane signaling adaptors that harbor ITAMs. Vivier et al. (2004) described the mechanism by which these positive pathways are antagonized by intracytoplasmic PTPs (protein tyrosine phosphatases) that are activated upon engagement of cell surface receptors with intracytoplasmic ITIMs. The tyrosine phosphorylation status of several signaling components that are substrates for both PTKs and PTPs is thus key to the propagation of the NK cell effector pathways. Additional cell surface receptors that are not directly coupled to ITAMs also participate in NK cell activation, such as NKG2D (which is noncovalently associated with the DAP10 transmembrane signaling adaptor), adhesion molecules, and cytokine receptors (Vivier et al. 2004).

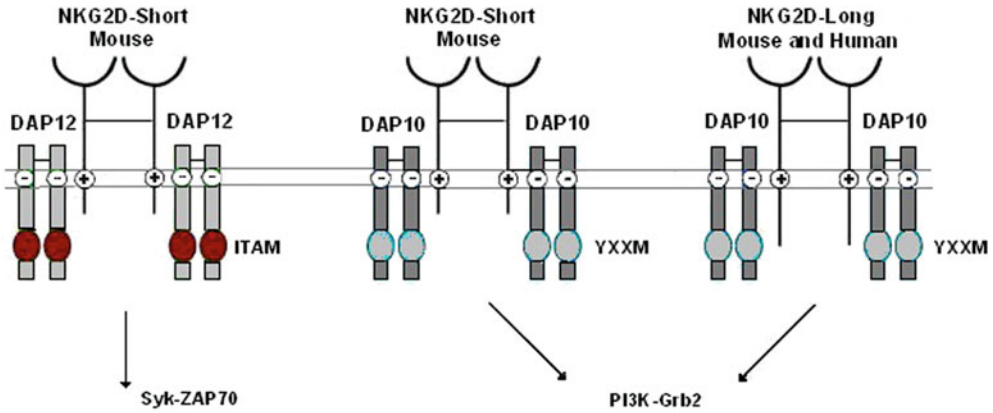

Fig. 31.2 Schematic representation of the NKG2D receptor complexes and ITAM-mediated signaling in NK cells: Hexameric NKG2D–DAP10 or NKG2D–DAP12 receptor complexes: Murine NKG2D exists as a homodimer on the surface of NK cells (Fig. 31.1a). The side view shows that the dimmer is formed as an extension of an anti parallel β-sheet across the interface with a two-fold crystallographic axis perpendicular to the extended sheet. The NKG2D dimmer interface is primarily composed of a central hydrophobic core encased by a hydrogen bond and a salt bridge network (Call et al. 2010). Each TM domain of human NKG2D assembles with one DAP10 dimer. This interaction involves both TM aspartic acids of DAP10 and the TM arginine (Garrity et al. 2005). NKG2D signals via association with adapter molecules through charged residues in the transmembrane domain. Mouse NKG2D associates with both DAP10 and DAP12 signaling molecules, whereas human NKG2D associates with DAP10 only. The pairing of NKG2D with DAP12 results in the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) and triggering of the Syk and/or Zap70 cascade. Association with DAP10 leads to tyrosine phosphorylation on the YINM motif and triggering of the PI3K and Grb2 signaling cascade. ITAM-bearing signaling subunits are phosphorylated, probably by Src family kinases, after receptor engagement. Syk and/or ZAP-70 (both of which are expressed by human and mouse NK cells) are recruited to the phosphorylated ITAMs, initiating a cascade of downstream signaling as depicted. The signaling pathways depicted are hypothetical and were deduced by synthesizing results from many studies investigating ITAM-coupled receptor signaling in human and mouse NK cells (Reproduced with permission from Champsaur and Lanier 2010 © John Wiley and Sons)

Findings suggest how NKG2D-DAP10 mediates cytotoxicity and provides a framework for evaluating activation by other receptor complexes that lack ITIM (Upshaw et al. 2006).

Isoforms of PLC-γ in NK Cell Cytotoxicity and Innate Immunity: The two isoforms of PLC-γ couple immune recognition receptors to important calcium- and PKC-dependent cellular functions. It has been assumed that PLC-γ 1 and PLC-γ2 have redundant functions and that the receptors can use whichever PLC-γ isoform is preferentially expressed in a cell of a given hemopoietic lineage. The ITAM-containing immune recognition receptors can use either PLC- γ1 or PLC-γ2, whereas the NK cell-activating receptor NKG2D preferentially couples to PLC-γ2. Experimental models evaluating signals from either endogenous receptors (FcR verus NKG2D-DAP10) or ectopically expressed chimeric receptors (with ITAM-containing cytoplasmic tails vs DAP10-containing cytoplasmic tails) showed that PLC-γ1 and PLC-γ 2 both regulate the functions of ITAM-containing receptors, whereas only PLC-γ2 regulates the function of DAP10-coupled receptors. These data suggest that specific immune recognition receptors can differentially couple to the two isoforms of PLC-γ (Upshaw et al. 2005). Cytotoxicity was completely abrogated in PLC-γ2-deficient cells, regardless of whether targets expressed NKG2D ligands or others. It was suggested that exocytosis of cytotoxic granules, but not cellular polarization toward targets, depends on intracellular Ca^{2+} rise during NK cell cytotoxicity. In vivo, PLC-γ2 regulates selective facets of innate immunity because it is essential for NK cell responses to malignant and virally infected cells but not to bacterial infections (Caraux et al. 2006). Hence PLCγ1 and PLCγ2 play non-redundant and obligatory roles in NK cell ontogeny and in its effector functions (Regunathan et al. 2006).

Phosphatidylinositol 3-kinase Activation in the Formation of NKG2D Immunological Synapse: In human cells, NKG2D signaling is mediated through the associated DAP10 adapter. However, Giurisato et al. (2007) showed that engagement of NKG2D by itself is sufficient to stimulate the formation of the NK immunological synapse (NKIS), with recruitment of NKG2D to the center synapse. Mutagenesis studies of DAP10 revealed that the phosphatidylinositol 3-kinase binding site, but not the Grb2 binding site, was required and sufficient for recruitment of DAP10 to the NKIS. Surprisingly, in the absence of the Grb2 binding site, Grb2 was still recruited to the NKIS. Since the recruitment of Grb2 was dependent on phosphatidylinositol-trisphosphate (PIP3), the possibility that recruitment to the NKIS is mediated by a pleckstrin homology (PH) domain-containing binding partner for Grb2 was explored. It was found that the PH domain of SOS1, but not that of

Vav1, was able to be recruited by PIP3. These results demonstrate how multiple mechanisms can be used to recruit the same signaling proteins to the plasma membrane (Giurisato et al. 2007).

Many NK Cell Receptors Activate ERK2 and JNK1 to Trigger Microtubule Organizing Center: The NK activating receptors on NK cell signal the polarization of the microtubule organizing center (MTOC) together with cytolytic granules to the synapse with target cells. After ligation of any one of these receptors, Src family kinases initiate activation of two signal pathways, the phosphoinositide-3 kinase —>ERK2 and the phospholipase Cγ —>JNK1 pathways. Both are required for polarization of the MTOC and cytolytic granules, a prerequisite for killing the targets. Crosslinking of CD28, NKG2D, NKp30, NKp46, NKG2C/CD94, or 2B4 leads to the phosphorylation of both ERK2 and JNK1, although they use different proximal signaling modules. Thus, many, if not all, activating receptors stimulate these two distal pathways, independent of the proximal signaling module used. By contrast, CD2, DNAM-1, and β1-integrin cross-linking do not activate either pathway; they may be costimulatory molecules or have another function in the synapse (Chen et al. 2007).

In search of signaling pathway involved after NKG2D ligation, Li et al. either incubated NKG2D-bearing human NKL tumor cell line with K562 target cells or cross-linked with NKG2D mAb induced strong activation of the MAP kinases. Selective inhibition of JNK MAP kinase greatly reduced NKG2D-mediated cytotoxicity toward target cells and blocked the movement of the MTOC, granzyme B, and paxillin (a scaffold protein) to the immune synapse. NKG2D-induced activation of JNK kinase was also blocked by inhibitors of Src protein tyrosine kinases and phospholipase PLCγ, upstream of JNK. A second MAP kinase pathway through ERK was also shown to be required for NK cell cytotoxicity. Thus, activation of two MAP kinase pathways is required for cytotoxic granule and MTOC polarization and for cytotoxicity of human NK cells when NKG2D is ligated (Li et al. 2008).

Regulation of NK Cell-Mediated Cytotoxicity by the Tyrosine Kinase Itk: In activated human NK cells, the tyrosine kinase IL-2-inducible T cell kinase (Itk) differentially regulates distinct NK-activating receptors. Itk plays a complex role in regulating the functions initiated by distinct NK cell-activating receptors. Enhanced expression of Itk leads to increases in calcium mobilization, granule release, and cytotoxicity upon stimulation of the ITAM-containing FcR, suggesting that Itk positively regulates FcR-initiated cytotoxicity (Khurana et al. 2007). Gross et al. (2008) identified a molecular basis for the segregation of NK cell receptor-induced signals for cytokine release and target cell killing and suggested the roles for CARD-protein/Bcl10/Malt1 complexes in ITAM receptor signaling in innate and adaptive immune cells (Gross et al. 2008).

31.6 Functions of NKG2D

31.6.1 Engagement of NKG2D on γδ T Cells and Cytolytic Activity

Engagement of NKG2D on NK cells and γδ T cells can trigger cytolytic activity. The number of γδ T cells has been found to increase in some tumor tissues. This was demonstrated in transfectants that express the MIC proteins (Bauer et al. 1999; Cosman et al. 2001), the ULBPs (Cosman et al. 2001), the Rae-1 proteins (Cerwenka et al. 2000; Diefenbach et al. 2000) and H-60 (Cerwenka et al. 2000; Diefenbach et al. 2000). The parent cell lines were resistant to NK lysis while the transfectants were readily susceptible to NK lytic activity. The results also indicated that the NKG2D activating signal can overcome the inhibitory signals resulting from MHC class I recognition (Bauer et al. 1999; Cosman et al. 2001). Populations of γδ T cells that occur in tumors and in the intestinal epithelium also killed target cells, which expressed MIC proteins (Girardi et al. 2001). Most tumor cells express comparable levels of MICA and MICB as well as ULBP (Groh et al. 1999). Most epithelial tumors were susceptible to allogeneic γδT-cell lysis and in the case of an established ovarian carcinoma to autologous γδT cell killing. The NKG2D pathway appears to be involved in the lysis of different melanomas, pancreatic adenocarcinomas, squamous cell carcinomas of the head and neck, and lung carcinoma (Wrobel et al. 2007). The $CD56^+$ γδ T cells are potent anti-tumor effectors capable of killing squamous cell carcinoma and may play an important therapeutic role in patients with head and neck cancer and other malignancies (Alexander et al. 2008). Despite the increase in γδ T cells in some cancers, the levels of NKG2D receptors, responsible for the cytolytic effect of γδ T cells, were lower in cancer patients than in healthy adults (Bilgi et al. 2008) (see Section 31.7.2 on Immune Evasion Mechanisms). Based on the surface expression of CD56 and CD16 or inhibitory and activatory receptors, NK cells from cord blood (CB) could be divided into four subsets. Interestingly, CB NK cells, similar to the naïve T cells, express CD45RA but not CD45RO molecules. Moreover, CD27, a memory T cell marker, highly expressed on $CD56^{hi}CD16^-$ NK cells. The killing-associated molecules, NKG2A, NKG2D, CD95 and the intracellular granzyme B and perforin are heterogeneously expressed among the 4 subsets. Addition of IL-12 into cultures resulted in the induction of IFN-γ expression by

$CD56^{hi}$ $CD16^{-}$ and $CD56^{lo}$ $CD16^{-}$ subsets and the enhancement of NK cytolytic activity (Fan et al. 2008).

Activation of Vγ 9Vδ 2T Cells by NKG2D: Human γδT cells expressing a Vγ 9Vδ2 T-cell receptor (TCR) kill various tumor cells including autologous tumors. In addition to TCR-dependent recognition, activation of NKG2D-positive γδT cells by tumor cell-expressed NKG2D ligands can also trigger cytotoxic effector function. Human Vγ9 Vδ2 T cells recognize phosphoantigens, certain tumor cells, and cells treated with aminobisphosphonates. Evidences suggest that Vγ9 Vδ2T cells may also be directly activated by NKG2D. Culture of PBMC with immobilized NKG2D-mAb or NKG2D ligand MICA induces the up-regulation of CD69 and CD25 in NK and Vγ9 Vδ2 but not in CD8 T cells. Furthermore, NKG2D triggers the production of TNF-α but not of IFNγ, as well as the release of cytolytic granules by Vγ9 Vδ2 T cells. Due to remarkable similarities in NKG2D function in NK and Vγ9 Vδ2 T cells, it suggests a new perspective for Vγ9 Vδ2 T cell-based immunotherapy based on NKG2D ligand-expressing tumors (Rincon-Orozco et al. 2005). Trichet et al. (2006) reported that activating NKRs have less affinity for their MHC ligands than homologous inhibitory NKRs. Despite this, activating NKRs recognizing MHC class I molecules play an important role in the increased killing by Vγ9Vδ2 T cells of tumor cells with down-regulated MHC class I molecule expression, and suggest that these T cells will best lyse tumor cells combining MHC class I molecule expression down-regulation with up-regulated NKG2D ligand expression (Trichet et al. 2006).

31.6.2 NKG2D: A Co-stimulatory Receptor for Naive $CD8^+$ T Cells

$CD8^+$ T cells require a signal through a co-stimulatory receptor in addition to TCR to become activated. The role of CD28 in co-stimulating T cell activation is well established. The CD28 has a cytoplasmic YXXM motif that activates PI3-kinase via the DAP10 adapter protein (Wu et al. 1999) suggesting a similar co-stimulatory capacity of NKG2D. It is likely that NKG2D acts as a co-stimulatory molecule only under restricted conditions or requires additional cofactors. Whereas NKG2D can only function as a co-stimulatory receptor on $CD8^+$ αβ T cells under the domination of αβ TCR in spite of deficiency of co-stimulatory molecule CD28, NKG2D can directly activate NK cells even in the presence of inhibitory signals from MHC-I and corresponding receptor complexes. Results indicate that NKG2D can regulate the priming of human naive $CD8^+$ T cells, which may provide an alternative mechanism for potentiating and channeling the immune response (Maasho et al. 2005). In mice, however, two alternative splicing products NKG2D polypeptides associate differentially with the DAP10 and DAP12 signaling subunits and that differential expression of these isoforms and of signaling proteins determines whether NKG2D only functions as a co-stimulatory receptor in the adaptive immune system ($CD8^+$ T cells) or as both a primary recognition unit and a co-stimulatory receptor in the innate immune system (NK cells and macrophages) (Cao and He 2004; Diefenbach et al. 2002; Ogasawara and Lanier 2005). The 4-1BB co-stimulatory molecule expressed by resting $CD8^+$ T cells is an essential regulator of NKG2D in $CD8^+$ T cells. Kim et al. (2008) proposed that 4-1BB plays a critical role in protecting NKG2D from TGF-β1-mediated down-modulation.

Experiments with blocking antibodies revealed that NKG2D engagement contributed to cytolytic activity only during later stages of the infection when MHC class I expression was down-regulated and the MIC proteins were up-regulated. Lytic activity absolutely required the T cell receptor, and NKG2D provided a co-stimulatory signal. Same T cell clones secreted increased levels of cytokines in response to their target antigens when simultaneous NKG2D engagement occurred. Co-stimulation was also observed in mouse macrophages (Diefenbach et al. 2000; Groh et al. 2001). Transfectants expressing H-60 or Rae-1ß were used to stimulate activated macrophages. TNF-α transcription and nitric oxide production were enhanced in response to the signal, but only in the presence of LPS, which also provided an activating signal to macrophages (Ehrlich et al. 2005). NKG2D functions as a prototypic costimulatory receptor in a subset of human cytomegalovirus (HCMV)-specific $CD4^+$ T lymphocytes and thus may have a role in the response against infected HLA class II^+ cells displaying NKG2D ligands (Saez-Borderias et al. 2006).

31.6.3 NKG2D in Cytokine Production

The activating signal of NKG2D can also elicit cytokine production. NK cell production of MIP-1ß, TNF-α and IFN-γ was enhanced upon treatment with soluble ULBP (Kubin et al. 2001). A much more striking effect of ULBP was observed when IL-12 was included in the culture. Similarly, HCMV-specific γδ T cells produced IFN-γ, TNF-α, IL-2, and IL-4 when exposed to target antigen presented by C1R cells. Cytokine secretion was strongly enhanced if the C1R cells were transfected with MICA (Groh et al. 2001).

It was shown that dendritic epidermal T cells (DETCs), also known to express NKG2D, constitutively express NKG2D-S, NKG2D-L, DAP10, and DAP12 transcripts as

well as cell surface NKG2D protein. Blocking of NKG2D inhibited DETC-mediated cytotoxicity against target cells that do not express T cell receptor ligands. Cross-linking of NKG2D on DETCs induced IFNγ production. Thus, DETCs constitutively express NKG2D that acts as a primary activating receptor, and plays important role in cutaneous immune surveillance (Nitahara et al. 2006). Chan et al. (2006) described a third DC lineage, termed interferon-producing killer DCs (IKDCs), distinct from conventional DCs and plasmacytoid DCs. They produce substantial amounts of type I IFN and IL-12 or IFNγ, depending on activation stimuli. Upon stimulation with CpG oligodeoxy-nucleotides, ligands for Toll-like receptor (TLR)-9, IKDCs kill typical NK target cells using NK-activating receptors. Their cytolytic capacity subsequently diminishes, associated with the loss of NKG2D receptor and its adaptors, DAP10 and DAP12. By virtue of their capacity to kill target cells, followed by antigen presentation, IKDCs provide a link between innate and adaptive immunity (Chan et al. 2006).

31.6.4 Heterogeneity of NK Cells in Umbilical Cord Blood

NK cells from umbilical cord blood (CB) play an important role in allogeneic stem cell transplantation and defending infections of newborn. The proportion of NK cells is high in CB, but the IFN-γ production low compared to later part in life. In contrast, the proportion of T cells is low in CB, an observation that indicates a deviation of the regulatory function of NK cells in CB compared to later in life. Additionally, the level of expression of NKG2D, NKp30 and NKp46 decreased with age. These age related changes in NK cell populations defined by the expression of activating and inhibitory receptors may be the result of pathogen exposure and/or a continuation of the maturation process that begins in the bone marrow (Sundström et al. 2007). Human NK cells from cord blood are the key effector cells involved in graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL). The activity of CB NK cells was shown to be lower than that of adult peripheral blood (PB) NK cells. Though, the expression of activating NK receptors, CD16, NKG2D and NKp46 did not show significant difference between CB and PB NK cells, the expression of inhibitory receptor NKG2A/CD94 was significantly higher on CB NK cells. The high expression of NKG2A/CD94 and low expression of granzyme B may be related with the reduced activity of CB NK cells (Wang et al. 2007).

31.7 Cytotoxic Effector Function and Tumor Immune Surveillance

31.7.1 Anti-Tumor Activity

Recent advances clearly implicate that NKG2D recognition plays an important role in tumor immune surveillance and that NKG2D primarily acts to trigger perforin-mediated apoptosis. NKG2D-mediated tumor rejection was demonstrated in a murine system using the tumor-forming RMA cell line (Cerwenka et al. 2001). RMA transfectants expressing Rae-1 proteins were rejected. In vivo depletion experiments demonstrated that tumor rejection was mediated by NK cells, and studies in CD1 deficient mice demonstrated that CD1-restricted NKT cells were not involved in the rejection. A second report using similar methods confirmed that ectopic expression of the NKG2D ligands, Rae-1ß or H60, in several tumor cell lines resulted in potent rejection of the tumor cells by syngeneic mice (Diefenbach et al. 2001). Cell depletion experiments demonstrate that both NK cells and $CD8^+$ T cells are involved in this rejection. These authors also report a potent immune memory in contrast to observation of Cerwenka et al. (2001). NKG2D ligands might be useful in the design of tumor vaccines. Though the ligands for the NKG2D stimulatory receptor are frequently upregulated on tumors, rendering them sensitive to NK cells killing, Guerra et al. (2008) provided the evidence in NKG2D-deficient mice that NKG2D was not necessary for NK cell development but was critical for immunosurveillance of epithelial and lymphoid malignancies in transgenic models. These findings provided important genetic evidence for surveillance of primary tumors by an NK cell receptor.

The importance of the NKG2D pathway was further illustrated in mice deficient for either IFNγ or TNF-related apoptosis-inducing ligand, whereas mice depleted of NK cells, T cells, or deficient for perforin did not display any detectable NKG2D phenotype. Furthermore, IL-12 therapy preventing MCA-induced sarcoma formation was also largely dependent on the NKG2D pathway. The data begin to place the NKG2D pathway into the context of other recognition-effector systems used by NK cells (Hayakawa and Smyth 2006a, b). Ewing sarcoma (EWS) cells are potentially susceptible to NK cell cytotoxicity due to the expression of activating NK cell receptor ligands. The use of cytokine-activated NK cells rather than resting NK cells in immunotherapy may be instrumental to optimize NK cell reactivity to EWS (Verhoeven et al. 2008; von Strandmann et al. 2006).

Tumor-Derived Exosomes Down-Modulate NKG2D Expression: Soluble NKG2D ligands and growth factors, such as TGFβ1 emanating from tumors, are mechanisms for down-regulating NKG2D expression. Cancers thereby impair the capacity of lymphocytes to recognize and destroy them (Groh et al. 2002). Exosomes derived from cancer cells express ligands for NKG2D and express TGF β1. Exosomes produced by various cancer cell lines, or by mesothelioma patients triggered down-regulation of surface NKG2D expression by NK cells and $CD8^+$ T cells. Other markers remained unchanged, indicating the selectivity and non-activatory nature of the response. Exosomal NKG2D ligands were partially responsible for this effect. Exosomally expressed TGF β1 was the principal mechanism. NKG2D is a likely physiological target for exosome-mediated immune evasion in cancer (Clayton et al. 2008).

NKR-P2, the rat orthologue of human NKG2D, is a potent activation receptor on rat DCs. A potential agonistic anti-NKR-P2 mAb (1A6) mimics the NKR-P2 ligand and induces activation and maturation of DCs. Interaction of DCs with 1A6 enhances nitric oxide-mediated apoptosis in tumor cells. Cross-linking of NKR-P2 with 1A6 up-regulates MHC II and decreases endocytic activity of DC, thus suggeting a pivotal role in adaptive immune responses. Blocking of 1A6-mediated activation and maturation with inhibitors of PI3K, p38K and ERK1/2K suggests the involvement of MAP kinase in signal transduction. 1A6 cross-linking activates NF-kB, which acts as key executioner of DC activation (Srivastava et al. 2007).

NK Cell-Mediated Cytotoxicity in Human Lung Adenocarcinoma: Although adenocarcinoma (ADC) cells express heterogeneous levels of NKG2DLs, they are often resistant to NK cell-mediated killing. Resistance of ADC-Coco to allogeneic polyclonal NK cells and autologous NK cell clones are correlated with shedding of NKG2DLs resulting from a matrix metalloproteinase (MMP) production. Treatment of ADC-Coco cells with a MMP inhibitor (MMPI) combined with IL-15 stimulation of autologous NK cell clones lead to a potentiation of NK cell-mediated cytotoxicity. This lysis is mainly NKG2D mediated, since it is abrogated by anti-NKG2D-neutralizing mAb. These results suggest that MMPIs, in combination with IL-15, may be useful for overcoming tumor cell escape from the innate immune response (Le Maux Chansac et al. 2008).

31.7.2 Immune Evasion Mechanisms

Acute Myeloid Leukemia (AML): Little is known regarding the regulation of NKp46 and NKG2D expression in AML. Mononuclear and polymorphonuclear phagocytes down-regulate the cell surface density of NKp46 and NKG2D on NK cells with $CD56^{dim}$ phenotype in vitro by a mechanism that is dependent on the availability of phagocyte-derived reactive oxygen species (ROS). Histamine maintained NKp46 and NKG2D expression despite the presence of inhibitory phagocytes by targeting an H2 receptor on phagocytes. By contrast, NKp46 and NKG2D expression by the $CD56^{bright}$ subset of NK cells was resistant to inhibition by phagocytes. These findings are suggestive of a mechanism of relevance to the regulation of NKp46/NKG2D receptor expression (Romero et al. 2006).

Malignant blood cells in AML display low levels of ligands for NKG2D and can thus evade NK immunosurveillance. Rohner et al. (2007) attempted to up-regulate NKG2D-L ULBP using anti-neoplastic compounds with myeloid differentiation potential. Combinations of 5-aza-2′-deoxycytidine, trichostatin A, vitamin D3, bryostatin-1, and all-trans-retinoic acid, used together with myeloid growth factors and IFNγ, increased cell surface ULBP expression in the AML cell line HL60 and in primary AML blasts. Up-regulation of ULBP ligands was associated with induction of myelomonocytic differentiation of AML cells. Higher ULBP expression increased NKG2D-dependent sensitivity of HL60 cells to NK-mediated killing (Rohner et al. 2007).

Myelodysplastic Syndromes: (MDS) are characterized by ineffective hematopoiesis with potential for progression to acute myeloid leukemia (AML). Findings suggest that impairment of NK cytolytic function derives in part from reduced activating NK receptors such as NKG2D in association with disease progression. Evasion of NK immunosurveillance may have importance for MDS disease progression (Epling-Burnette et al. 2007).

Human Melanomas: Several human melanomas (cell lines and freshly isolated metastases) do not express MICA on the cell surface but have intracellular deposits of this NKG2DL. Fuertes et al. (2008) identified a strategy developed by melanoma cells to evade NK cell-mediated immune surveillance based on the intracellular sequestration of immature forms of MICA in the endoplasmic reticulum. The low expression of CD161 and activating NKG2D receptors, without increased expression of KIR receptors CD158a and CD158b, as well as a decrease in the cytotoxic, $CD16^{bright}$ NK cell subset, is associated with a significant impairment in NK cell activity in metastatic melanoma patients. Furthermore, the predictive pretherapy finding that IL-2, IFN, IFN and RA, unlike RA alone, can enhance NK cell activity of metastatic melanoma patients against FemX melanoma tumor cell line can be of help in the design and development of therapeutic regimens (Konjević et al. 2007).

Risk of Colorectal Cancer with the High NK Cell Activity NKG2D Genotype: Cancer patients with advanced disease display signs of immune suppression, which constitute a major obstacle for effective immunotherapy. Both T cells and NK cells are affected by a multitude of mechanisms of which the generation of reactive oxygen species is of major importance. Two weeks of high-dose treatment with the anti-oxidant vitamin E may enhance NK cell function in cancer patients by protecting from oxidative stress. Vitamin E treatment was associated with a minor, but consistent, induction of NKG2D expression in patients with colorectal cancer (Hanson et al. 2007; Melum et al. 2008).

It is reported that there are two haplotypes of NKG2D, HNK1 (high NK activity) and LNK1 (low NK activity). The HNK1 is reported to reduce the overall cancer risk. To elucidate its impact on colorectal cancer (CRC), Furue et al. (2008) found a reduced risk of CRC with the NKG2D HNK1. It was suggested that the HNK1 genotype, associated with high NK cell activity, might be an independent protective factor for CRC among the Japanese population.

Differing Phenotypes Between Intraepithelial and Stromal Lymphocytesin Tongue Cancer: The significance of tumor-infiltrating lymphocytes (TIL) has attracted much attention in relation to the prognosis of patients. The tumor nest-infiltrating (intraepithelial) $CD8^+$ T cells frequently expressed PD-1, an inhibitory receptor, in sharp contrast to those in the stroma or in the lichen planus. Conversely, the intraepithelial $CD8^+$ T cells only infrequently expressed NKG2D, an activating receptor, in contrast to those in the stroma or in the lichen planus. Furthermore, the intraepithelial NK cells expressed NKG2A, an inhibitory receptor, more frequently than those in the stroma or the lichen planus. Collectively, the intraepithelial $CD8^+$ T cells and NK cells are phenotypically inactivated, whereas stromal counterparts are phenotypically just as active as those in the lichen planus. These results suggest the first-step occurrence of an immune evasion mechanism in the tumor nest of oral squamous cell carcinoma (Katou et al. 2007). The expression of NKG2D on $CD8^+$ T lymphocytes from esophageal cancer patients was significantly lower than in those of normal controls. The decreased NKG2D expression on $CD8^+$ T cells was correlated with disease severity (Osaki et al. 2009).

Cholangiocarcinoma in Primary Sclerosing Cholangitis: Melum et al. (2008) investigated the influence of genetic variations in the NKG2D-MICA receptor-ligand pair on the risk of cholangiocarcinoma (CCA) in patients with primary sclerosing cholangitis (PSC). Seven SNPs covering the NKG2D gene were genotyped in Scandinavian PSC patients and controls. This suggested that genetic variants of the NKG2D receptor are associated with development of CCA in PSC patients and the interaction between NKG2D and MICA is involved in protection against CCA in PSC. Patients who are homozygous for the nonrisk alleles are unlikely to develop CCA; this finding could be helpful in identifying PSC patients with a low CCA risk.

Gastric Cancer: Some studies suggest that the NKG2D expression on $CD8^+$ T cells is down-regulated and this reduction may be involved in immune evasion in cancer patients. In gastric cancer patients, NKG2D expression on circulating $CD8^+$ T cells was down-regulated and significantly correlated with IFN-γ production in gastric cancer patients. There was no difference in soluble MICA between gastric cancer patients and normal controls. Decreased NKG2D expression may be one of the key factors responsible for immune evasion by tumors in gastric cancer (Osaki et al. 2007).

MIF in the Immune Escape of Ovarian Cancer: The proinflammatory cytokine macrophage migration inhibitory factor (MIF) stimulates tumor cell proliferation, migration, and metastasis; promotes tumor angiogenesis; suppresses p53-mediated apoptosis; and inhibits antitumor immunity by unknown mechanisms. Functionally, MIF may contribute to the immune escape of ovarian carcinoma by transcriptionally down-regulating NKG2D in vitro and in vivo which impairs NK cell cytotoxicity toward tumor cells. Perhaps inhibitors of MIF can be used for the treatment of MIF-secreting cancers (Krockenberger et al. 2008).

31.8 NKG2D in Immune Protection and Inflammatory Disorders

31.8.1 NKG2D Response to HCMV

Human cytomegalovirus (HCMV) infection is a paradigm of the complexity of host-pathogen interactions. To avoid recognition by cytotoxic T lymphocytes (CTL) HCMV inhibits the expression of HLA-I molecules. As a consequence, engagement of KIR, CD94/NKG2A, and CD85j (ILT2 or LIR-1) NKR specific for HLA-I molecules is impaired, and infected cells become vulnerable to an NK cell response driven by activating receptors. In addition to the well-defined role of the NKG2D, the involvement of other triggering receptors (i.e., activating KIR, CD94/NKG2C, NKp46, NKp44, and NKp30) in the response to HCMV is being explored (Gumá et al. 2006). To escape from NK cell-mediated surveillance, HCMV interferes with the expression of NKG2D ligands in infected cells. In addition, the virus may keep NK inhibitory receptors engaged preserving HLA-I molecules with a limited role in antigen presentation (i.e., HLA-E) or, alternatively, displaying class I surrogates. Despite considerable progress in the field, a number of issues

regarding the involvement of NKR in the innate immune response to HCMV remain uncertain.

31.8.2 HTLV-1-Associated Myelopathy

Human T cell lymphotropic virus type I (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic inflammatory disease of the CNS that resembles multiple sclerosis. Disease progression involves production of IL-15 and its receptor through transactivation. Substantial proportions of HAM/TSP patient CD4 T and CD8 cells are positive for NKG2D and both subsets express MIC. Engagement of MIC by NKG2D promotes spontaneous HAM/TSP T cell proliferation and, apparently, CTL activities against HTLV-1-infected T cells. Perhaps it is the viral strategy that exploits immune stimulatory mechanisms to maintain a balance between promotion and limitation of infected host T cell expansions (Azimi et al. 2006).

NKG2D in Clearance of Picornavirus from Infected Murine Brain: The role of NKG2D-mediated augmentation in the clearance of viral infections from CNS was explored using Theiler's murine encephalomyelitis virus model. Studies suggest that NKG2D-positive $CD8^+$ cytotoxic T cells enter the brain, and that NKG2D ligands are expressed in the brain during acute infection. The interruption of NKG2D ligand recognition via treatment with a blocking antibody attenuates the efficacy of viral clearance from the CNS (Deb and Howe 2008).

31.8.3 Protection Against Bacteria

The NKG2D-activating receptor interacts with ligands expressed on the surface of cells stressed by pathogenic and nonpathogenic stimuli. Lysis of bronchial epithelial cells is not MHC I restricted but depends on NKG2D signaling. It appears that respiratory epithelium has antigen presenting function and directly alloactivates cytotoxic $CD8^+$ T cells, which show nonclassical effector function (Kraetzel et al. 2008). In response to acute pulmonary *Pseudomonas aeruginosa* infection, the expression of mouse NKG2D ligands (Rae1) increased in airway epithelial cells and alveolar macrophages in vivo and also increased the cell surface expression of human ULBP2 on airway epithelial cells in vitro. NKG2D receptor blockade inhibited the pulmonary clearance of *P. aeruginosa* and also resulted in decreased production of Th1 cytokines and nitric oxide in the lungs of *P. aeruginosa*-infected mice. These results suggest the importance of NKG2D-mediated immune activation in the clearance of acute bacterial infection and that epithelial cell-lymphocyte interactions mediate pulmonary cytokine production and bacterial clearance (Borchers et al. 2006).

Expression of the activating receptors NKp30, NKp46, and NKG2D is enhanced on NK cells after exposure to *M. tuberculosis*-infected monocytes, whereas expression of DNAX accessory molecule-1 and 2B4 was not. Anti-NKG2D and anti-NKp46 inhibited NK cell lysis of M. tuberculosis-infected monocytes, but Abs to NKp30, DNAX accessory molecule-1, and 2B4 had no effect. Following infection of monocytes, the expression of the ULBP1, but not of ULBP2, ULBP3, or MICA/B was up-regulated. Therefore, NKp46 and NKG2D are the principal receptors involved in lysis of *M. tuberculosis*-infected mononuclear phagocytes, and that ULBP1 on infected cells is the major ligand for NKG2D. It appears that TLR2 contributes to up-regulation of ULBP1 expression (Vankayalapati et al. 2005).

31.8.4 Autoimmune Disorders

Studies have shown that NK-DC interaction plays an important role in the induction of immune response against tumors and certain viruses. The effect of this interaction is bidirectional. Coculture with NK cells causes several fold increase in IL-12 production by *Toxoplasma gondii* lysate Ag-pulsed DC. In vitro blockade of NKG2D neutralizes the NK cell-induced up-regulation of DC response. Results emphasize the critical role played by NKG2D in the NK-DC interaction (Guan et al. 2007). A role for NKG2D has been indicated in several autoimmune diseases in humans and in animal models of type 1 diabetes (T1DM) and multiple sclerosis, and treatment with monoclonal antibodies to NKG2D attenuated disease severity in these models.

NKG2D in Progression of Autoimmune Diabetes: RAE-1 is present in prediabetic pancreas islets of NOD mice and the autoreactive $CD8^+$ T cells infiltrating the pancreas express NKG2D. Treatment with a nondepleting anti-NKG2D monoclonal antibody (mAb) during the prediabetic stage completely prevented disease by impairing the expansion and function of autoreactive $CD8^+$ T cells. These results suggest that NKG2D is associated with disease progression and suggest a new therapeutic target for autoimmune type I diabetes (Ogasawara et al. 2004).

Rheumatoid Arthritis: Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis has been suggested by Groh et al. (2003). With the reported expansion of peripheral $CD4^+CD28^-NKG2D^+$ T cells in rheumatoid arthritis (RA), this interaction is important in triggering autoimmunity. But in studies of Schrambach et al. (2007), despite occasional and indiscriminate expansion of incriminated T cell subpopulation, no correlation could be observed between the $CD4^+CD28^-NKG2D^+$ T cells and auto-immunity.

Moreover, in situ, the presence of NKG2D matched that of $CD8^+$, but not that of $CD4^+$ T cells (Schrambach et al. 2007).

NKG2D Does Not Differ in Systemic Lupus Erythematosus: Schepis et al. (2009) studied NK cells in SLE, a B-cell-driven systemic autoimmune disease and found that $CD56^{bright}$ NK cells increase in SLE patients, regardless of disease activity, in association with some increased expression of NKp46/CD335 on NK cells. Other receptors on lymphocytes or NKRs, including CD94, NKG2C/CD159c, NKG2D/CD314, NKp30/CD337, NKp44/CD336, CD69 did not differ between patient groups.

Sjögren's Syndrome: Izumi et al. (2006) found that NK cell number, NK cell killing activity, and NKG2D were significantly decreased, and the expression of NKp46, as well as the percentage of apoptotic NK cells, were significantly increased in primary Sjögren's syndrome (SS) patients compared with healthy controls. Data suggested that reduced NK cell numbers, probably a result of apoptotic death, might contribute to impaired NK cell activity in patients with primary SS.

NKG2D and Its Ligands in Allograft Transplant Rejection: The early "danger" signals associated with transplantation lead to rapid up-regulation of NKG2D ligands. A second wave of NKG2D ligand up-regulation is mediated by the adaptive immune response to allografts. Treatment with an Ab to NKG2D was highly effective in preventing CD28-independent rejection of cardiac allografts. Notably, NKG2D blockade did not deplete $CD8^+$ T cells or $NK1.1^+$ cells nor affect their migration to the allografts. Results suggest a functional role of NKG2D and its ligands in the rejection of solid organ transplants (Kim et al. 2007; Zhang and Stastny 2006).

Role of CD45 for Cytokine and Chemokine Production from Killing in Primary NK Cells: Engagement of receptors on the surface of NK cells initiates a biochemical cascade ultimately triggering cytokine production and cytotoxicity. Huntington et al. (2005) investigated the role of cell surface phosphatase CD45 in NK cell development and intracellular signaling from activating receptors. Stimulation via MHC class I-binding receptor, Ly49D on $CD45^{-/-}$ primary NK cells resulted in the activation of phosphoinositide-3-kinase and normal cytotoxicity but failed to elicit a range of cytokines and chemokines. This blockage was associated with impaired phosphorylation of Syk, Vav1, JNK, and p38, and mimics results obtained using inhibitors of the src-family kinases (SFK). These data, supported by analogous findings after CD16 and NKG2D stimulation of $CD45^{-/-}$ primary NK cells, place CD45 upstream of SFK in NK cells after stimulation via immunoreceptor tyrosine-based activation motif-containing receptors. Thus CD45 is a pivotal enzyme in eliciting a subset of NK cell responses.

Interaction Between Human NK Cells and Stromal Cells: It appears that HLA-I molecules do not protect bone marrow stromal cells (BMSC) from NK cell-mediated injury. But NK cells, activated upon binding with BMSC, may regulate BMSC survival (Poggi et al. 2005). While the NK receptors NKp30 and NKp46 are responsible for the delivery of lethal hit to APC, the NKG2D-activating receptor, the MICA, and the ULBP are involved in stromal cell killing. These events are dependent on the activation of phosphoinositol 3-kinase and consequent release of perforins and granzymes. Altogether, studies support the notion that NK cells can recognize self-cells possibly affecting both APC function and interaction between lymphocytes and microenvironment leading to autoreactivity (Poggi et al. 2007). Activated T cells become susceptible to autologous NK lysis via NKG2D/NKG2DLs interaction and granule exocytosis, suggesting that NK lysis of T lymphocytes via NKG2D may be an additional mechanism to limit T-cell responses (Cerboni et al. 2007; Coudert and Held 2006; Poggi et al. 2005).

31.9 Decidual/Placental NK Cell Receptors in Pregnancy

Inhibitory and Activating Receptors on Decidual/Placenta NK Cells: The interaction of decidual KIRs with on HLA-C trophoblast cells appears to block NK cytotoxicity against trophoblast cells. NK cell receptors protect the trophoblasts and the outcome of pregnancy depends on women's NKRs. Peripheral blood NK cells express CD56 surface antigen in low ($CD56^{dim}$) or high ($CD56^{bright}$) density. In contrast to $CD56^{bright}$ NK cells, $CD56^{dim}$ cells express KIR such as CD158a, CD158b, and NKB1. Evidences indicate that $CD56^{bright}$ cells are specialized NK cells that regulate immunological response mechanisms rather by cytokine supply than by their cytotoxic potential. The poor cytolytic capacity of $CD56^{bright}$ NK cells can be explained by weak ability in forming conjugates with target cells and low contents of perforin and granzyme A in their granules (Jacobs et al. 2001). The balance of inhibitory and activating NKRs on maternal decidual NK cells, most of which are $CD56^{bright}$, is thought to be crucial for the proper growth of trophoblasts in placenta.

In Recurrent Spontaneous Abortions (RSA): Women with alloimmune abortions have a limited inhibiting KIR repertoire and such miscarriages could occur because

trophoblastic HLA class I molecules are recognized by decidual NK cells lacking the appropriate inhibitory KIRs (Varla-Leftherioti et al. 2003). Ntrivalas et al. (2005) suggested that CD158a and CD158b inhibitory receptor expression by $CD56^{dim}/CD16^{+}$ and $CD56^{bright}/CD16^{-}$ NK cells were significantly decreased, and CD161-activating receptor expression by $CD56^{+}/CD3^{+}$ NKT cells was significantly increased in women with implantation failures when compared with normal controls, suggesting an imbalance between inhibitory and activating receptor expression on NK cells of women with implantation failures. The expression of CD94/NKG2, the receptor for HLA-E, on trophoblasts, motivated Kusumi et al. (2006) to investigate the expression patterns of inhibitory receptor CD94/NKG2A, and activating receptor, CD94/NKG2C, on decidual NK cells in early stage of normal pregnancy and to compare them with those on peripheral NK cells, most of which are $CD56^{dim}$. The rate of NKG2A-positive cells was significantly higher for decidual $CD56^{bright}$ NK cells than for peripheral $CD56^{dim}$ NK cells, but the rates of NKG2C-positive cells were comparable between the two cell types. Interestingly, peripheral $CD56^{dim}$ NK cells reciprocally expressed inhibitory NKG2A and activating NKG2C, but decidual $CD56^{bright}$ NK cells that expressed activating NKG2C simultaneously expressed inhibitory NKG2A. The co-expression of inhibitory and activating NKG2 receptors may fine-tune the immunoregulatory functions of the decidual NK cells to control the trophoblast invasion in constructing placenta (Kusumi et al. 2006).

Down-Regulation of NKG2D on PBMC by MICA and MICB: A novel mechanism for fetal evasion of maternal immune attack, based on the down-regulation of the activating NKR NKG2D on peripheral blood mononuclear cells (PBMC) by soluble MHC class I chain-related proteins A and B has been proposed. A similar immune escape pathway has been described in tumors. The MIC mRNA is constitutively expressed by human placenta and restricted to the syncytiotrophoblast on the apical and basal cell membrane and in cytoplasmic vacuoles as MIC-loaded microvesicles/exosomes. Simultaneously, the cell surface NKG2D expression on maternal PBMC was down-regulated compared with nonpregnant controls. The soluble MIC molecules in pregnancy serum were shown to interact with NKG2D and down-regulate the receptor on PBMC from healthy donors, with the consequent inhibition of the NKG2D-dependent cytotoxic response. These results suggested a new physiological mechanism of silencing the maternal immune system that promotes fetal allograft immune escape and supports the view of the placenta as an immunoregulatory organ (Mincheva-Nilsson et al. 2006).

Decidual NK Cells Are Not Cytotoxic: In early pregnancy, invading fetal trophoblasts encounter abundant maternal decidual (d) dNK cells. dNK express perforin, granzymes A and B and the activating receptors NKp30, NKp44, NKp46, NKG2D, and 2B4 as well as LFA-1. Even though they express essential molecules required for lysis, fresh dNK displayed very low lytic activity on classical MHC I negative targets K562 and 721.221. dNK formed conjugates and activating immune synapses with 721.221 and K562 cells in which CD2, LFA-1 and actin were polarized toward the contact site. However, in contrast to peripheral NK cells, they failed to polarize their microtubule organizing centers and perforin-containing granules to the synapse, accounting for their lack of cytotoxicity (Kopcow et al. 2005).

31.10 Regulation of NKG2D Functions

31.10.1 NKG2D Induction by Chronic Exposure to NKG2D Ligand

It seems that signaling capacity of NKG2D receptor complexed with ligands is altered. The prolonged encounter with tumor cell-bound, but not soluble, ligand can completely uncouple the receptor from mobilization of $(Ca^{2+})_i$ and the exertion of cell-mediated cytolysis. However, cytolytic effector function is intact since NKG2D ligand-exposed NK cells can be activated via Ly49D receptor. While NKG2D-dependent cytotoxicity is impaired, prolonged ligand exposure results in constitutive IFNγ production, suggesting sustained signaling. The functional changes are associated with a reduced presence of the relevant signal transducing adaptors DAP-10 and KARAP/DAP-12 (Coudert et al. 2005). Coudert et al. (2008) found that sustained NKG2D engagement induces cross-tolerization of several unrelated NK cell activation receptors. It was observed that receptors that activate NK cells via the DAP12/KARAP and DAP10 signaling adaptors cross-tolerize preferentially NK cell activation pathways that function independent of DAP10/12, such as antibody-dependent CMC and missing-self recognition. These results identify NK cell activation receptors that can tolerize mature NK cells (Coudert et al. 2008).

Inhibitory Role of Ly49 Receptors on NKG2D: The inhibitory role of Ly49 receptors on NKG2D-mediated activation has only started emerging. Evidence suggests that NKG2D-mediated cytotoxicity and cytokine production result from the fine balance between activating and inhibitory receptors, thereby defining the NK cell-mediated immune responses (Regunathan et al. 2005). It is hypothesised that NKG2D-mediated activation is a function of 'altering the balance' in the signaling strength between the activating NKG2D and inhibiting Ly49 receptors. Balance in the signaling strength depends on the expression levels of activating ligands on the

target cells. Qualitative and quantitative variations of MHC class I molecules expressed on the target cells also plays a major role in determining this 'altered-balance'. Consequently, the nature of Ly49 receptors expressed on specific NK subsets determines the level of NKG2D-mediated NK cell activation. These observations provide a firm basis of 'altered-balance' in NK signaling and describe an active interplay between inhibitory Ly49 and activating NKG2D receptors (Malarkannan 2006; Regunathan et al. 2005)

31.10.2 Effects of Cytokines and Other Factors

Elevated TGF-β1 Down-Regulates NKG2D Surface Expression: Lee et al. (2004) showed that TGF-β1 secreted by tumors is responsible for down-regulating of NKG2D and poor NK lytic activity. The elevated plasma level of TGF-β1in human lung and colorectal cancer patients compared with in normal controls was inversely related with surface expression of NKG2D on NK cells in these patients. In a murine model of head and neck squamous cell carcinoma (SCC VII/SF), SCC VII/SF tumors expressed high levels of TGF-β1, which were down-modulated by vaccination with rvv-IL-2 (Dasgupta et al. 2005). TGF-β is known to play a major role for the reduced expression of NKG2D in cancer patients. The protected expression of NKG2D by IL-2/IL-18 provides insight into the mechanism of NKG2D regulation (Song et al. 2006). An inverse correlation does exist between NK cell activation and $CD4^+CD25^+$ regulatory T (T_{reg}) cells expansion in tumor-bearing patients. Results indicated that T_{reg} cells expressed membrane-bound TGF-β, which directly inhibited NK cell effector functions and down-regulated NKG2D receptors on the NK cell surface. Thus T_{reg} cells generated during tumor growth can blunt the NK cell functions of the innate immune system (Ghiringhelli et al. 2005).

Interleukin-15 stimulates NKG2D: IL-15 is the major physiologic growth factor responsible for NK cell differentiation, survival and cytolytic activity of mature NK cells. The IL-15 treatment increases NKG2D transcripts and surface expression in NK cells. The up-regulatory effect of IL-15 on NK cytolysis is partly dependent on the interaction of NKG2D and MICA (Zhang et al. 2008b). IL-15 activates peripheral blood mononuclear cells to lyse *Cryptosporidium*-infected epithelial cells. Flow cytometry revealed that IL-15 increased expression of NKG2D on NK cells. Thus, IL-15 has an important role in activating an NK cell-mediated pathway that leads to the elimination of intracellular protozoans from the intestines (Dann et al. 2005). Results also indicate that the pro-inflammatory cytokines (IL-2, IL-15 and IL-21) can modify the peripheral repertoire of NK cells (de Rham et al. 2007).

Interleukin-12, 18 and 21: IL-12 improves cytotoxicity of NK cells and expression of NKG2D. IL-12, produced primarily by APCs, plays an important role in the interaction between the innate and adaptive arms of immunity acting upon T and NK cells to generate CTLs. Ortaldo et al. (2006) demonstrated that cross-linking of NKG2D and NK1.1 results in a synergistic NK IFN- IFNγ response when combined with IL-12 or IL-18. Multiple convergent signals maximize the innate immune response by triggering complementary biochemical signaling pathways (Ortaldo et al. 2006; Zhang et al. 2008a).

IL-21 mediates its biological effects via the IL-21R in conjunction with the common receptor γ-chain that is also shared by members of the IL-2 family. Human primary NK and $CD8^+$ T cells with IL-21 in combination with IL-2- results in significant reduction of surface expression of NKG2D, compared with that in cells treated with IL-2 alone. This was attributed to a dramatic reduction in DAP10 promoter activity. In contrast to NKG2D expression, IL-21 was able to induce the expression of the NK activation receptors NKp30 and 2B4 as well as the co-stimulatory receptor CD28 on $CD8^+$ T cells (Burgess et al. 2006). IL-21 has a synergistic effect by increasing the numbers of NK cells on a large scale. IL-2 and IL-15 may induce the expression of KIRs, the NKG2D and NKp31. Findings indicated that the pro-inflammatory cytokines IL-21 and others (IL-2 and IL-15) can modify the peripheral repertoire of NK cells (de Rham et al. 2007). IL-21 can promote the anti-tumor responses of the innate and adaptive immune system. Mice treated with IL-21 reject tumor cells more efficiently, and a higher percentage of mice remain tumor-free compared with untreated controls. In certain tumor models, IL-21-enhanced tumor rejection is NKG2D dependent. IL-21 therapy may work optimally against tumors that can elicit a NKG2D-mediated immune response (Takaki et al. 2005).

Other Factors: NKG2D, expressed on γδ T cells participates in demyelination since blockade of NKG2D interactions in vivo resulted in 60% reduction in demyelination (Dandekar et al. 2005). All-trans retinoic acid (ATRA), a metabolite of vitamin A plays an important role in regulating immune responses. ATRA suppressed cytotoxic activities of NK92 cells without affecting their proliferation. It suppressed NF-kB activity and prevented IkBα degradation in a dose-dependent way, inhibited IFN-γ production and gene expression of granzyme B and NKp46 (Li et al. 2007). Activation of NK cells is triggered by multiple receptors. SLP-76 is required for CD16- and NKG2D-mediated NK cell cytotoxicity, while MIST negatively regulates these responses in an SLP-76-dependent manner (Hidano et al. 2008). Tryptophan (Trp) catabolism mediated by indoleamine 2,3-dioxygenase (IDO) plays a central role in the regulation of T-cell-mediated

immune responses and NK-cell functions. The effect of l-kynurenine appears to be restricted to NKp46 and NKG2D, while it does not affect other surface receptors such as NKp30 or CD16 (Della Chiesa et al. 2006).

31.11 Role in Immunotherapy

Requirement of DNAM-1, NKG2D, and NKp46 in NK Cell-Mediated Killing of Myeloma Cells: Ovarian carcinoma cells display ubiquitous expression of DNAX accessory molecule-1 (DNAM-1) ligand PVR and sparse/heterogeneous expression of MICA/MICB and ULBP1, ULBP2, and ULBP3. In line with the NK receptor ligand expression profiles, antibody-mediated blockade of activating receptor pathways revealed a dominant role for DNAM-1 and a complementary contribution of NKG2D signaling in tumor cell recognition. Thus, resting NK cells are capable of directly recognizing ovarian carcinoma as a potential target for adoptive NK cell-based immunotherapy (Carlsten et al. 2007). DNAM-1 is also important in the NK cell-mediated killing of myeloma cells expressing the cognate ligands (El-Sherbiny et al. 2007). Heat shock proteins (HSPs) are known activate both adaptive and innate immune responses. Vaccination with autologous tumor-derived HSP96 of colorectal cancer patients, radically resected for liver metastases, induced a significant boost of NK activity detected as cytokine secretion and cytotoxicity in the presence of NK-sensitive targets. Increased NK activity was associated with an enhanced expression of NKG2D and/or NKp46 receptors (Pilla et al. 2005).

Immunotherapy with Chimeric NKG2D Receptors: Adoptive transfer of tumor-reactive T cells is a promising antitumor therapy for many cancers. Adoptive transfer of selected populations of alloreactive HLA class I-mismatched NK cells in combination with pharmacologic induction of NKG2D-ligands merits clinical evaluation as a new approach to immunotherapy of human acute myeloid leukemia (AML) (Diermayr et al. 2008). Barber et al. designed a chimeric receptor linking NKG2D to the CD3ζ chain of the T-cell receptor to target ovarian tumor cells. More than 80% of primary human ovarian cancer samples express ligands for NKG2D on the cell surface. Engagement of chimeric NKG2D receptors (chNKG2D) with ligands for NKG2D leads to T-cell secretion of proinflammatory cytokines and tumor cytotoxicity. ChNKG2D-expressing T cells lysed ovarian cancer cell lines. T cells from ovarian cancer patients that express chNKG2D secrete proinflammatory cytokines when cultured with autologous tumor cells. chNKG2D T cells can be used therapeutically in a murine model of ovarian cancer and offer potential application as immunotherapy for ovarian cancer (Barber et al. 2007). Most myeloma tumor cells express NKG2D ligands and respond to human chNKG2D T cells. The chNKG2D T cells can be generated with serum-free media, and maintained effector functions on cryopreservation (Barber et al. 2008a). The chNKG2D T-cell-mediated therapeutic effects were mediated by both cytokine-dependent and cytotoxic mechanisms in vivo (Zhang et al. 2007). Specifically, chNKG2D T cell expression of perforin, GM-CSF, and IFN-γ were essential for complete antitumor efficacy (Barber et al. 2008b).

Regulatory T Cells Suppress NK Cell-Mediated Immunotherapy: $CD4^+CD25^+$ regulatory T cells (Treg) that suppress T cell-mediated immune responses may also regulate other arms of an effective immune response. In particular, T_{reg} directly inhibit NKG2D-mediated NK cell cytotoxicity in vitro and in vivo, effectively suppressing NK cell-mediated tumor rejection. In vitro, T_{reg} were shown to inhibit NKG2D-mediated cytolysis largely by a TGF-β-dependent mechanism and independently of IL-10. Adoptively transferred T_{reg} suppressed NK cell antimetastatic function in RAG-1-deficient mice. Depletion of T_{reg} before NK cell activation via NKG2D and the activating IL-12 cytokine dramatically enhanced NK cell-mediated suppression of tumor growth and metastases. The study shows at least one mechanism by which T_{reg} can suppress NK cell antitumor activity and requirement of subsequent NK cell activation to promote strong innate anti-tumor immunity (Smyth et al. 2006; Roy et al. 2008).

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KLRC4, KLRG1, and Natural Cytotoxicity Receptors

32

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In earlier Chapters we discussed mouse and human NK cell lectin receptors which can deliver inhibitory or activatory NK cell cytotoxicity in presence or absence of MHC-I molecules. Among non-lectin inhibitory receptors, killer immunoglobulin (Ig)-like receptors (KIRs) recognize different allelic groups of HLA-A, -B or -C molecules. Immunoglobulin-like transcript 2 (ILT2 or LIR1 or CD85j) receptors are more 'promiscuous', as they recognize a large number of HLA class I alleles, while CRD containing CD94-NKG2A recognizes HLA-E, an HLA class I molecule with a limited polymorphism. It is well known that various HLA class I alleles provide signal sequence peptides that bind HLA-E and enable it to be expressed on the cell surface. Importantly, each type of KIR is expressed only by a subset of NK cells (Braud et al. 1998). A common characteristic of the various HLA class I-specific inhibitory receptors is the presence, in their cytoplasmic tail, of ITIM that enable them to recruit and activate SHP-1 and SHP-2-phosphatases (Moretta et al. 2001; Lanier 1998). In turn, these phosphatases switch off the activating signaling cascade initiated by the various activating receptors (Moretta and Moretta 2004). In this chapter we continue discussing cytotoxic receptors which do not depend on HLA antigens for their action and do not contain ITIM motif in their cytoplasmic tail as well as activating NK cell receptors which are of recent origin but do not contain lectin-like domain till date.

32.1 Killer Cell Lectin-Like Receptor F-1 (NKG2F/KLRC4/*CLEC5C*/NKp80)

32.1.1 NKp80 or Killer Cell Lectin-Like Receptor Subfamily F-Member 1 (KLRF1)

Killer cell lectin-like receptor subfamily F-member 1 (KLRF1), also known as KLRC4 or NKp80, is a human gene which is localized 25 kb from NKG2-A (Roda-Navarro et al. 2000) and related to NKG2-D cDNA on the high-resolution physical map of chromosome 12p. It encodes a putative type II transmembrane glycoprotein. The 3′ end of NKp80/KLRC4/KLRF1 transcript includes the first non-coding exon found at the 5′ end of the adjacent D12S2489E gene transcript (Renedo et al. 2000). The genomic structure of the NKp80/KLRF1 gene and the existence of one spliced variant have been described. It is expressed at the mRNA level in peripheral blood leukocytes, activated NK cells, monocytes and NK and myeloid cell lines. NKp80, a C-type lectin-like receptor, stimulates the cytotoxicity of NK cells and releases cytokine. Roda-Navarro et al. described the existence of one alternative spliced form, lacking the stalk region of the extracellular domain and two novel KLRF1 alternative spliced variants coding for truncated proteins lacking the C-type lectin-like domain (Plougastel and Trowsdale 1998a, b; Roda-Navarro et al. 2001). A conserved 24-amino acid sequence, present in all members of the NKG2 family, suggests that NKG2-F is also able to form heterodimers with CD94 (Plougastel and Trowsdale 1998a, b).

NKp80 is a type II transmembrane protein of 231 amino acids identical to the putative protein encoded by a cDNA termed KLRF1 (Vitale et al. 2001) (Fig. 32.1). NKp80 is protein with a C-type lectin domain in its extracellular region. NKp80 is expressed at the cell surface as a dimer of ~80 kDa on virtually all NK cells and by a minor subset of T cells characterized by the CD56 surface antigen and shown to function as a co-receptor rather than as true receptor with natural cytotoxicity receptors (NCR) to induce activation of NK cell-mediated cytotoxicity. NKp80 surface expression was also detected in all $CD3^-$ and in 6/10 $CD3^+$ large granular lymphocyte expansions derived from patients with lympho-proliferative disease of granular lymphocytes. mAb-mediated cross-linking of NKp80 resulted in induction of cytolytic activity and Ca^{2+} mobilization. The NKp80 recognizes a ligand on normal T cells that may be down-regulated during tumor transformation. Indeed, it induces natural cytotoxicity only when co-engaged with a triggering receptor (Vitale et al. 2001). NK cells in monkeys were generally identified by negative selection of peripheral

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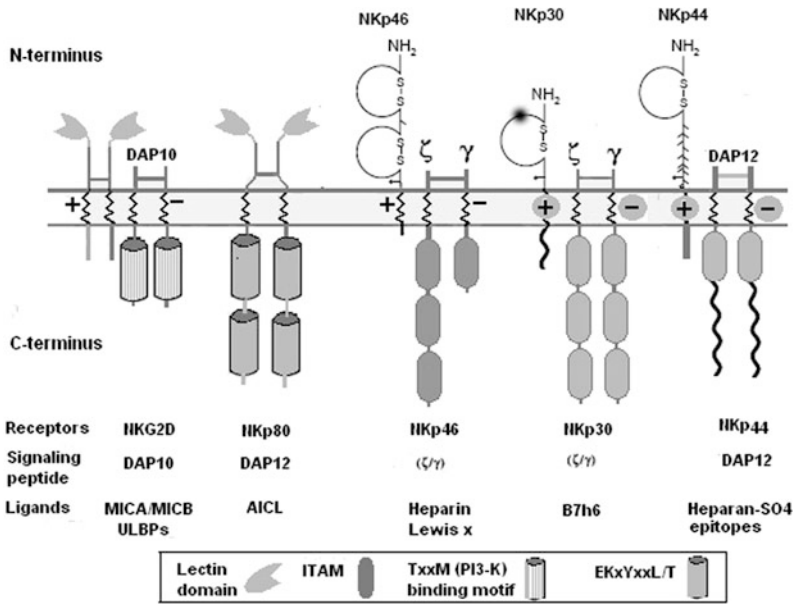

Fig. 32.1 Activating NK cell receptors and co-receptors and their cellular ligands. The Figure illustrates the molecular structure of the NK receptors NKG2D, NKp80, NKp46, NKp30, NKp44. Their interaction with signaling polypeptides or with relevant cytoplasmic molecules is also shown

blood mononuclear cells (PBMCs) for the absence of T-cell, B-cell, and monocyte markers.

32.1.1.1 Triggering Receptors in Sub-Human Primate NK Cells

Mavilio et al. (2005) indicated that mAb-mediated ligation of NKp80 induced NK cell cytotoxicity, while NKG2A inhibited the lysis of target cells by NK cells from macaques, as well as from humans. This phenotypic and functional characterization of NKG2A and NKp80 in rhesus and pig-tailed macaque NK cells is useful in the analysis of their innate immune system. Both M. fascicularis and M. mulatta NK cells express NKp80, which displayed a high degree of sequence homology with their human counterpart. Analysis of NK cells in simian HIV-infected M. fascicularis revealed reduced surface expression of selected NK cell-triggering receptors associated with a decreased NK cell function only in some animals (Biassoni et al. 2005). Functional characterization for NKp46, NKp30, NKp80, and NKG2D on Pan troglodytes NK cells revealed that, in this AIDS-resistant species, relevant differences to human NK cells involve NKp80 and particularly NKp30, which is primarily involved in NK-DC interactions (Rutjens et al. 2007).

32.1.2 NKp80/KLRF1 Associates with DAP12

The presence of two ITIM within the cytoplasmic tail of NKp80/KLRF1 suggests an inhibitory role in NK cell and monocyte activity. NKG2F/KLRF1 is an orphan gene within the NKG2 family whose translated product would contain both a positively charged residue in its transmembrane region, an intracellular ITIM-like sequence (YxxL) and an extracellular domain (62 residues) that is truncated relative to other NKG2 molecules. Expression of NKp80/KLRF1 appears to be confined to intracellular compartments probably due to its inability to associate with CD94. It can, however, associate with DAP12 thereby providing activation signaling potential. Since it was not possible to demonstrate phosphorylation of Tyr residue in the ITIM-like motif, it was suggested that it is a mock ITIM. NKG2F, a receptor component with an unidentified partner(s), can regulate cell activation through competition for DAP12 with other receptors, such as NKG2C and -E/H, or it could simply be a vestigial gene product (Kim et al. 2004). Using the NK cell line NK92MI, Dennehy et al. (2011) demonstrated that NKp80, but not NKp80 mutated at tyrosine 7 (NKp80/Y7F), is tyrosine phosphorylated. Accordingly, NKp80/Y7F, but not NKp80/Y30F or NKp80/Y37F, failed to induce cytotoxicity. Altogether, report suggests that NKp80 uses an atypical hemi-ITAM and Syk kinase to trigger cellular cytotoxicity (Fig. 32.1).

32.2 Activation-Induced C-Type Lectin (CLECSF2): Ligand for NKp80

Thomas et al. (2008) demonstrated that the ligand for NKp80 is the myeloid-specific CTLR activation-induced C-type lectin (AICL), which is encoded in the natural killer gene complex (NKC) adjacent to NKp80. Hamann et al. (1997) cloned a cDNA of a molecule, designated activation-induced C-type lectin (AICL), whose gene maps to the human NKC proximal to the CD69 gene. AICL is a myeloid-specific activating orphan receptor that is upregulated by Toll-like receptor stimulation. AICL is a 149-amino acid polypeptide with a

short cytoplasmic tail of seven amino acids and a C-type lectin domain separated from the transmembrane region by only nine amino acids. The AICL shows highest sequence similarity to the domains of CD69 and the 17.5 chicken-like lectin. NKp80 binds to the genetically linked receptor AICL, which, like NKp80, is absent from rodents. AICL-NKp80 interactions promote NK cell-mediated cytolysis of malignant myeloid cells. In addition, during crosstalk between NK cells and monocytes, NKp80 stimulated the release of proinflammatory cytokines from both cell types. Thus, NKp80-AICL interactions may contribute to the initiation and maintenance of immune responses at sites of inflammation (Welte et al. 2006). The Kaposi's sarcoma-associated herpesvirus (KSHV) immune evasion gene, K5, reduces cell surface expression of AICL. Down-regulation of AICL requires the ubiquitin E3 ligase activity of K5 to target substrate cytoplasmic tail lysine residues. The down-regulation of ligands for both the NKG2D and NKp80 activation pathways provides KSHV with a powerful mechanism for evasion of NK cell antiviral functions (Thomas et al. 2008).

The AICL is also expressed on a minor fraction of human $CD8^+$ T cells that exhibit a high responsiveness and an effector memory phenotype. The $NKp80^+$ T-cell subset is characterized by the co-expression of other NK receptors and NKp80 ligation augmentes CD3-stimulated degranulation and IFNγ secretion by effector memory T cells. Hence, NKp80 may enable effector memory CD8 T cells to interact functionally with cells of myeloid origin at sites of inflammation (Kuttruff et al. 2009).

32.3 KLRG1 (Rat MAFA/CLEC15A or Mouse 2F1-Ag)

32.3.1 Mast Cell Function-Associated Antigen (MAFA)

The mAb G63 identified a membrane component of mast cells, called as mast cell function-associated antigen (MAFA). Mast cell antigen is a glycoprotein with an M_r of 28–40 kDa, and is present on the surface of rat mucosal and serosal mast cells. Its density on cells of the mucosal mast cell line RBL-2H3 is $1–2 \times 10^4$ copies per cell. Cross-linking of this protein by mAb G63 results in the inhibition of Fc ε RI-mediated secretion of RBL-2H3 cells and inhibits biochemical processes initiated by FcεRI aggregation, such as the hydrolysis of phosphatidylinositides, the influx of Ca^{2+} ions, and the synthesis and release of inflammatory mediators and in redistribution of the membrane component recognized by G63 (Ortega et al. 1991). Based on MAFA density the rat mucosal mast cell line, RBL-2H3, can be classified into two subpopulations: cells carrying either high or low density of a MAFA (Cohen-Dayag et al. 1992). The MAFA is expressed at a ratio of approximately 1:30 with respect to the Type I FcεRI on rat mast cell line. Despite this stoichiometry, clustering MAFA by its specific mAb G63 substantially inhibits secretion of both granular and de novo synthesized mediators induced upon FcεRI aggregation. Since the FcεRIs apparently signal from within raft micro-environments, Barisas et al. (2007) investigated co-localization of MAFA within these membrane compartments containing aggregated FcεRI and suggested for constitutive interactions between FcεRI and MAFA.

32.3.2 MAFA Is a Lectin

The recombinant MAFA (rMAFA) was expressed as a monomeric and disulfide-linked homodimeric glycoprotein in the membrane of insect cells. The rMAFA binds specifically the terminal mannose residues in a Ca^{2+}-dependent manner. Results support the notion that the extracellular domain of the MAFA is able to bind ligands, which may be modulatory for the mast cell response (Binsack and Pecht 1997). The MAFA interferes with the coupling cascade of the type 1 FcεR upstream to PLCγ1 activation by protein-tyrosine kinases. The MAFA cDNA shows that MAFA contains a single ORF, encoding a 188-amino acid-long type II integral membrane protein. The 114C-terminal amino acids display sequence homology with the CRD of calcium-dependent animal lectins. The cytoplasmic tail of MAFA contains a YXXL (YSTL) motif, which is conserved among related C-type lectins and is an ITIM. Changes in the MAFA tyrosyl- and seryl-phosphorylation levels are observed in response to binding of mAb G63, antigenic stimulation, and a combination of both treatments (Guthmann et al. 1995b). The high conservation of cysteinyl residues suggests an important role for intrachain disulfide bonds in attaining its structure and biological activity. The MAFA clustering by G63 inhibits the de novo synthesis and secretion of IL-6 induced by the FcεRI stimulus. Experiments suggested that MAFA might have a role in cell adhesion in addition to its immunomodulatory capacity (Guthmann et al. 1995a).

32.3.3 Interactions of MAFA with FcεR

The nature of MAFA-FcεRI interactions giving rise to this inhibition remains unclear. By time-resolved phosphorescence anisotropy, Song et al. (2002) studied the rotational behavior of both MAFA and FcεRI as ligated by various reagents involved in FcεRI -induced degranulation and MAFA-mediated inhibition there of. Clustering the FcεRI-IgE complex by antigen or by anti-IgE increases the

phosphorescence anisotropy of G63 Fab and slows its rotational relaxation. Studies indicated that unperturbed MAFA associates with clustered FcεRI (Song et al. 2002).

Clustering of MAFA Induces Tyrosyl Phosphorylation of the FcεRI-β Subunit: Clustering of MAFA on the surface of rat mucosal mast cells RBL-2H3 leads to suppression of the secretory response induced by FcεRI. The MAFA is located in the vicinity of the FcεRI on resting cells, and clustering of Fcε RI leads to no significant change in the proximity of the two molecular species. Reports suggest that the secretory response inhibition by MAFA interferes with the signal transduction cascade initiated via the FcεRI (Jürgens et al. 1996). The inhibition was found to take place upstream to the production of inositol phosphates and the transient increase in free cytosolic Ca^{2+} ion concentration. Hence it probably interferes with the cascade at the level of the protein tyrosyl kinases (PTK) activity (Schweitzer-Stenner et al. 1999).

Studies indicate that MAFA's inhibitory action involves at least two different enzymes: Following the tyrosyl-phosphorylation of MAFA ITIM by the PTK Lyn, two phosphatases SHIP and SHP2 are recruited at the plasma membrane where they propagate the inhibitory signals. The inhibition has been shown to take place upstream to the production step of inositol phosphates in the FcεRI coupling cascade. Syk tyrosine phosphorylation and activation, as well as LAT (linker for activation of T cells) tyrosine phosphorylation, both induced by FcεRI clustering, were found to be reduced upon MAFA clustering. In contrast, the activity of the SH2-containing protein tyrosine phosphatase (SHP-2) increased. Reports suggest that one possible mechanism by which MAFA affects the FcεRI stimulation cascade is suppression of Syk activity, i.e. MAFA clustering leads SHP-2 to act on Syk, thereby reducing its tyrosine phosphorylation and its activity (Abramson et al. 2002; Xu et al. 2001). MAFA has also a capacity of modulating the cell cycle of RBL-2H3 line and cell proliferation rate by inhibiting RasGTP formation in the Ras signaling pathway (Abramson et al. 2002; Licht et al. 2006).

Inhibition of FcεRI-Induced de Novo Synthesis of Mediators by MAFA: Aggregation of the type 1 Fc-ε-RI on mast cells initiates a network of biochemical processes culminating in secretion of both granule-stored and de novo-synthesized inflammatory mediators. A strict control of this response is obviously a necessity. MAFA selectively regulates the FcεRI stimulus–response coupling network and the subsequent de novo production and secretion of inflammatory mediators. Specifically, MAFA suppresses the PLC-γ2-[Ca^{2+}]i, Raf-1-Erk1/2, and PKC-p38 coupling pathways, while the Fyn-Gab2-mediated activation of PKB and Jnk is essentially unaffected. Hence, the activities of several transcription/nuclear factors for inflammatory mediators (NF-kB, NFAT) are markedly reduced, while those of others (Jun, Fos, Fra, p90rsk) are unaltered. This results in a selective inhibition of gene transcription of cytokines including IL-1beta, IL-4, IL-8, and IL-10, while that of TNF-alpha, MCP-1, IL-3, IL-5, or IL-13 remains unaffected. These results illustrate the capacity of an ITIF-containing receptor to cause tight and specific control of the production and secretion of inflammatory mediators by mast cells (Abramson et al. 2006).

32.3.4 MAFA Gene

MAFA is encoded by a single-copy gene that spans 13 kb in the rat genome and is composed of five exons. Three separate exons encode the CRD of the MAFA, defining its close homology to the genes of CD23, CD69, CD72, NKR-P1, and Ly49. Analysis of the 5′ flanking region of the gene reveals that a cell type-specific promoter is located within the first 664 bp upstream of the transcription origin. The promoter lacks any obvious TATA box and drives gene transcription originating from multiple start sites. Examination for possible polymorphism of the MAFA transcripts revealed two transcripts, generated by alternative splicing. The transcription of the MAFA gene was detected in normal rat lungs, where both transmembranal and soluble MAFA appear to be expressed. Lung immunohistochemical analysis further suggests that MAFA expression is restricted to mast cells.

32.4 KLRG1 (OR 2F1-AG): A Mouse Homologue of MAFA

32.4.1 KLRG1: A Mouse Homologue of MAFA

The KLRG1or formarly known as 2F1-AG or MAFA is expressed by NK cells and memory T cells in man and mice. A mAb (2F1), specific for mouse NK cells, recognized the mouse homolog of MAFA that is expressed on rat mast cells. The 2F1 antigen (2F1-Ag) and rat MAFA are highly conserved and contain a cytoplasmic ITIM used in inhibitory signaling. The human homologue is closely related to the rodent MAFA/2F1-Ag proteins. Like rat MAFA, 2F1-Ag is probably encoded by a single gene, which exhibits relatively little polymorphism. While rat MAFA is considered a mast cell antigen, it was not possible to detect cell surface expression of 2F1-Ag by mouse mast cell lines, bone marrow-derived mast cells, or peritoneal mast cells. However, mouse bone marrow-derived mast cells were devoid of 2F1-Ag mRNA. Instead, 40% of mouse NK cells express 2F1-Ag (Hanke et al. 1998).

The mouse MAFA (mMAFA) gene expression is strongly induced in effector $CD8^+$ T cells and lymphokine-activated NK cells but not in effector $CD4^+$ T cells and in mouse mast cells. Moreover, mMAFA gene expression was only found in effector $CD8^+$ T cells that had been primed in vivo with live virus because in vitro activated $CD8^+$ T cells did not express mMAFA. Sequence comparison revealed a high degree of conservation (89% similarity) between rat MAFA and mMAFA (Blaser et al. 1998). Using a mAb (2F1), Corral et al. (2000) characterized a mouse NK cell KLRG. KLRG1 is expressed on 30–60% of murine NK cells, and a small fraction of T cells, and is composed of a homodimer of glycosylated 30–38-kDa subunits. Cell surface expression of KLRG1 by NK cells is down-regulated in mice deficient for expression of class I molecules, in contrast to the Ly49 lectin-like NK receptors, which are up-regulated in class I-deficient mice. KLRG1 does not bind class I molecules in a cell-cell adhesion assay. Transgenic expression of KLRG1 was unaffected by class I deficiency, indicating that class I molecules do not affect the KLRG1 protein directly, and suggesting that regulation is at the level of expression of the endogenous KLRG1 gene. Evidence showed that class I molecules regulate KLRG1 via interactions with inhibitory Ly49 molecules and SHP-1 signaling (Corral et al. 2000). The KLRG1 is an inhibitory C-type lectin expressed on NK cells and activated CD8 T cells. The mouse gene spans about 13 kb and consists of five exons. Short interspersed repeats of the B1 and B2 family, a LINE-1-like element, and a (CTT)170 triplet repeat were found in intron sequences. In contrast to human KLRG1 and to the murine KLR family members, mouse KLRG1 locates outside the NK complex on Chromosome 6 between the genes encoding CD9 and CD4 (Voehringer et al. 2001).

32.4.1.1 KLRG1 on Effector and Memory CD8 T Cells

KLRG1 was present on 1–3% of adult splenic CD8 cells that expressed CD8αβ heterodimers as well as a polyclonal TCR Vβ repertoire indicative of conventional CD8 cells and $CD8^+$ effector/memory cells that can secrete cytokines but have poor proliferative capacity. Spontaneous IFN-γ production by approximately 20% of $KLRG1^+$ CD8 cells identified them as pro-inflammatory effector cells. In contrast to NK cells, Ly49 and KLRG1 expression on CD8 cells was found to be mutually exclusive. KLRG1 triggering interferes with TCR αβ -mediated Ca^{2+} mobilization and cytotoxicity, raising the possibility that KLRG1 functionally participates in down-regulation of CD8 T cell responses (Beyersdorf et al. 2001). Furthermore, $CD8^+$ cells expressing CD57, a marker of replicative senescence, also expressed KLRG1 and a population of $CD57^-KLRG1^+$ cells has also been identified. The combination of KLRG1 and CD57 expression might thus aid in refining functional characterization of $CD8^+$ T cell subsets (Ibegbu et al. 2005). KLRG1 expression is dramatically induced on $CD8^+$ T cells during both a viral and a parasitic infection. The engagement of KLRG1 on a transfected NK cell line inhibits both cytokine production and NK cell-mediated cytotoxicity (Robbins et al. 2002, 2003).

The acquisition of the NK cell inhibitory markers NK1.1 and KLRG on T cells exposed to high numbers of DCs suggests a role for these molecules in the protection of antigen-responsive T cells from exhaustion by overstimulation. Using a mAb for human KLRG1, Voehringer et al. (2002) identified human T cells that are capable of secreting cytokines but fail to proliferate after stimulation. Furthermore, the lack of proliferative capacity of CD8 T cells correlates better with KLRG1 expression than with absence of the CD28 marker.

32.4.1.2 Characterization of Mouse CD4 T Cell Subsets Defined by Expression of KLRG1

In normal mice, while a polyclonal TCR repertoire suggests thymic origin of $KLRG1^+$ $CD4^+$ cells, KLRG1 expression was found to be restricted to peripheral $CD4^+$ T cells. Based on phenotypic analyses, a minority of $KLRG1^+$ $CD4^+$ cells are effector/memory cells with a proliferative history. The majority of $KLRG1^+$ $CD4^+$ cells are, however, bonafide Treg cells that depend on IL-2 and/or CD28 and express both FoxP3 and high levels of intracellular CD152. KLRG1 expression represents a distinctive subset of senescent effector/memory and potent regulatory $CD4^+$ T cells (Beyersdorf et al. 2007).

$CCR7^+$ central memory (T_{CM}) $CD4^+$ T cells play a central role in long-term immunological memory. Reports indicate that a proportion of $CD4^+$ T_{CM} is able to produce effector cytokines. Stubbe et al. (2008) characterized cytokine-producing human $CD4^+$ T_{CM} specific for cleared protein and persistent viral Ag. The type of Ag stimulation is the major determinant of $CD4^+$ T_{CM} differentiation. CMV-specific T_{CM} were significantly more differentiated than protein Ag-specific T_{CM} and included higher proportions of IFN-γ-producing cells. The expression of KLRG1 by protein Ag- and CMV-specific T_{CM} was associated with increased production of effector cytokines. $KLRG1^+$ T_{CM} expressing high levels of CD127 suggests that they can survive long term under the influence of IL-7 (Stubbe et al. 2008). Studies with hepatitis C virus (HCV)-specific $CD8^+$ T also demonstrated central memory ($CCR7^+$) and early-differentiated phenotypes of HCV-specific $CD8^+$ T cells (Bengsch et al. 2007).

32.4.1.3 KLRG1 in Cord Blood

Umbilical cord blood T cells in humans represent a homogenous pool of naive T cells. However, analysis of KLRG1 expression in cord blood revealed an unexpected heterogeneity of human T cells in newborns. A substantial subsets of

CD4 (30%) and CD8 (20%) αβ T cells in cord blood expressed KLRG1. In contrast to T cells in adult, $KLRG1^+$ T cells in cord blood exhibited predominantly a naive $CCR7^+CD45RA^+$ and $CD11a^{low}$ phenotype. After birth, KLRG1 expression in T cells from peripheral blood decreased rapidly to reappear in effector/memory T cells in adults. $KLRG1^+$ T cells in cord blood expressed a diverse T cell receptor β repertoire and the cells proliferated normally, in contrast to $KLRG1^+$ T cells from adults (Marcolino et al. 2004). In naive C57BL/6 mice KLRG1 is expressed on a subset of $CD44^{high}CD62L^{low}$ T cells. KLRG1 expression can be detected on a small number of Vα14*i* NK T cells but not on $CD8\alpha\alpha^+$ intraepithelial T cells that are either $TCR\gamma\delta^+$ or $TCR\alpha\beta^+$ (Eberl et al. 2005).

32.4.1.4 ER Stress Regulator XBP-1 Contributes to Effector CD8+ T Cell Differentiation During Acute Infection

The transcription factor X-box-binding protein-1 (XBP-1) plays an essential role in activating the unfolded protein response in the ER. The IRE-1/XBP-1 pathway is activated in effector $CD8^+$ T cells during acute infection. An XBP-1 splicing reporter was enriched in terminal effector cells expressing high levels of KLRG1. Over-expression of the spliced form of XBP-1 in $CD8^+$ T cells enhanced KLRG1 expression during infection, whereas XBP-$1^{-/-}$ $CD8^+$ T cells or cells expressing a dominant-negative form of XBP-1 showed a decreased proportion of $KLRG1^{high}$ effector cells. These results suggest that, in the response to pathogen, activation of ER stress sensors and XBP-1 splicing contribute to the differentiation of end-stage effector $CD8^+$ T cells (Kamimura and Bevan 2008).

32.4.1.5 Viral and Bacterial Infections Induce Expression of Multiple NK Cell Receptors in Responding $CD8^+$ T Cells

Viral infections are often accompanied by extensive proliferation of reactive CD8 T cells. NK cell receptors are also expressed by certain memory phenotype $CD8^+$ T cells, and in some cases are up-regulated in T cells responding to viral infection. The majority of pathogen-specific $CD8^+$ T cells initiated expression of the inhibitory CD94/NKG2A heterodimer, the KLRG1 receptor, and a murine NK cell marker (10D7); very few Ag-specific T cells expressed Ly49 family members. The up-regulation of these receptors was independent of IL-15 and persisted long after clearance of the pathogen. Thus, CD94/NKG2A expression is a common consequence of $CD8^+$ T cell activation (McMahon et al. 2002).

After a defined number of divisions, normal somatic cells enter a nonreplicative stage termed senescence. The KLRG1 is a unique marker for replicative senescence of murine CD8 T cells. KLRG1 expression is induced in a substantial portion (30–60%) of CD8 T cells in C57BL/6 mice infected with lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus, or vaccinia virus. Similarly, KLRG1 was found on a large fraction of LCMV gp33 peptide-specific TCR-transgenic (tg) effector and memory cells activated in vivo using an adoptive transfer model. Thus study demonstrates that senescent CD8 T cells are induced in abundant numbers during viral infections in vivo (Voehringer et al. 2001).

The frequency of $CD8^+$ T cells expressing KLRG1, unable to undergo further clonal expansion, was markedly elevated in $CD8^+$ T cells from old donors. Moreover, the elevated frequency of CMV-specific $CD8^+$ T cells in the elderly was due to an accumulation of cells bearing this dominant negative receptor. The fraction of CMV-specific T cells able to secrete IFN-γ after specific antigenic stimulation was significantly lower in the elderly than in the young, although the total number of functional cells was comparable. Therefore, the majority of the clonally expanded virus-specific $CD8^+$ cells in the elderly was dysfunctional. Thus, T cell responses are altered in the aged by an accumulation of replicatively senescent dysfunctional T cells carrying receptors for persistent herpes viruses (Ouyang et al. 2003).

The repetitive and persistent antigen stimulation leads to an increase in KLRG1 expression of virus-specific $CD8^+$ T cells in mice and that virus-specific $CD8^+$ T cells are mostly $KLRG1^+$ in chronic human viral infections (HIV, cytomegalovirus, and Epstein-Barr virus) while $CD8^+$ T cells targeting resolved viral antigens (influenza virus) typically display high CD127 and low KLRG1 expressions. Thus, by using KLRG1 as a T-cell marker, results suggest that the differentiation status and function of virus-specific $CD8^+$ T cells are directly influenced by persistent antigen stimulation (Thimme et al. 2005).

As a result of clonal expansion T lymphocytes, some T lymphocytes acquire a senescent phenotype, fail to replicate in response to further antigenic stimulation, and express KLRG1 and/or CD57. The T lymphocytes with a senescent phenotype are mobilized and subsequently removed from the bloodstream in response to acute high-intensity exercise (Simpson et al. 2007). There is a greater proportion of senescent $CD3^+/CD8^+$ T-lymphocytes in the blood of older adults compared to young at rest and immediately after exhaustive exercise, indicating that the greater frequency of $KLRG1^+$/CD8+ T-lymphocytes in older humans is ubiquitous and not localised to the peripheral blood (Simpson et al. 2008).

32.4.2 NK Cell Maturation and Homeostasis Is Linked to KLRG1 Up-Regulation

KLRG1 expression is acquired during periods of NK cell division such as development and homeostatic proliferation. $KLRG1^+$ NK cells are mature in phenotype, and these cells have a slower in vivo turnover rate, reduced proliferative

response to IL-15, and poorer homeostatic expansion potential compared with mature NK cells lacking KLRG1. Results indicate that NK cells acquire KLRG1 on their surface during development, and this expression correlates with functional distinctions from other peripheral NK cells in vivo (Huntington et al. 2007). While both IL-7 and IL-15 induced proliferation of KLRG1^{low} CD8^{+} T cells, KLRG1^{high} cells exhibited an extraordinarily high level of resistance to cytokine-driven proliferation in vivo despite their dramatic accumulation upon IL-15 administration. Thus, IL-15 and IL-2 greatly improve the survival of KLRG1^{high} CD8^{+} T cells, which are usually destined to perish during contraction, in the absence of proliferation (Rubinstein et al. 2008).

32.4.3 Cadherins as Ligands of KLRG1

Cadherins have been identified as ligands for mouse and human KLRG1, which binds three of the classical cadherins (E-, N-, and R-). Cadherins are ubiquitously expressed in vertebrates and mediate cell–cell adhesion by homotypic or heterotypic interactions. By expression cloning using the mouse KLRG1 tetramer as a probe, human E-cadherin was found to be a xenogeneic ligand with a syngeneic interaction between mouse KLRG1 and mouse E-cadherin. KLRG1 also binds N- and R-cadherins. E-cadherin binding of KLRG1 prevents the lysis of E-cadherin–expressing targets by KLRG1^{+} NK cells (Ito et al. 2006; Tessmer et al. 2007). E-cadherin is expressed by normal epithelial cells, Langerhans cells, and keratinocytes and is usually down-regulated on metastatic cancer cells. KLRG1 ligation by E-cadherin in healthy tissue may thus exert an inhibitory effect on primed T cells (Gründemann et al. 2006). E-cadherin function is often inactivated during development of human carcinomas and splice-site mutations resulting in in-frame loss of exon 8 or 9 occur frequently in diffuse type gastric carcinomas. KLRG1 ligation by E-, N-, or R-cadherins may regulate the cytotoxicity of killer cells to prevent damage to tissues expressing the cadherins (Ito et al. 2006; Schwartzkopff et al. 2007; Tessmer et al. 2007).

Li et al. (2009) determined the structure of KLRG1 in complex with E-cadherin. KLRG1 mediates missing self recognition by binding to a highly conserved site on classical cadherins, enabling it to monitor expression of several cadherins (E-, N- and R-) on target cells (Fig. 32.2). This site overlaps the site responsible for cell–cell adhesion, but is distinct from the integrin $\alpha_E\beta_7$ binding site. Li et al. (2009) proposed that E-cadherin might co-engage KLRG1 and $\alpha_E\beta_7$, and that KLRG1 overcomes its exceptionally weak affinity for cadherins through multipoint attachment to target cells, resulting in inhibitory signaling.

32.5 MAFA-Like Receptor in Human NK Cell (MAFA-L)

32.5.1 Characteristics and Biological Properties

Butcher et al. (1998) identified a receptor gene in NK cell gene complex on human chromosome 12p12-13 encoding a putative type II transmembrane glycoprotein. The product was 54% identical to the rat MAFA, which inhibits mast cell activation by IgE. The human MAFA-like receptor (MAFA-L) and the rat MAFA protein are expressed by basophils and both have an ITIM in the cytoplasmic tail, consistent with an inhibitory role in basophil activation. Like other genes in the NK cell gene complex, MAFA-L is also expressed by NK cells as well as the monocyte-like cell-line U937. Expression in NK cells is restricted to peripheral blood NK cells, where as decidual NK cells did not express MAFA-L. While MAFA-L and rat MAFA might have a similar role in basophils, the expression of MAFA-L in other cell types implies additional functions for this molecule.

Human MAFA cDNA product is similar to the rat MAFA having an intracellular domain containing a putative ITIM and an extracellular C type lectin-like domain. However, in contrast to rat MAFA, the amino acid sequence suggests the presence of two additional extracellular N-linked glycosylation sites. In addition, alternative mRNA transcripts are observed that differ substantially from those found in the rat (Lamers et al. 1998).

32.6 Killer Cell Lectin Like Receptor Subfamily E, Member 1 (KLRE1)

A novel NK receptor, killer cell lectin like receptor subfamily E, member 1 (KLRE1) (also known as NKG2I), belonging to KLRs family has been characterized (Koike et al. 2004; Westgaard et al. 2003; Wilhelm and Mager 2003). A series of experiments using anti–KLRE-1 mAbs indicate that this receptor plays a role in the cytotoxicity mediated by NK cells in vitro (Koike et al. 2004; Westgaard et al. 2003). KLRE1 receptor is a type II transmembrane protein with a C-terminal lectin-like domain and belongs to the NKG2 family. Rat *Klre1* gene was mapped to the NK gene complex. KLRE1 was shown to be expressed by NK cells and a subpopulation of CD3^{+} cells, with pronounced inter-strain variation. KLRE1 can be expressed on the NK cell surface as a disulphide-linked dimer. The predicted proteins do not contain ITIMs or a positively charged amino acid in the transmembrane domain. Despite absence of a signaling motif, KLRE1 has nevertheless been shown to regulate NK cell-mediated cytotoxicity. Thus, KLRE1 may form a functional heterodimer with ITIM-

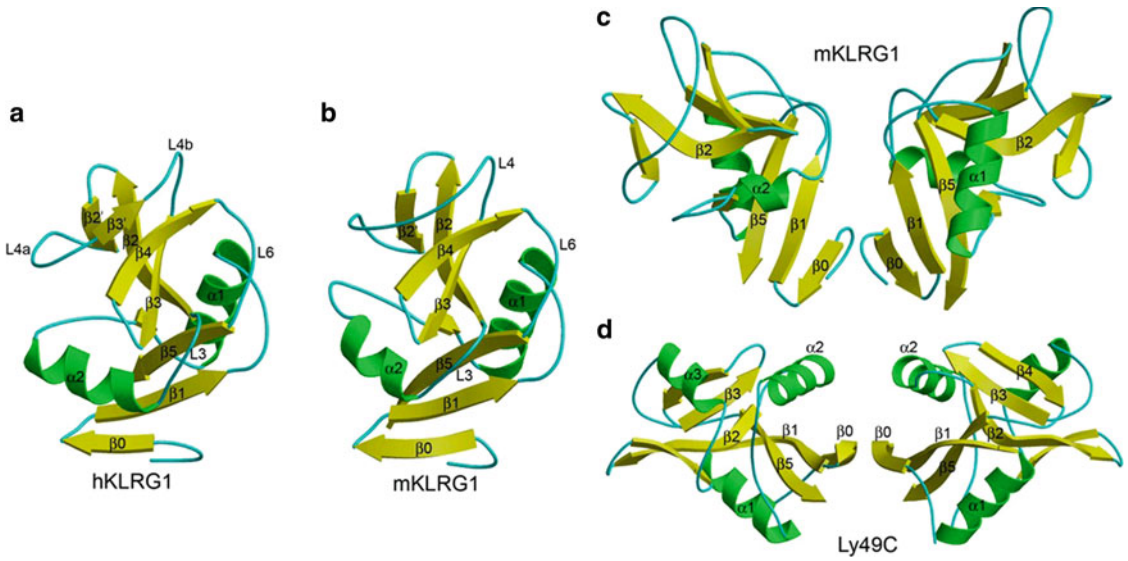

Fig. 32.2 Structure of KLRG1 (**a**) Ribbon diagram of human KLRG1, as observed in the hKLRG1–hEC1 complex. Secondary structure elements are labeled. α-helices are colored in *green*, β-stands in *yellow*, and loops in *cyan*. (**b**) Structure of mouse KLRG1 in unbound form. (**c**) Mouse KLRG1 homodimer, as observed in the mKLRG1–hEC1 complex. This dimer was not observed in structures of mKLRG1 alone or in the hKLRG1–hEC1 complex. (**d**) Structure of the Ly49C homodimer (PDB ID: 3C8J). Secondary structure elements are colored as in (**a**) (Adapted by permission from Li et al. 2009 © Elsevier)

bearing partner that recruits SHP-1 to generate an inhibitory receptor complex (Westgaard et al. 2003). Murine NK receptor, NKG2I, which was composed of 226 amino acids, showed ~40% homology to the murine NKG2D and CD94 in the C-type lectin domain. The expression of NKG2I was largely confined to NK and NKT cells, but was not seen in T cells. Furthermore, anti-NKG2I mAb inhibited NK cell–mediated cytotoxicity, whereas cross-linking of NKG2I enhanced IL-2 and IL-12–dependent IFN-γ production. NKG2I is an activating receptor mediating recognition and rejection of allogeneic target cells (Koike et al. 2004).

KLRE/I1 and KLRE/I2: A Pair of Heterodimeric Receptors: Saether et al. (2008) demonstrated that KLRE1 forms functional heterodimers with either KLRI1 or KLRI2. Cotransfection with KLRE1 was necessary for surface expression of the NKR chains KLRI1 and KLRI2 in 293T cells and can be co-precipitated with KLRI1 or KLRI2 from transfected NK cell lines. Unlike other KLRs, KLRE1/KLRI1 and KLRE1/KLRI2 heterodimers predominantly migrate as single chains in SDS-PAGE, indicating noncovalent association. KLRI1 showed activity of SH-2 domain-containing phosphatase 1. Ab to KLRE1 induced inhibition in KLRI1-transfected cells but increased cytotoxicity in KLRI2 transfectants, demonstrating that KLRE/I1 is a functional inhibitory heterodimer in NK cells, whereas KLRE/I2 is an activating heterodimeric receptor (Saether et al. 2008).

KLRE-1 in BM Allograft Rejection: KLRE-1-deficient mice were born at an expected frequency and showed no aberrant phenotype on growth and lymphoid development. However, KLRE-1-deficient cells showed a severely compromised allogeneic cytotoxic activity compared with the wild-type cells. Allogeneic bone marrow (BM) transfer culminated in colony formation in the spleen of KLRE-1-deficient mice, whereas no colony formation was observed in wild-type recipient mice. Therefore, KLRE-1 is a receptor mediating recognition and rejection of allogeneic target cells in the host immune system (Shimizu et al. 2004). These results indicate that NKG2I on NK cells recognizes putative ligands present on allogeneic BM cells and induces signals leading to the rejection of allografts. It should be mentioned that administration of anti-NK1.1, anti–asialo GM1, or anti–Ly-49D mAb also abrogated the rejection of allogeneic BM grafts, but these effects were primarily due to the depletion of NK cells expressing these molecules

32.7 KLRL1 from Human and Mouse DCs

KLRL1 belongs to the KLR family and is a novel inhibitory NK cell receptor. The KLRL1 from human and mouse DCs is a type II transmembrane protein with an ITIM and a C-type lectin-like domain. The KLRL1 gene is located in the central region of the NK gene complex in both humans and mice, on human chromosome 12p13 and mouse chromosome 6F3, adjacent to the other KLR genes. KLRL1 is preferentially expressed in lymphoid tissues and immune cells, including NK cells, T cells, DCs, and monocytes or macrophages. KLRL1 forms a heterodimer with an as yet

unidentified partner. Human and mouse KLRL1 both contain a putative ITIMs, and associates with the tyrosine phosphatases SHP-1 and SHP-2 (Han et al. 2004).

32.8 Natural Cytotoxicity Receptors

The function of NK cells are finely regulated by a series of inhibitory or activating receptors. The inhibitory receptors, specific for MHC class I molecules, allow NK cells to discriminate between normal cells and cells that have lost the expression of MHC class I (e.g., tumor cells). The general molecular strategies that allow NK cells to spare normal cells and kill tumor or virally infected cells have been clarified. The major receptors responsible for NK cell triggering are NKp46, NKp30, NKp44 and NKG2D. The NK-mediated lysis of tumor cells involves several such receptors, while killing of DCs involves only NKp30. The target-cell ligands recognized by some receptors have been identified, but those to which major receptors bind are not yet known. Nevertheless, functional data suggest that they are primarily expressed on cells upon activation, proliferation or tumor transformation. Thus, the ability of NK cells to lyse target cells requires both the lack of surface MHC class I molecules and the expression of appropriate ligands that trigger NK receptors.

32.8.1 NK Cell Triggering: The Activating Receptors

Provided that turning NK cells 'off' represents the major fail-safe device to prevent the NK-mediated attack of normal HLA class I^+ autologous cells, an 'on' signal must be generated upon interaction of NK cells with potential target cells. This signal is extinguished whenever appropriate interactions occur between inhibitory receptors and MHC class I molecules. On the other hand, the 'on' signal can be readily detected when NK cells interact with target cells that lack MHC class I molecules. The receptors responsible for NK cell activation in the process of natural cytotoxicity were identified recently and characterized. Collectively termed natural cytotoxicity receptors (NCRs), NKp46 (Sivori et al. 1997; Pessino et al. 1998), NKp44 (Vitale et al. 1998; Cantoni et al. 1999) and NKp30 (Pende et al. 1999) possess limited homology with known human molecules and no homology to each other (Moretta et al. 2000, 2001) (Fig. 32.1). As their expression is restricted to NK cells, they represent the most accurate surface markers for human NK cell identification. NCRs play a major role in the NK-mediated killing of most tumor cell lines (Moretta et al. 2001). Moreover, their surface density on NK cells correlates with the magnitude of the cytolytic activity against NK-susceptible target cells (Sivori et al. 2000). The ligands of NCRs are still not defined. However, as revealed by cytolytic assays, they are expressed by cells belonging to different histotypes (Moretta et al. 2001; Sivori et al. 2000; Costello et al. 2002; Pende et al. 2002). While NKp46 and NKp30 enable a precise identification of all NK cells, regardless of whether these cells are resting or activated (which is not true for other widely used NK cell markers including CD56 and CD16), NKp44 is selectively expressed by activated NK cells (Vitale et al. 1998; Cantoni et al. 1999; Moretta et al. 2001). This might explain, at least in part, the higher levels of cytolytic activity of activated NK cells cultured in IL-2.

NKG2D, a surface receptor of the NKG2 family, also plays a role in NK-mediated cytolysis. NKG2D is not restricted to NK cells, but is also expressed by cytolytic T lymphocytes. NKG2D is specific for the stress-inducible MICA and MICB or ULBP proteins. These ligands are expressed predominantly, but not exclusively, by cells of epithelial origin (Chap. 31).

32.8.2 Activating Receptors and Their Ligands

Natural cytotoxicity receptors (NCRs) (Moretta et al. 1999) are involved in NK cell triggering upon recognition of non-HLA ligands. These receptors appear to complement each other in the induction of target cell lysis by NK cells. Other triggering surface molecules expressed by NK cells (but shared by other leukocyte types) appear to function primarily as co-receptors (Fig. 32.1). That is, their ability to signal depends on the simultaneous co-engagement of one or another triggering receptor (Moretta et al. 2001). They may function to amplify signaling by true receptors. Other molecules that function as a triggering co-receptor in NK cells were found to be 2B4, NTB-A, the Poliovirus receptor (PVR, CD155) and Nectin-2 (CD112); the last two are members of the nectin family.

32.8.2.1 NKp46, NKp44, and NKp30

Sivori et al. (1997) identified a surface molecule (p46), which induces strong NK cell triggering and, unlike other NK cell antigens, is expressed exclusively by NK cells. NKp46 is expressed by all resting or activated NK cells. Unlike other NK cell antigens, the expression of p46 was strictly confined to NK cells. The p46-mediated induction of Ca^{2+} increases or triggering of cytolytic activity was down-regulated by the simultaneous engagement of inhibitory receptors including p58, p70, and CD94/NKG2A. Both the unique cellular distribution and functional capability of p46 molecules suggest a possible role in the mechanisms of non-MHC-restricted cytolysis mediated by human NK cells.

Vitale et al. (1998) identified a 44-kD surface molecule (NKp44) that is absent in freshly isolated peripheral blood

lymphocytes but is progressively expressed by all NK cells in vitro after culture in IL-2. Different from other markers of cell activation, NKp44 is absent in activated T lymphocytes or T cell clones. NKp44 was not detected in any of the other cell lineages analyzed. The mAb-mediated cross-linking of NKp44 in cloned NK cells resulted in strong activation of target cell lysis in a redirected killing assay. NKp44 functions as a triggering receptor selectively expressed by activated NK cells and that together with p46, may be involved in the process of non-MHC-restricted lysis. Both p46 and NKp44 are coupled to the intracytoplasmic transduction machinery via the association with CD3 or KARAP/DAP12, respectively; and these associated molecules are tyrosine phosphorylated upon NK cell stimulation (Fig. 32.1).

Pende et al. (1999) identified NKp30, a 30-kD triggering receptor selectively expressed by all resting and activated human NK cells. Although mAb-mediated cross-linking of NKp30 induces strong NK cell activation, mAb-mediated masking inhibits the NK cytotoxicity against normal or tumor target cells. NKp30 cooperates with NKp46 and/or NKp44 in the induction of NK-mediated cytotoxicity against the majority of target cells, whereas it represents the major triggering receptor in the killing of certain tumors. This receptor is associated with CD3 ζ chains that become tyrosine phosphorylated upon sodium pervanadate treatment of NK cells. Molecular cloning of NKp30 cDNA revealed a member of the immunoglobulin superfamily, characterized by a single V-type domain and a charged residue in the transmembrane portion. Moreover, NKp30 is encoded by the 1C7 gene. Although the identification of different NCRs constitutes a major step forward in our understanding of the NK cell physiology, both the nature and the distribution of the NCR ligands on target cells remain to be determined. The occupational exposure to mixture of toxics is one of the important factors in the diminution of the NK cell receptor expression (De Celis et al. 2008). Following exposure of women to toxic compounds, NKp30 and NKG2D receptor expression was diminished.

Similar to NKp46, NKp30 is selectively expressed by all NK cells, both freshly isolated and cultured in IL-2, thus representing an optimal marker for NK cell identification. Although both belong to the Ig-SF, NKp30 does not display any substantial homology with previously identified NK receptors. In many respects, NKp30 appeared similar to NKp42. Indeed, their parallel expression on all NK cells (including the rare $CD16^-$ cells), the existence for both of a high or low density pattern of surface expression, together with their similar functional characteristics, led to the thought that the surface molecule recognized by the new mAbs could be identical or strictly related to NKp42. However, NKp30 and NKp46 displayed different molecular masses and, functionally, appeared to play a complementary role in the induction of natural cytotoxicity. Moreover, molecular cloning revealed that NKp30 is a protein with very limited homology with NKp46, as the two molecules display only 13% identity and 15% similarity, and are encoded by genes located on different chromosomes.

Both NKp46 and NKp44 receptors are involved in recognition and lysis of a variety of tumor targets, but neither displays significant identity. NKp46 and NKp44 were shown to cooperate in the process of tumor cell lysis by human NK cells. However, studies indicated the existence of additional receptor(s) cooperating with NKp46 and NKp44. Indeed, NKp30 represents a receptor that may cooperate with NKp46 and NKp44 in the induction of cytotoxicity against a variety of target cells. Perhaps, NKp30 represents the major receptor in inducing NK-mediated killing of certain tumor target cells, the lysis of which is largely NKp46/NKp44 independent. Molecular cloning revealed that NKp30 is the product of 1C7, a gene mapped on human chromosome 6 in the HLA class III region. Persistent high risk Human papillomavirus (HPV) infection can lead to cervical cancer. NK cells play a crucial role against tumors and virus-infected cells through a fine balance between activating and inhibitory receptors. Expression of triggering receptors NKp30, NKp44, NKp46 and NKG2D on NK cells correlates with cytolytic activity against tumor cells and their down-regulation represents an evasion mechanism associated to low NK cell activity, HPV-16 infection and cervical cancer progression (Garcia-Iglesias et al. 2009).

mAbs to NCR block to differing extents the NK-mediated lysis of various tumors. Moreover, lysis of certain tumors can be virtually abrogated by the simultaneous masking of the three NCRs. There is a coordinated surface expression of the three NCRs, their surface density varying in different individuals and also in the NK cells isolated from a given individual. A direct correlation exists between the surface density of NCR and the ability of NK cells to kill various tumors. NKp46 is the only NCR involved in human NK-mediated killing of murine target cells. Accordingly, a homologue of NKp46 has been detected in mouse. Molecular cloning of NCR revealed novel members of the Ig superfamily displaying a low degree of similarity to each other and to known human molecules. NCRs are coupled to different signal transducing adaptor proteins, including CD3 ζ, Fc εRI γ, and KARAP/DAP12. Besides different NCRs, several other surface molecules that can mediate NK cell triggering have been identified in humans and rodents. These include CD2, CD69, CD28, 2B4, and NKR-P1 (Pende et al. 1999).

32.8.2.2 Ligands of Activating Receptors

Although many core questions regarding NK cell receptors have been answered in recent years, there are still relevant issues that remain to be tackled in NK cell physiology. One

major problem is with regard to the identification of the cellular ligands for the major triggering receptors (NKp46, NKp30 and NKp44) as well as for other receptors or co-receptors (Fig. 32.1). Available information is compatible with the concept that, similar to MICA/B, PVR or Nectin-2, they may also be represented by molecules primarily expressed by cells that have been 'stressed' by cytokine activation, proliferation, high temperature, viral infection or tumor transformation. NKp46 ligands are expressed in reticular dermis, and their expression is enhanced in the active proliferation zone (dermoepidermal junction) of nevi and melanomas. The physiological role of NKp46 ligands in the progression of malignancy is unknown (Cagnano et al. 2008). Myeloid DCs express ligands for NKp46 and NKp30 natural cytotoxicity receptors, which are partially reduced on HCMV infection; yet. The studies stress the importance of the dynamics of viral immune evasion mechanisms (Magri et al. 2011). NKp46 binds to heparin and heparan sulfate (HS); and also to multimeric sialyl Lewis X expressing transferrin secreted by human hepatoma HepG2 cells (HepTF). NKp46 binds to sulfate- and 2,3-NeuAc-containing glycans mainly via ionic interactions. The HS GAG are not ligands for NKp30 (Ito et al. 2011).

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Part XI

C-Type Lectins: Endocytic Receptors

Asialoglycoprotein Receptor and the Macrophage Galactose-Type Lectin

33

Anita Gupta

33.1 Asialoglycoprotein Receptor: The First Animal Lectin Discovered

Mammalian liver expresses endocytic cell surface receptors that specifically bind natural or synthetic molecules containing terminal galactosyl or N-acetylgalactosaminyl sugars. One of these hepatocyte receptors is the asialoglycoprotein receptor (ASGP-R), which mediates the endocytosis and subsequent lysosomal degradation of these glyco-molecules. The ASGP-R was the first mammalian lectin discovered by Morell et al. (1968) and is a member of the C-type lectin family, which require Ca^{2+} for ligand binding and to contain disulfide bridges in the CRD (Zelensky and Gready 2005). ASGP-R is an integral membrane protein, predominately expressed on the sinusoidal surface of mammalian hepatocytes. Morphometric analysis estimated that approximately 90% of the receptor molecules were at the sinusoidal face, approximately 10% at the lateral face, and approximately 1% at the bile canalicular face. Nonhepatic cells such as endothelial and Kupffer cells had no receptor specific for asialoglycoproteins (Matsuura et al. 1982). At cellular level, the basic molecular function of the receptor is to mediate the uptake and ultimate degradation of galactosyl/N-acetylgalactosaminyl-containing molecules (ligands). At the organism level, however, the physiological function is uncertain. Weigel (1994) proposed physiological role of ASGP-R in regulating the dynamic flux of galactosyl/N-acetylgalac tosaminyl glycoconjugates in mammals. The ASGP-R is one of the best characterized systems for receptor-mediated endocytosis via the clathrin-coated pit pathway (Breitfeld et al. 1985; Weigel and Yik 2002; Stockert 1995).

33.2 Rat Asialoglycoprotein Receptor

33.2.1 Characteristics

Hepatic plasma membrane receptors mediate the specific binding and uptake of partially deglycosylated glycoproteins. These receptors, referred to as hepatic lectins, have been isolated from several mammalian liver, peritoneal macrophages (Ashwell and Harford 1982) and avian species (Baenziger and Maynard 1980). While the mammalian (rabbit, rat, and human) receptors recognize galactose residues exposed upon removal of terminal sialic acid, the avian (chicken) receptor recognizes terminal N-acetylglucosamine exposed upon further removal of the galactose. In spite of this difference in binding specificity, the mammalian and avian receptors share a number of properties, such as a strict Ca^{2+} requirement for ligand binding and similar pH profiles for binding and release of ligand (Brown et al. 1983). The complete amino acid sequence of the chicken hepatic lectin (Drickamer and Mamon 1982) suggests a unique organization for this membrane protein with its NH2 terminus in the cytoplasm and COOH terminus outside the cell.

Rat ASGP-R contains three subunits, designated rat hepatic lectins (RHL) 1, 2, and 3 where as human ASGP-R contains two subunits, HHL1 and HHL2. Peptide mapping experiments suggest that the three polypeptides of rat RHL may be structurally related to each other (Schwartz et al. 1981; Warren and Doyle 1981). The predominant polypeptide RHL-l has an M_r of 41.5 kDa, while two less abundant species appeared to be of higher molecular weight (RHL-2 of 49.0 kDa and RHL-3 of 54 kDa); the RHL-1 represents 70–80% of the total mass of the receptor. Each of the RHL-1 and RHL-2/3 polypeptides is associated into homo-oligomers but are physically unlinked to each other. The complete sequence of the major RHL species (RHL-1) has been established (Drickamer et al. 1984; Schwartz 1984). The complete sequence of RHL-1 is 283 residues long, although 20% of the protein as isolated is missing the first two residues at NH2 terminus. The overall arrangement of the polypeptide, similar to chicken ASGP-R, consists of an NH2-terminal stretch of hydrophilic amino acids, a segment of about 30 uncharged residues, and a COOH-terminal portion which contains three oligosaccharide attachment sites. The COOH terminus of the rat and chicken receptors

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showed 28% identity. Evidence shows that there must be at least two distinct genes for RHL receptor polypeptides. ASGP-Rs are covalently modified by fatty acylation (Zeng et al. 1995; Zeng and Weigel 1996). The single Cys residue in the cytoplasmic domain of each RHL or HHL subunit is fatty acylated. Deacylation ofASGP-Rs with hydroxylamine results in the spontaneous formation of dimers through reversible disulfide bonds, indicating that deacylation concomitantly generates free thiol groups. Cys^{57} within the transmembrane domain of HHL1 is not normally palmitoylated. In conclusion, Cys^{35} in RHL1, Cys^{54} in RHL2 and RHL3, and Cys^{36} in HHL1 are fatty acylated. Cys^{57} in HHL1 and probably Cys^{56} in RHL1 are not palmitoylated.

The second subunit, RHL-2/3, consists of two species that are differentially glycosylated forms of a second, homologous polypeptide. The structure of RHL-2/3 polypeptide reveals that this protein is homologous to RHL-1 throughout its length but contains one major insertion of 18 amino acids near its NH2 terminus. RHL-1 and RHL-2/3 bind carbohydrate ligands through COOH-terminal, Ca^{2+}-dependent CRDs. Results indicate that RHL-1 and RHL-2/3 polypeptides are self-associated into two distinct molecules, each of which has galactose-binding activity (Halberg et al. 1987).

Structural analysis of wild-type and mutant forms of homologous C-type CRD from serum MBP suggests that carbohydrate ligands bind to the CRDs of RHL-1 at a conserved Ca^{2+}designated site two. The primary interaction with sugar is through hydroxyl groups that form coordination bonds with the $Ca^{2+}$and hydrogen bonds with protein side chains that also ligate the Ca^{2+} (Weis et al. 1992). The molecular basis for galactose binding to C-type CRDs has been studied in a mutant CRD from serum MBP which has been engineered to bind galactose. Three single amino acid replacements ($E^{185}Q$, $N^{187}D$, and $H^{189}W$) and insertion of a glycine-rich loop result in a modified CRD designated QPDWG, which has affinity and selectivity similar to RHL-1 (Iobst and Drickamer 1994). The crystal structures of this modified CRD and of a further mutant that binds *N*-acetylgalactosamine with high affinity have been solved (Kolatkar and Weis 1996; Kolatkar et al. 1998). These structures provide a basis for modeling the CRD of RHL-1. Murine ASGP-R cDNA exhibits homology with rat and human receptors. The membrane-bound M (mouse) HL polypeptide does not contain a cleavable N-terminal signal sequence and is probably anchored to the membrane via an internal insertion sequence (Sanford and Doyle 1990).

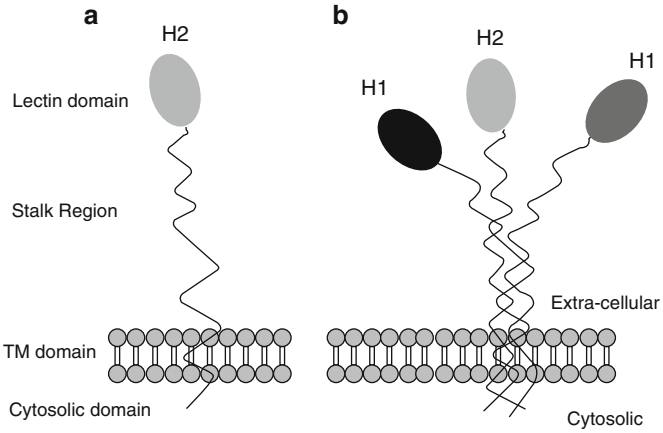

Fig. 33.1 Schematic presentation of asialoglycoprotein receptor (ASGP-R). (**a**) Each subunit, H1 and H2, consists of four domains; a *N*-terminal cytoplasmic domain, a single-pass transmembrane domain, an extracellular stalk segment and a C-type lectin carbohydrate recognition domain. (**b**) A hetero-oligomeric complex of two H1 and one H2 subunits has been proposed as the minimum size of an active ASGP-R

33.3 Human Asialoglycoprotein Receptor

33.3.1 Structural Characteristics

The human liver ASGP-R is a multichain heterooligomer composed of two homologous subunits, the major H1 and the minor H2, sharing 58% sequence identity. The ASGP-R from human liver and from the human hepatoma cell line HepG2 migrates as a single species of 45 kDa. The receptor encoding a cDNA clones has been isolated in H1 and H2 forms, with a protein sequence homology of 58%. Each subunit consists of four domains, a *N*-terminal cytoplasmic domain, a single-pass transmembrane domain, an extracellular stalk segment and a CRD, the latter being responsible for the ligand binding (Fig. 33.1a) (Weigel and Yik 2002; Chiacchia and Drickamer 1984; Shia and Lodish 1989). The H1 is a single-spanning membrane protein with amino terminus facing the cytoplasm and the carboxy terminus exposed on the exoplasmic side of plasma membrane. The transmembrane segment, residues 38–65, functions as an internal signal directing protein synthesis to the endoplasmic reticulum and initiating membrane insertion (Spiess 1987; Spiess and Handschin 1987). A notable difference between H1 and H2 is an 18-amino acid insert present in the cytoplasmic domain of only H2. Comparison of sequences of rat RHL-l and RHL-2 indicates that H1 is more homologous to R1 than to H2, and H2 is more similar to R2 than to H1 (Weigel and Yik 2002; Spiess and Lodish 1985).

Furthermore, while H1 only occurs as one protein isoform, H2 transcripts exist in multiple variants both in HepG2

cells and in the normal human liver. Some of these appear to be the result of alternative splicing events. Three splice variants of H2, designated H2a, H2b and H2c, have been isolated from human liver and HepG2 cells. However, H2a does not occur in native ASGP-R complexes. In contrast, both H2b and H2c associate with H1 in functional ASGP-Rs, but never together in the same receptor complex (Yik et al. 2002).

Though, H1 and H2 are highly homologous, the major H1 subunit is stably expressed by itself, whereas in the absence of H1 most of the H2 subunits are degraded in ER. Since the internal pool of H2 was also able to traffick to the cell surface, it was suggested that H2 recycles between the surface and intracellular compartments, similar to the constitutive recycling of hetero-oligomeric ASGP-R complexes. However, unlike H1, which can bind the ligand asialo-orosomucoid (ASOR), H2 failed to bind or endocytose ASOR. It seems that the H2 subunit of the human ASGP-R contains functional, although weak, signal(s) for endocytosis and recycling and has the ability to oligomerize. H2 homo-oligomers, however, do not create binding sites for desialylated glycoproteins, such as ASOR, that contain tri- and tetra-antennary N-linked oligosaccharides (Saxena et al. 2002).

The X-ray crystal structure of the carbohydrate recognition domain of the major subunit H1 was studied at 2.3 A resolution (Meier et al. 2000). While the overall fold of this and other known C-type lectin structures are well conserved, the positions of the bound calcium ions are not, indicating that the fold is stabilised by alternative mechanisms in different branches of the C-type lectin family. It was the first CRD structure where three calcium ions form an intergral part of the structure. In addition, the structure provided direct confirmation for the conversion of the ligand-binding site of the mannose-binding protein to an asialoglycoprotein receptor-like specificity suggested by Drickamer and colleagues. In agreement with the prediction that the coiled-coil domain of the ASGP-R is separated from the CRD and its N-terminal disulphide bridge by several residues, these residues are indeed not alpha-helical, while in tetranectin they form an alpha-helical coiled-coil (Drickamer 1988; Meier et al. 2000).

33.3.1.1 Position of Cysteine Is Critical for Palmitoylation of H1

The two subunits, H1 and H2 ASGP-R are palmitoylated at the cytoplasmic Cys residues near their transmembrane domains (TMD). The cytoplasmic Cys^{36} in H1 is located at a position that is five amino acids from the transmembrane junction. Neither the native amino acid sequence surrounding Cys^{36} nor the majority of the cytoplasmic domain sequence is critical for palmitoylation. Palmitoylation was also not dependent on the native TMD of H1. In contrast, the attachment of palmitate was abolished if the Cys residue was transposed to a position that was 30 amino acids away from the transmembrane border. Thus, the spacing of a Cys residue relative to the TMD in the primary protein sequence of H1 is the major determinant for successful palmitoylation (Yik and Weigel 2002).

33.3.1.2 Macrophage ASGP-Binding Protein

The primary structure of macrophage asialoglycoprotein-binding protein, M-ASGP-BP consists of 306 amino acid residues with a molecular mass of 34,242 Da. The sequence was highly homologous with that of the rat liver asialoglycoprotein receptor (RHL), particularly that of RHL-1 (the major form of RHL), throughout its whole length, and especially so in its putative membrane-spanning region and CRD. There were two N-glycosylation sites in M-ASGP-BP, the location of which were identical to those in RHL-1 (Ii et al. 1990). However, M-ASGP-BP was characteristic in having a shorter cytoplasmic tail, and an inserted segment of 24 amino acids containing an Arg-Gly-Asp sequence between the membrane-spanning region and carbohydrate recognition domain (Ii et al. 1990).

Ligand specificity and binding affinity depend on the arrangement of the subunits in the complex. Presence of both subunits is a prerequisite for the ASGP-R to reach full functionality. Although the subunit composition of the native receptor remains unspecified (Weigel and Yik 2002), a stoichiometry of 2:1 of H1 and H2 has been proposed as the minimum size of an active ASGP-R complex, exhibiting high affinity ligand binding (Fig. 33.1b) (Hardy et al. 1985; Schwartz 1984). Results suggest that the stalk segments of the receptor subunits oligomerize to constitute an α-helical coiled coil stalk on top of which the carbohydrate binding domains are exposed for ligand binding. This subunit ratio was partially supported by another study in which the functional receptor was suggested to be a 2:2 heterotetramer. Extracellular stalk segments of H1 and H2, responsible for the subunit oligomerization, were expressed and shown to associate in homo- and heterooligomers in vitro (Bider et al. 1996). A study of e binding of three different ^{125}I-labeled, galactose-terminated ligands to the hepatic galactose/N-acetylgalactosamine-specific lectin revealed that the different ligands manifest different physical parameters of binding. For example, there were many more total binding sites for di-tris-lac on the surface of rabbit hepatocytes than there were for asialoorosomucoid, although the dissociation constants were similar for these ligands (Hardy et al. 1985).

33.4 Ligand Binding Properties of ASGP-R

The ASGP-Rs specifically recognize galactose- or N-acetylgalactosamine-terminated oligosaccharides, present on desialylated glycoproteins. The ASGP-R recognizes with

high affinity (K_D in nM range) tri- and tetra-antennary N-linked sugar side chains with terminal galactose residues. Glycoproteins with such glycosylation patterns are rapidly endocytosed by the ASGP-R via clathrin-coated pits and vesicles. The specificity and affinity of ligand binding of ASGP-R depends on the number and spatial arrangement of several galactose-binding sites within the receptor complex. A single Gal residue exhibits only modest binding affinity with a K_D in the range of 1 mM. Additional sugars, from a mono- to a triantennary structure, result in a significant increase in affinity with K_D ~10 nM for the native receptor. Hence, high affinity binding is achieved through multiple interactions between the CRDs in a receptor complex and multiple sugar residues (Westerlind et al. 2004). Furthermore, the sugar spacing has been shown to be important for optimal receptor recognition. A spacer length of >20 Å, separating the sugar moieties from the branching point of the ligand, results in high affinity binding to the ASGP-R of Gal containing ligand constructs (Westerlind et al. 2004; Rensen et al. 2001). In addition, the ASGP-R clearly favors binding of Gal*N*Ac over Gal, showing an approximate 50-fold higher affinity for the former (Rensen et al. 2001; Baenziger and Maynard 1980). Studies indicate that both subunits are required for high-affinity ligand binding, i.e. for the simultaneous interaction with three galactose residues within an N-linked glycan. However, asialoorosomucoid (ASOR) and asialofetuin (ASF) bind to transfected COS-7 cells expressing subunit H1 in absence of the second subunit H2. It was found that, at a sufficiently high density of H1 on the cell surface, high-affinity binding of ASOR and ASF is the result of two or more glycans interacting with H1 oligomers with low affinity in a bivalent manner. ASOR displays a K_D of ⟨10 nM for the native receptor. It has also been observed to bind H1, overexpressed in transfected COS-7 cells, in the absence of H2 with a K_D of ⟨40 nM (Bider et al. 1996). In order to develop the non-viral Bioplex vector system for targeted delivery of genes to hepatocytes, Westerlind et al. (2004) evaluated the structure-function relationship for a number of synthetic ligands designed for specific interaction with the hepatic lectin ASGP-R. Uptake efficiency increased with number of displayed GalNAc units per ligand, in a receptor dependent manner. Thus, a derivative displaying six GalNAc units showed the highest uptake efficacy both in terms of number of internalizing cells and increased amount of material taken up by each cell. However, this higher efficiency was shown to be due not so much to higher number of sugar units, but to higher accessibility of the sugar units for interaction with the receptor (longer spacer) (Westerlind et al. 2004).

Analogous binding specificity can be engineered into the homologous rat MBP-A by changing three amino acids and inserting a glycine-rich loop (Iobst and Drickamer 1994). Crystal structures of this mutant complexed with β-methyl galactoside and N-acetylgalactosamine (GalNAc) revealed that as with wild-type MBPs, the 3- and 4-OH groups of the sugar directly coordinate Ca^{2+} and form hydrogen bonds with amino acids that also serve as Ca^{2+} ligands. The different stereochemistry of the 3- and 4-OH groups in mannose and galactose, combined with a fixed Ca^{2+} coordination geometry, leads to different pyranose ring locations in the two cases. The glycine-rich loop provides selectivity against mannose by holding a critical tryptophan in a position optimal for packing with the apolar face of galactose but incompatible with mannose binding. The 2-acetamido substituent of GalNAc is in the vicinity of amino acid positions identified by site-directed mutagenesis (Iobst and Drickamer 1996) as being important for the formation of a GalNAc-selective binding site (Kolatkar and Weis 1996). ASGP-R was also postulated to account for the low density lipoprotein (LDL) receptor-independent clearance of lipoproteins including chylomicron remnants (Ishibashi et al. 1996). Later, immunoglobulin A (Rifai et al. 2000) and fibronectin (Rotundo et al. 1998) emerged as likely candidates of natural ligands for ASGP-R. The clearance of apoptotic cells or a subpopulation of lymphocytes in the liver has been also attributed to ASGP-R (Stockert 1995).

33.4.1 Interaction with Viruses

It is particularly noteworthy that ASGP-R has also been proposed to be utilized as entry sites into hepatocytes by several hepatotropic viruses including hepatitis B virus (Treichel et al. 1994), Marburg virus (Becker et al. 1995), and hepatitis A virus (Dotzauer et al. 2000). The liver is one of the main target organs of Marburg virus (MBG), a filovirus causing severe haemorrhagic fever with a high fatality rate in humans and non-human primates. MBG grown in certain cells does not contain neuraminic acid, but has terminal galactose on its surface glycoprotein. The lack of neuraminic acid together with the marked hepatotropism of the MBG infection led to suggest that the ASGP-R of hepatocytes may serve as a receptor for MBG in the liver. MBG lacking sialic acid specifically binds to the ASGP-R. This observation may at least in part explain why the liver is a central target for MBG and may thus contribute to an understanding of the pathogenesis of this infection (Becker et al. 1995). Structural proteins of hepatitis C virus (HCV) genotype 1a bind to cultured human hepatic cells. This binding was decreased by calcium depletion and was partially prevented by ligands of ASGP-R, thyroglobulin, asialothyroglobulin, and antibody against a peptide in CRD of ASGP-R but not preimmune antibody (Saunier et al. 2003).

33.5 The Crystal Structure of H1-CRD

The structure of ASGP-R H1-CRD was solved in 2000 by X-ray crystallography (Meier et al. 2000). It is a globular protein, containing two α-helices and eight β-strands. The β-strands are arranged in a bent plane and form the core of the protein, while the α-helices are positioned on either side of the plane. Three calcium ions can be seen in the structure, forming an integral part of the protein as they pin together several loops. The two calcium ions at site 1 and 2 are seen in close proximity, both coordinated by Glu^{252}. Site 2 is of particular interest as it is essential for sugar binding and is present in all C-type lectins. Calcium binding site 3 is found close to the *N*- and *C*-terminus of the protein (Fig. 33.2).

C-type CRDs can be classified into short-form CRDs, with two disulfide bridges, or long-form CRDs, containing a conserved extension with an additional disulfide bond at the *N*-terminus. H1-CRD falls into the latter category, containing seven cysteines out of which six are engaged in three disulfide bridges. One bridge constitutes part of the sugar binding site, seen in the crystal structure between Cys^{254} and Cys^{268}. A second bond is formed between Cys^{181} and Cys^{276}, bringing the *C*- and *N*-terminus close together and contributes to the tertiary structure of the subunit. Finally, a third typical long-form CRD disulfide bridge is found at the *N*-terminus between Cys^{153} and Cys^{164}. The *N*-terminal residues 147–152, including the seventh odd cysteine (Cys^{152}), and the *C*-terminal residues 281–290 could not be positioned into the electron density and hence, cannot be seen in the crystal structure (Meier et al. 2000; Johansson 2007).

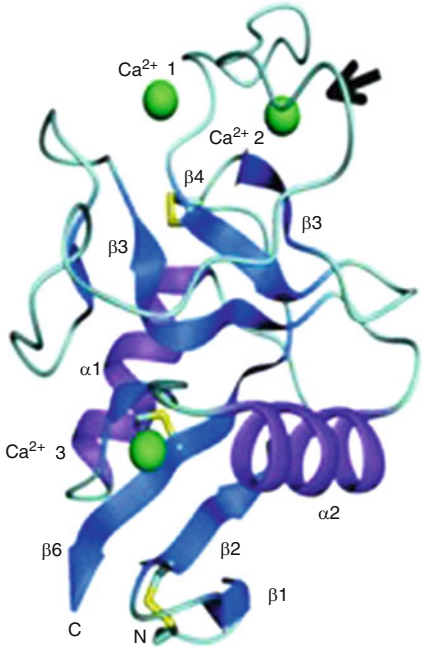

Fig. 33.2 Ribbon diagram of the carbohydrate recognition domain of the H1 subunit. The three calcium ions can be seen in *green*. The second calcium binding site, which is part of the sugar binding site, is denoted by a *black arrow*. The disulfide bridges are marked in *yellow* (PDB ID: 1DV8) (Adapted with permission from Meier et al. 2000 © Elsevier)

33.5.1 The Sugar Binding Site of H1-CRD

The specificity and affinity of ligand binding of ASGP-R depends on the number and spatial arrangement of several galactose-binding sites within the receptor complex. Studies indicate that both subunits are required for high-affinity ligand binding, i.e. for the simultaneous interaction with three galactose residues within an N-linked glycan. However, asialoorosomucoid (ASOR) and asialofetuin (ASF) bind to transfected COS-7 cells expressing subunit H1 in the absence of the second subunit H2. It was found that, at a sufficiently high density of H1 on the cell surface, high-affinity binding of ASOR and ASF is the result of two or more glycans interacting with H1 oligomers with low affinity in a bivalent manner. The region around the sugar binding site in H1-CRD is formed by one continuous stretch of the polypeptide chain from Arg^{236} to Cys^{268} (Johansson 2007).

Mutagenesis studies have been carried out with closely related protein RHL-1, aiming to deduce the residues giving rise to it's ligand binding specificity. RHL-1 has been used as a reference in attempts to mimic its Gal- and Gal*N*Ac-binding properties in other lectins, e.g. the MBP (Iobst and Drickamer 1994) and the macrophage galactose receptor (MGR) (Iobst and Drickamer 1996). Such studies form the basis for explanation of H1-CRDs sugar preference as well as its distinction between high- and low-affinity ligands. Introduction of two point mutations in MBP, $E^{185}Q$ and $N^{187}D$, corresponding to Q^{239} and D^{241} in RHL-1, was sufficient to achieve galactose binding in the protein, which is normally binding mannose (Iobst and Drickamer 1994). However, as the Gal affinity of the MBP mutant, designated QPD, was rather low it was concluded that other residues in H1-CRD also must contribute to the ligand binding. An additional mutation, $H^{189}W$, showed to increase the affinity significantly, making the galactose binding of mutant QPDW comparable to that of RHL-1. Selectivity for galactose over mannose was achieved by incorporating a glycine-rich stretch following the QPDW sequence, referred to as the QPDWG mutant. Stepwise mutations within the glycine rich loop, corresponding to residues Y^{244}, G^{245} and H^{246} in RHL-1, showed that they all contribute to the selectivity for Gal (Iobst and Drickamer 1994). However, the influence of the amino acids on the

selectivity could equally be attributed to stabilization and support of the protein structure as to an actual effect on the ligand binding.

RHL-1, as well as H1-CRD, shows preferential binding to Gal*N*Ac over Gal. The selectivity for Gal*N*Ac has been probed by mutations studies of MGR, which binds Gal and Gal*N*Ac with roughly equal affinity. The CRDs of RHL-1 and MGR are highly homologues (77%) and the difference in selectivity is likely to stem from divergences in the sequences. It was concluded that substitution of four amino acids in MGR, $V^{230}N$, $A^{258}R$, $K^{260}G$ and $S^{281}T$, is sufficient to induce Gal*N*Ac binding comparable with that of RHL-1. N^{230} increased the selectivity 20-fold, while the contributions by R^{258} and G^{260} were less prominent. The latter two residues only showed marginal or no effect at all when substituted individually, but a twofold increase when present together. It is possible that glycine contributes to the affinity by positioning arginine, which has the potential for forming hydrogen bonds with the ligand. T^{281} significantly increased the selectivity for Gal*N*Ac when inserted into MGR simultaneously with R^{258} and G^{260}, but is more likely to play an indirect role for ligand binding as it is predicted to be positioned at a considerable distance (10 Å). The residue appearing most important for achieving Gal*N*Ac binding is a histidine (His, H), found in both RHL-1 and MGR at position 256 and 278 respectively. Initial studies exchanging H^{278} in MGR for an alanine (Ala, A) resulted in an almost complete loss of Gal*N*Ac selectivity, but without any apparent effect on the galactose binding. The importance of the histidine was further investigated by repeating the substitution in RHL-1, causing a 25-fold loss in affinity for Gal*N*Ac, but without affecting that for Gal (Iobst and Drickamer 1996). Corresponding residue in MBP, T^{202}, was also substituted by histidine and resulted in a 14-fold increase in the relative affinity for Gal*N*Ac of the protein (referred to as the QPDWGH mutant) (Kolatkar et al. 1998).

33.5.2 Sugar Binding to H1-CRD

A model for sugar binding to H1-CRD has been proposed based on the mutagenesis experiments with MBP (Kolatkar and Weis 1996; Kolatkar et al. 1998). Crystallographic data of the QPDWG mutant could show that Gal and Gal*N*Ac bind directly to the Ca^{2+} in the second calcium binding site. The 3-OH and 4-OH groups of the sugar replaces two water molecules, normally coordinated by the calcium. In addition, the same OH groups also forms hydrogen bonds with amino acid chains that are Ca^{2+} site 2 ligands (Q^{239}, D^{241}, E^{252}, N^{264} in H1-CRD). Further interactions between the ligand and the protein are formed by stacking of the apolar face of the sugar against the side-chain of a tryptophan (W^{243} in H1-CRD) (Kolatkar and Weis 1996). The Gal*N*Ac-specific MBP mutant QPDWGH was also analyzed by crystallography.

Regions of the CRD of the receptor believed to be important in preferential binding to N acetylgalactosamine were inserted into the homologous CRD of a MBP mutant that was previously altered to bind galactose. Introduction of a single histidine residue corresponding to residue 256 of the hepatic ASGP-R was found to cause a 14-fold increase in the relative affinity for N-acetylgalactosamine compared with galactose. The relative ability of various acyl derivatives of galactosamine to compete for binding to this modified CRD suggest that it is a good model for the natural N-acetylgalactosamine binding site of the ASGP-R. Crystallographic analysis of this mutant CRD in complex with N-acetylgalactosamine reveals a direct interaction between the inserted histidine residue and the methyl group of the N-acetyl substituent of the sugar. The structure of this mutant reveals that the beta-branched valine side chain interacts directly with the histidine side chain, resulting in an altered imidazole ring orientation (Kolatkar et al. 1998).

33.5.2.1 Histidine 256: Responsible for pH Dependent Ligand Binding

Efficient release of ligands from CRD of the hepatic ASGP-R at endosomal pH requires a small set of conserved amino acids that includes a critical histidine residue. Mutagenesis studies of RHL-1 have shown that histidine 256 plays an important role in pH dependent ligand binding exhibited by the subunit (Wragg and Drickamer 1999; Feinberg et al. 2000). When these residues are incorporated at corresponding positions in an homologous galactose-binding derivative of serum MBP, the pH dependence of ligand binding becomes more like that of the receptor. The modified CRD displays 40-fold preferential binding to N-acetylgalactosamine compared with galactose, making it a good functional mimic of ASGP-R. In the crystal structure of the modified CRD bound to N-acetylgalactosamine, the His^{202} contacts the 2-acetamido methyl group and also participates in a network of interactions involving Asp^{212}, Arg^{216}, and Tyr^{218} that positions a water molecule in a hydrogen bond with the sugar amide group. These interactions appear to produce the preference for N-acetylgalactosamine over galactose and are also likely to influence the pK_a of His^{202}. Protonation of His^{202} would disrupt its interaction with an asparagine that serves as a ligand for Ca^{2+} and sugar. The structure of the modified CRD without sugar displays several different conformations that may represent structures of intermediates in the release of Ca^{2+} and sugar ligands caused by protonation of His^{202} (Feinberg et al. 2000).

In another work (Johansson 2007), His^{256} of H1-CRD was substituted by glutamate, the corresponding residue in H2-CRD. Binding of Gal*N*Ac to mutant $H^{256}E$ and WT H1-CRD was investigated at different pH ranging from 7.4 to 5. Results showed that binding of the Gal*N*Ac-polymer decreased at pH 6 or lower. $Hist^{256}$ clearly renders H1-CRD more sensitive to low pH compared to a glutamate in the

same position. His^{256} has been proposed to interact with Asn^{264}, which in turn is involved in both calcium- and ligand binding. The histidine stabilizes Asn^{264} by a hydrogen bond formed between the imidazole and the amide. Protonation of imidazole, caused by a drop in pH during endocytosis, will disrupt the hydrogen bond. As a consequence, the asparagine is destabilized and both ligand and calcium binding is disturbed.

33.6 Physiological Functions

A number of diverse physiological roles have been proposed for ASGP-R over the years. Among them, hepatic clearance of the desialylated and senascent serum proteins was most originally proposed. Later physiological and pathophysiological functions to this lectin include: the removal of apoptotic cells, clearance of lipoproteins, and the sites of entry for hepatotropic viruses. The primary function of the ASGP-R has been considered to be the removal and degradation of desialylated glycoproteins from the circulation. Normally, many oligosaccharide chains on glycoproteins carry terminal sialic acid residues. Upon removal of the sialic acid, caused by the action of neuraminidases, penultimate galactose residues are exposed and recognized by ASGP-R (Tozawa et al. 2001). The assembly of two homologous subunits, H1 and H2, is required to form functional, high affinity receptors on the cell surface. However, the importance of the individual subunits for receptor transport to the cell surface is controversial. To explore the significance of the minor H2 subunit for receptor expression and function in vivo, homozygous H2-deficient mice (*MHL-2*$^{-/-}$) are superficially normal. However, H1 expression in the liver is greatly reduced, indicating that H2 may H1 is strictly required for the stable expression of H2. Although these mice are completely unable to clear asialoorosomucoid, a high affinity ligand for asialoglycoprotein receptor, they do not accumulate desialylated glycoproteins or lipoproteins in their circulation (Ishibashi et al. 1994; Tozawa et al. 2001). Results suggest that ASGP-R is not ultimately responsible for the clearance of plasma glycoproteins, but is likely to possess other functions.

ASGP-R has been proposed to be involved in the metabolism of plasma lipoproteins and cellular fibronectin (Rotundo et al. 1998). However, studies with ASGP-R knock-out mice could not confirm this result, as the plasma levels of fibronectin and lipoprotein appeared unaffected in the absence of the receptor (Tozawa et al. 2001). ASGP-R has also been implicated in the clearance of apoptotic cells by the liver. Studies showed that the uptake of apoptotic bodies was blocked in the presence of an ASGP-R specific antibody or following the addition of receptor specific ligands such as ASF or Gal*N*Ac (Dini et al. 1992). Immunoglobulin A has also been proposed as a ligand for the receptor (Rifai et al. 2000). Finally, ASGP-R is thought to act as an entry point for a few specified pathogenic viruses to the hepatocytes. Experimental data indicate that the Marburg virus (Becker et al. 1995), the hepatitis B virus (Owada et al. 2006; Treichel et al. 1994) and the hepatitis C virus (Saunier et al. 2003) are capable of binding to the receptor, followed by infection of the host cell. Results on the infectivity of HBV in vitro showed that ASGP-R may be a specific HBV receptor once viral particles are desialylated (Owada et al. 2006).

Geuze et al. (1984) compared the endocytotic pathways of the receptors for ASGP-R, mannose-6-phosphate ligands (M6PR), and polymeric IgA (IgA-R). All three were found within the Golgi complex, along the entire plasma membrane, in coated pits and vesicles, and within a compartment of uncoupling of receptors and ligand (CURL). The receptors occurred randomly at the cell surface, in coated pits and vesicles. Within CURL tubules ASGP-R and M6PR were co-localized, but IgA-R and ASGP-R displayed microheterogeneity. Thus, in addition to its role in uncoupling and sorting recycling receptor from ligand, CURL serves as a compartment to segregate recycling receptor (e.g. ASGP-R) from receptor involved in transcytosis (e.g. IgA-R).

33.6.1 Impact of ASGP-R Deficiency on the Development of Liver Injury

Function of ASGP receptor is impaired in pathological states such as liver disease among alcoholics. The knockout ASGP-receptor$^{-/-}$mice, after various toxic challenges (alcohol, anti-Fas, CCl_4 and LPS/galactosamine), consistently showed more liver injury than the wild-type animals. This suggested that ASGP receptor functions as a protective agent. Thus, receptor-mediated endocytosis may be a novel mechanism that may be involved in the induction of toxin-induced liver injury. However, it is likely that impaired clearance of apoptotic bodies, perturbations in extracellular matrix deposition, oxidative stress, and cytokine dysregulation may play roles in the progression of disease (Lee et al. 2009). Overall, results suggest a possible link between hepatic receptors and liver injury. In particular, adequate function and content of the ASGP receptor may provide protection against various toxin-mediated liver diseases.

33.7 ASGP-R: A Marker for Autoimmune Hepatitis and Liver Damage

33.7.1 Autoimmune Hepatitis

Antibodies to ASGP-R have prognostic value. Circulating anti-ASGP-R autoantibodies in autoimmune hepatitis is independent of geographic or ethnic criteria. Results in different ethnic groups suggest highest frequency (76%) of

anti-human ASGP-R in autoimmune hepatitis patients (11/24 U.S.; 21/25 European; 28/30 Japanese), particularly in those with active disease before treatment (53/62, 85%), and decreased in titer with response to immunosuppressive therapy.

The research has been focused on the hepatocytes by examining endocytosis using the ASGP-R pathway as a model and identified multiple ethanol-induced impairments in receptor function. The altered uptake of apoptotic cells via ASGP-R may result in the release of proinflammatory mediators, the introduction of autoimmune responses, and inflammatory injury to the tissue. It was found that uptake of apoptotic bodies is impaired in hepatocytes isolated from ethanol-fed animals compared to controls, and that this impairment is linked to altered ASGP-R function. There is an attempt to examine a link between ethanol-impaired ASGP-R function, apoptotic body accumulation, and inflammation in the liver (Casey et al. 2008; McVicker et al. 2002). Hemolysis in patients with advanced alcoholic liver disease is a common clinical problem and indicates an unfavorable prognosis. All patients with liver disease have a soluble variant of the human s-ASGP-R in their serum, as well as high titers of autoantibodies against this receptor. Examination of patients with alcoholic liver disease reveal a high incidence for s-ASGP-R (36%) and anti-ASGP-R (27%) in patients with alcoholic liver cirrhosis compared to patients with cirrhosis due to viral hepatitis. This suggests a non-immunological mechanism for hemolysis in patients with alcoholic liver disease, mediated through agglutination by a soluble variant of the human ASGP-R and mechanical shear stress (Hilgard et al. 2004).

33.7.2 Hepatocellular Carcinoma

ASGP-Rs expression is decreased in various chronic liver diseases and hepatoma. Although the active shedding of the receptors clearly occurs in vitro, the significance of these shed receptors in blood will require further studies. The expression of ASGP-R on human hepatocellular carcinoma (HCC) cells might be exploited to reduce the extrahepatic toxicity of DNA synthesis inhibitors by their conjugation with galactosyl-terminating peptides. The results clearly demonstrated that DNA synthesizing cancer cells expressed the ASGP-R on their surface. The presence of ASGP-R on cell plasma membrane in the majority of differentiated HCCs and its maintenance on proliferating cells encourages studies in order to restrict the action of the inhibitors of DNA synthesis of HCC cells by their conjugation with galactosyl-terminating carriers internalized through this receptor (Trerè et al. 1999). Both acute and chronic phenobarbital administration decreased the number of ASGP-Rs per cell. Partial hepatectomy had a similar effect on the number of receptors per cell (Evarts et al. 1985).

33.7.3 Extra-Hepatic ASGP-R

In humans, ASGP-R is predominantly expressed by hepatocytes, at an estimated density of 100,000–500,000 binding sites per cell (Wall and Hubbard 1981; Matsuura et al. 1982) but does occur extrahepatically in thyroid, in small and large intestines, and in the testis. There is evidence of expression of the receptor in the T-cell line Jurkat. Moreover, Tera-1 cells derived from human testis gave rise to a weak signal, indicating presence of ASGP-R (Park et al. 1998). In the kidney, there has been evidence both for and against its existence in mesangial cells. Studies support ASGP-R expression in cells originating from human bone intestine and kidney (Park et al. 2006; Seow et al. 2002). Primary renal proximal tubular epithelial cells have a functional ASGP-R, consisting of the H1 and H2 subunit which are capable of specific ligand binding and uptake (Seow et al. 2002).

33.8 ASGP-R: A Model Protein for Endocytosis

Transport of macromolecules into the cell by receptor-mediated endocytosis follows a complex series of intracellular transfers, passing through distinct environments. The ASGP-R constitutively enters cells via coated pits and delivers ligand to these intracellular compartments. In addition to being a model of receptor-mediated endocytosis, the presence of the receptor on hepatocytes provides a membrane-bound active site for cell-to-cell interactions, has made possible the selective targeting of chemotherapeutic agents and foreign genes, and has also been implicated as a site mediating hepatitis B virus uptake. Regulated expression of receptor subunits and their intracellular trafficking during biosynthesis and endocytosis has provided insights into the relationship of receptor structure to its overall function. As a marker of hepatocellular differentiation, its study has uncovered a unique response to intracellular guanosine 3′, 5′-cyclic monophosphate and translational regulation of the receptor.

Receptor-mediated endocytosis serves as a mechanism by which cells can internalize macromolecules like peptides and proteins. The ASGP-R has been the focus of several studies aiming to understand endocytosis via the clathrin-coated pit pathway. Following binding of ligand to the ASGP-R at the cell surface, the receptor-ligand complex is endocytosed via clathrin-coated pits and directed to endosomes, where the

complex dissociates. The carbohydrate ligand is targeted for degradation in lysosomes while the receptor recycles to the cell surface with a vacant binding site (Spiess 1990). Upon ligand binding, the ASGP-Rs cluster into clathrin-coated domains of the membrane, which in turn invaginates. Clathrin-coated pits are then formed and subsequently turned into coated vesicles, which are internalized by the cell. With few exceptions, receptor-mediated endocytosis of specific ligands is mediated through clustering of receptor-ligand complexes in coated pits on the cell surface, followed by internalization of the complex into endocytic vesicles. During this process, ligand-receptor dissociation occurs, most probably in a low pH prelysosomal compartment. In most cases the ligand is ultimately directed to the lysosomes, wherein it is degraded, while the receptor recycles to the cell surface. In a human hepatoma cell line, Ciechanover et al. (1983) gained some insight into the complex mechanisms which govern receptor recycling as well as ligand sorting and targeting and explained why transferrin is exocytosed intact from the cells, while asialoglycoproteins are degraded in lysosomes. The receptor appears to be a transmembrane protein and is localized both to the cell surface as well as to several membranous intracellular compartments (Schwartz 1984). The ASGP-Rs meet a less grim fate as they are recycled and transported back to the plasma membrane (Geffen and Spiess 1992; Spiess 1990).

Recycling of the ASGP-R is a continuous process and occurs several hundred times during the life span of an individual receptor, the time of which is estimated to approximately 30 h (Schwartz 1984). Internalization of the ASGP-R takes place independently of ligand binding, but was shown to increase 2-fold upon binding of ASOR. Speculations attribute this enhanced internalization rate to a possible conformational change induced by ligand binding (Bider and Spiess 1998). Furthermore, a tyrosine residue in H1 has been shown to be of critical importance for efficient endocytosis of the ASGP-R. The corresponding residue in H2, a phenylalanine, does not appear to contribute to the internalization of hetero-oligomeric receptor complexes (Fuhrer et al. 1991, 1994).

Endosomal pH is an important determinant of recycling of the asialoglycoprotein receptor as well as other endocytic receptors (Mellman et al. 1986). The dissociation process that occurs in endosomes can be mimicked in vitro by ligand release at pH 5.4. In the case of the chicken hepatic lectin, an homologous endocytic receptor, pH modulates structural transitions between several distinct states of the CRD (Loeb and Drickamer 1988). At endosomal pH, the structural change causes an approximately 10-fold reduction in affinity for $Ca^{2+}$with concomitant loss of ligand binding activity. Site-directed mutagenesis of the CRD to residues His^{256}, Asp^{266}, and Arg^{270} singly and in combination indicate that these residues reduce the affinity of the CRD for Ca^{2+}, so that ligands are released at physiological Ca^{2+} concentrations. The proximity of these three residues to the ligand-binding site at Ca^{2+} site 2 of the domain suggests that they form a pH-sensitive switch for Ca^{2+} and ligand binding. Introduction of histidine and aspartic acid residues into the mannose-binding protein CRD at positions equivalent to His^{256} and Asp^{266} raises the pH for half-maximal binding of ligand to 6.1. The results, as well as sequence comparisons with other C-type CRDs, confirm the importance of these residues in conferring appropriate pH dependence in this family of domains (Wragg and Drickamer 1999).

Glycans as Endocytosis Signals Animal cells internalize specific extracellular macromolecules (ligands) by using specialized cell surface receptors that operate through a complex and highly regulated process known as receptor-mediated endocytosis, which involves the binding, internalization, and transfer of ligands through a series of distinct intracellular compartments. For the uptake of a variety of carbohydrate-containing macromolecules, such as glycoproteins, animal cells use specialized membrane-bound lectins as endocytic receptors that recognize different sugar residues or carbohydrate structures present on various ligands. Studies of how the asialoglycoprotein receptor functions have led to the discovery of two functionally distinct, parallel pathways of clathrin-mediated endocytosis (called the State 1 and State 2 pathways), which may also be utilized by all the other endocytic recycling receptor systems. Weigel and Yik (2002) discussed the characteristics and physiological importance of ASGP-R as an example of how lectins can function as endocytic receptors.

33.9 ASGP-R for Targeting Hepatocytes

The ASGP-R on mammalian hepatocytes provides a unique means for the development of liver-specific carriers, such as liposomes, recombinant lipoproteins, and polymers for drug or gene delivery to the liver, especially to hepatocytes (Wu et al. 1998). The abundant receptors on the cells specifically recognize ligands with terminal galactose or N-acetylgalactosamine residues, and endocytose the ligands for an intracellular degradation process. The use of its natural ligand, i.e. asialofetuin, or synthetic ligands with galactosylated or lactosylated residues, such as galactosylated cholesterol, glycolipids, or galactosylated polymers has achieved significant targeting efficacy to the liver. There are several examples of successful targeted therapy for acute liver injury with asialofetuin-labeled and vitamin E-associated liposomes or with a caspase inhibitor loaded in sugar-carrying polymer particles, as well as for the delivery of an antiviral agent, 9-(2-phosphonylmethoxyethyl) adenine. Liposome-mediated

gene delivery to the liver is more difficult than to other organs, such as to lungs. Galactosylated polymers are promising for gene delivery, but require further studies to verify their potential applications (Wu et al. 2002) (see Chap. 46).

Labeling conventional liposomes with asialofetuin was seen to result in a significant increase of liver uptake, compared to unlabeled liposomes, after intravenous injection into mice. The enhanced uptake was most likely mediated by the ASGP-R (Wu et al. 1998). Another study used glycolipids containing a cluster galactoside moiety for targeting to ASGP-R. The liver uptake of the glycolipid-liposomes was estimated to exceed 80% compared to less than 10% for conventional liposomes after injection. DNA-galactosylated cationic liposome complexes show higher DNA uptake and gene expression in the liver parenchymal cells in vitro than DNA complexes with bare cationic liposomes. In the in vitro gene transfer experiment, galactosylated liposome complexes are more efficient than DNA-galactosylated poly(amino acids) complexes but they have some difficulties in their biodistribution control. On the other hand, introduction of mannose residues to carriers resulted in specific delivery of genes to non-parenchymal liver cells. These results suggest advantages of these glycosylated carriers in cell-specific targeted delivery of genes (Hashida et al. 2001).

In order to reduce the extrahepatic side-effects of antiviral nucleoside analogues in the treatment of chronic viral hepatitis, these drugs were conjugated with galactosyl-terminating macromolecules. The conjugates selectively enter hepatocytes after interaction of the carrier galactose residues with the ASGP-R present in large amounts and high affinity only on these cells. The validity of this chemotherapeutic strategy has been endorsed by a clinical study (Fiume et al. 1997). Receptors like ASGP-R provide unique opportunity to target liver parenchymal cells. The results obtained so far reveal tremendous promise and offer enormous options to develop novel DNA based pharmaceuticals for liver disorders in near future. The ^{99m}Tc-labeled asialoglycoprotein analog, TcGSA (galactosyl-human serum albumin-diethylenetriamine -pentaacetic acid) has been applied to human hepatic receptor imaging. This method is unique and provides information that is totally independent of the ICG test or Child-Turcotte Score (Kokudo et al. 2003; Pathak et al. 2008).

33.10 Macrophage Galactose-Type Lectin (MGL) (CD301)

33.10.1 Human MGL (CD 301)

Serial analysis of gene expression in monocyte-derived DCs, monocytes, and macrophages revealed that 7 of the 19 C-type lectin mRNAs are present in immature DCs. Two of these, the macrophage mannose receptor (MMR) and the macrophage lectin specific for galactose/N-acetylgalactosamine (MGL), were found only in immature DCs (Steinman et al. 2003). Subcloning and sequencing the amplified mRNA, revealed nucleotide sequences encoding seven different human MGL (hMGL) subtypes, which were apparently derived from alternatively spliced mRNA. In addition, the hMGL gene locus on human chromosome 17p13 contains one gene. A single nucleotide polymorphism was identified at a position in exon 3 that corresponds to the cytoplasmic region proximal to the transmembrane domain. Of all the splicing variants, the hMGL variant 6 C was expressed at the highest levels on immature DCs from all donors tested.

Macrophage galactose-type lectins are a family of type II C-type lectins expressed in connective tissue macrophages and in bone marrow-derived DCs (Mizuochi et al. 1997; Denda-Nagai et al. 2002). The human macrophage C-type lectin was homologous to galactose- and N- acetylgalactosamine-specific C-type macrophage lectins of rodents. In the putative CRD, deduced amino acid sequence revealed 60 and 63% homology to galactose- and N- acetylgalactosamine-specific C-type macrophage lectins of mice and rats, respectively. The *Mgl* gene consists of ten exons and has been mapped to mouse chromosome 11 (Tsuiji et al. 1999). The *MGL* gene has been mapped to human chromosome 17p13.2 and contains one gene. The chromosome localization of MGL is distinct from that of many C-type lectins, MMR, DEC-205, DCIR, langerin, DC-SIGN, and Dectin-1, claimed to be specific for DCs. A single nucleotide polymorphism was identified at a position in exon 3 that corresponds to the cytoplasmic region proximal to the transmembrane domain. Of all the splicing variants, the hMGL variant 6 C was expressed at the highest levels on immature DCs. It was proposed that hMGL is a marker of immature DCs and that it functions as an endocytic receptor for glycosylated antigens (Higashi et al. 2002a). MGL is also called DC-asialoglycoprotein receptor (DC-ASGP-R) or human macrophage lectin (HML) (Higashi et al. 2002b; Valladeau et al. 2001; Suzuki et al. 1996). The liver-specific ASGP-R is closest homolog of MGL.

33.10.2 Murine MGL1/MGL2 (CD301)

Murine bone marrow–derived immature DCs also bind and internalize α-*N* acetylgalactosaminides conjugated to soluble polyacrylamide (α-GalNAc polymers), whereas mature DCs and bone marrow cells did not. It was suggested that mMGL is transiently expressed on bone marrow–derived DCs during their development and maturation and seemed to be involved in the uptake of glycosylated antigens for presentation (Denda-Nagai et al. 2002).

There are two MGL genes in mice: *Mgl1*, and *Mgl2* (Tsuiji et al. 2002; Onami et al. 2002; Dupasquier et al. 2006). The *Mgl* family is known to have two homologous

genes in mice, *Mgl1* and *Mgl2*, and these two lectins have distinct carbohydrate recognition specificities, although their distinct roles have not yet been defined (Tsuiji et al. 2002; Oo-Puthinan et al. 2008). MGL1 and/or MGL2 are mainly expressed on macrophages and immature DCs, and that these cells were observed mainly in the connective tissue of various organs, especially in skin, large intestines, and lymph nodes (Mizuochi et al. 1997). MGL'CD301 recognizes terminal galactose and (GalNAc) residues as monosaccharides in a calcium-dependent manner (Imai and Irimura 1994; Sato et al. 1992; Yamamoto et al. 1994; Denda-Nagai et al. 2010). These lectins were found to be involved in the uptake of mucin-like GalNAc-conjugated polymers by murine bone marrow-derived and human monocyte-derived DCs (Higashi et al. 2002b; Denda-Nagai et al. 2002), which was thought to be an important process of antigen processing. *Mgl1*-deficient mice did not show obvious defects in lymphoid and erythroid homeostasis (Onami et al. 2002). In an in vivo study with mouse embryos, MGL1 was shown to function as an endocytic receptor for X-irradiation-induced apoptotic cells, whereas *Mgl1*-deficient mice showed retarded clearance of apoptotic cells in neural tubes (Yuita et al. 2005). It is also suggested that MGL1 regulates trafficking of MGL1-expressing cells from skin to lymph nodes (Chun et al. 2000a, b; Kumamoto et al. 2004). Antigen-induced inflammatory tissue formation in skin was abrogated in *Mgl1*-deficient mice (Sato et al. 2005a), suggesting that MGL1 functioned under inflammatory conditions. MGL2 expresses in the skin and the cutaneous LNs. It was highly restricted to DDCs in the skin and the LNs. Evidence indicates that hapten-incorporated DDCs are sufficient to induce CHS in vivo. The availability of MGL2 as a marker for DDCs suggested the contribution of $MGL2^+$ DDCs for initiating contact hypersensitivity (Kumamoto et al. 2009).

The sequence of mMGL2 is highly homologous to the mMGL, which should now be called mMGL1. The ORF of mMGL2 contains a sequence corresponding to a type II transmembrane protein with 332 amino acids having a single extracellular C-type lectin domain. The 3′-untranslated region included long terminal repeats of mouse early transposon. The *Mgl*2 gene spans 7,136 base pairs and consists of ten exons, similar to the genomic organization of mMGL1. The mMGL2 mRNA was also detected in mMGL1-positive cells. The soluble recombinant proteins of mMGL2 exhibited carbohydrate specificity for α- and β-GalNAc-conjugated soluble polyacrylamides, whereas mMGL1 preferentially bound Lewis X-conjugated soluble polyacrylamides (Tsuiji et al. 2002).

The MGL, expressed by DC and macrophages, mediates binding to glycoproteins and lipids that contain terminal GalNAc moieties. MGL represents an exclusive marker for myeloid-type APC. Dexamethasone increased MGL expression on DC in a time- and dose-dependent manner. In contrast, DC generated in the presence of IL-10 did not display enhanced MGL levels. Furthermore, dexamethasone and IL-10 also differentially regulated expression of other C-type lectins, such as DC-SIGN and mannose receptor. Results indicate that depending on the local microenvironment, DC can adopt different C-type lectin profiles, which could have major influences on cell-cell interactions, antigen uptake and presentation (van Vliet et al. 2006b).

33.10.3 Ligands of MGL

Ligands of Human MGL: Van Die et al. (2003) identified an exclusive specificity for terminal α- and β-linked GalNAc residues that naturally occur as parts of glycoproteins or glycosphingolipids. Specific glycan structures containing terminal GalNAc moieties, expressed by the human helminth parasite *Schistosoma mansoni* as well as tumor antigens and a subset of gangliosides, were identified as ligands for MGL. The dendritic cell-specific DC-SIGN is a receptor for Schistosoma 8 MGL recognition of terminal GalNAc residues (Van Die et al. 2003).

Exclusive specificity of human MGL for rare terminal GalNAc structures was revealed on the tumor-associated mucin MUC1 and CD45 on effector T cells. Tumor glycoproteins, such as carcinoembryonic antigen and MUC-1/MUC1-Tn are known to interact with MGL on APCs. In vivo studies in mice demonstrated the potency of targeting antigens to C-type lectins on antigen-presenting cells for anti-tumor vaccination strategies (Aarnoudse et al. 2006). Glycosylation changes during malignant transformation create tumor-specific carbohydrate structures that interact with C-type lectins on DCs. The detection of MGL positive cells in situ at the tumor site together with the modified glycosylation status of MUC1 to target MGL on DC suggests that MGL positive antigen presenting cells may play a role in tumor progression (Saeland et al. 2007). Tumor-associated Tn-MUC1 glycoform is internalized through the MGL and delivered to the HLA class I and II compartments in dendritic cells (Napoletano et al. 2007). Soluble model antigens are efficiently internalized by MGL and subsequently presented to responder CD4+ T cells. The tyrosine-5 residue in the YENF motif, present in the MGL cytoplasmic domain, was essential for the MGL-mediated endocytosis in CHO cells. MGL contributes to the antigen processing and presentation capacities of DC and may provide a suitable target for the initiation of anti-tumor immune responses (van Vliet et al. 2007). Findings implicate MGL in the homeostatic control of adaptive immunity. van Vliet et al. (2008b) discussed the functional similarities and differences between MGL orthologs and compared MGL to its closest homolog, the liver-specific ASGP-R (van Vliet et al. 2008). Evidence

indicates that both N-linked glycoproteins and distinct lipooligosaccharide glycoforms of *C. jejuni* are ligands for the human MGL and that the *C. jejuni* N-glycosylation machinery can be exploited to target recombinant bacteria to MGL-expressing eukaryotic cells (van Sorge et al. 2009).

Studies indicate the involvement of a C-type lectin, the MMR and the macrophage surface molecule MGL that binds influenza virus, both of which are known to be endocytic. Binding of influenza virus to MMR and MGL occurred independently of sialic acid through Ca^{2+}-dependent recognition of viral glycans by the CRDs of the two lectins. Thus, lectin-mediated interactions of influenza virus with the MMR or the MGL are required for the endocytic uptake of the virus into macrophages, and these lectins can thus be considered secondary or coreceptors with sialic acid for infection of this cell type (Upham et al. 2010).

Ligands of Murine MGLs: Despite the high similarity between the primary sequences of MGL1 and MGL2, they have different carbohydrate specificities, respectively, for Lewis X and a/b-GalNAc structures (Tsuiji et al. 2002). Earlier studies on COS-1 transfectants of MGL suggested a specificity for galactose and *N*-acetylgalactosamine as monosaccharides (Suzuki et al. 1996). In contrast, recombinant MGL displayed restricted binding to GalNAc (Iida et al. 1999). MGL1 shows high affinity for the Lewis-X trisaccharide among 111 oligosaccharides tested, whereas MGL2 preferentially bound globoside Gb4. Molecular modeling illustrated potential direct molecular interactions of Leu^{61}, Arg^{89}, and His^{109} in MGL2 CRD with GalNAc (Oo-Puthinan et al. 2008). NMR analyses of the MGL1-Lewis-X complex presented a Lewis-X binding mode on MGL1 where the galactose moiety is bound to the primary sugar binding site, including Asp^{94}, Trp^{96}, and Asp^{118}, and the fucose moiety interacts with the secondary sugar binding site, including Ala^{89} and Thr^{111}. Ala^{89} and Thr^{111} in MGL1 are replaced with arginine and serine in MGL2, respectively. The hydrophobic environment formed by a small side chain of Ala^{89} and a methyl group of Thr^{111} is a requisite for the accommodation of the fucose moiety of the Lewis-X trisaccharide within the sugar binding site of MGL1 (Sakakura et al. 2008; Oo-Puthinan et al. 2008). Using a glycan array, murine MGL1 was highly specific for Lewis-X and Lewis-A structures, whereas mMGL2, more similar to the human MGL, recognized GalNAc and galactose, including the O-linked Tn-antigen, TF-antigen and core 2. Strikingly, MGL2 interacted strongly to adenocarcinoma cells, suggesting a potential role in tumor immunity (Singh et al. 2009).

Specific glycan structures containing terminal GalNAc moieties, expressed by the human helminth parasite *Schistosoma mansoni* as well as tumor antigens and a subset of gangliosides, were identified as ligands for MGL. Results indicated an endogenous function for DC-expressed MGL in the clearance and tolerance to self-gangliosides, and in the pattern recognition of tumor antigens and foreign glycoproteins derived from helminth parasites (van Vliet et al. 2005).

33.10.4 Functions of MGL

The MGL on immature DCs is involved in mediating down-regulation of effector T cell function and T cell death by interaction with CD45 avoiding potentially harmful T cell activation (van Vliet et al. 2006a). Several lines of evidence suggest that MGL may also be involved in the trafficking of APC that express this α/β-GalNAc-specific lectin (van Vliet et al. 2005; Dupasquier et al. 2006; Chun et al. 2000a, b). MGL1/2-positive cells represent a distinct sub-population of macrophages, having unique functions in the generation and maintenance of granulation tissue induced by antigenic stimuli (Sato et al. 2005a). It is highly likely that MGL1-positive cells are not involved in tissue remodeling when inflammation is driven by nonspecific stimuli (Sato et al. 2005b). Mice DCs have two MGL genes, *Mgl*1 and *Mgl*2. A report demonstrates the involvement of GalNAc residues in antigen uptake and presentation by DCs that lead to $CD4^+$ T cell activation.

Pregnant mice with $Mgl1^{+/-}$ genotype were mated with $Mgl1^{+/-}$ or $Mgl1^{-/-}$genotype males, and the embryos were used to assess a hypothesis that this molecule plays an important role in the clearance of apoptotic cells. After X-ray irradiation at 1 Gy of developing embryos at 10.5 days post coitus (d.p.c.), the number of $Mgl1^{-/-}$ pups was significantly reduced as compared with $Mgl1^{+/+}$ pups. Results strongly suggest that MGL1 is involved in the clearance of apoptotic cells (Yuita et al. 2005).

MGL induction in Activated Macrophages by Parasitic Infections: The expression of the two members of the mouse mMGL1 and mMGL2 is induced in diverse populations of alternatively activated macrophages (aaMF), including peritoneal macrophages elicited during infection with the protozoan *Trypanosoma brucei brucei* or the Helminth *Taenia crassiceps* and alveolar macrophages elicited in a mouse model of allergic asthma. In addition, interleukin-4 (IL-4) and IL-13 up-regulate mMGL1 and mMGL2 expression in vitro, and that in vivo, induction of mMGL1 and mMGL2 is dependent on IL-4 receptor signaling. Moreover, expression of MGL on human monocytes is also up-regulated by IL-4 (Raes et al. 2005).

Initiation of Contact Hypersensitivity In Vivo: The MGL, expressed in immature DCs, mediates binding to glycoproteins carrying GalNAc moieties. MGL ligands are

present on the sinusoidal and lymphatic endothelium of lymph node and thymus, respectively. MGL binding strongly correlated with the expression of the preferred MGL ligand, α-GalNAc-containing glycan structures. MGL^+ cells were localized in close proximity of the endothelial structures that express the MGL ligand. Strikingly, instead of inducing migration, MGL mediated retention of human immature DCs, as blockade of MGL interactions enhanced DC trafficking and migration. Thus, MGL^+ DCs are hampered in their migratory responses and only upon maturation, when MGL expression is abolished; these DCs will be released from their MGL-mediated restraints (van Vliet et al. 2008a).

Anti-Inflammatory Role in Murine Experimental Colitis: Inflammatory bowel disease is caused by abnormal inflammatory and immune responses to harmless substances, such as commensal bacteria, in the large bowel. MGL1 expressed on intestinal lamina propria macrophages functions through its inter-action with commensal bacteria by magnifying the IL-10 production by these cells. Results in $Mgl1^{-/-}$ mice and their wild-type littermates strongly suggest that MGL1/CD301a plays a protective role against colitis by effectively inducing IL-10 production by colonic lamina propria macrophages in response to invading commensal bacteria (Saba et al. 2009). Mgl1 is not required for the trafficking of type 2 Adipose tissue macrophages to adipose tissue. But MGL1 is a novel regulator of inflammatory monocyte trafficking to adipose tissue in response to diet-induced obesity (Westcott et al. 2009).

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Dectin-1 Receptor Family

34

Rajesh K. Gupta and G.S. Gupta

34.1 Natural Killer Gene Complex (NKC)

Natural killer (NK) cell receptors belong to two unrelated, but functionally analogous gene families: the immunoglobulin superfamily, situated in the leukocyte receptor complex (LRC) and the C-type lectin receptors (CLRs) superfamily, located in the natural killer gene complex (NKC). Wong et al. (2009) described the largest NK receptor gene expansion seen to date and identified 213 putative C-type lectin NK receptor homologs in the genome of the platypus. Many have arisen as the result of a lineage-specific expansion. Orthologs of OLR1, CD69, KLRE, CLEC12B, and CLEC16p genes were also identified. The NKC is split into at least two regions of the genome: 34 genes map to chromosome 7, two map to a small autosome, and the remainder are unanchored in the current genome assembly. No NK receptor genes from the LRC were identified. The massive C-type lectin expansion and lack of Ig-domain-containing NK receptors represents the most extreme polarization of NK receptors found to date. This new data from platypus was utilized to trace the possible evolutionary history of the NK receptor clusters.

The myeloid cluster within NK gene complex comprises several CLRs genes of diverse and highly important functions in the immune system such as LOX-1 and DECTIN-1. The type II transmembrane CLRs are best known for their involvement in the detection of virally infected or transformed cells, through the recognition of endogenous (or self) proteinacious ligands. However, certain CLR families within the NKC, particularly those expressed by myeloid cells, recognize structurally diverse ligands and perform a variety of other immune and homoeostatic functions. One such family is the 'Dectin-1 cluster' of CLRs, which includes MICL (CLEC12A), CLEC-2 (CLEC1B), CLEC12B, CLEC9A, CLEC-1(CLEC-1A), in addition to Dectin-1 (CLEC7A), and LOX-1 (Fig. 34.1). Current understanding of these CLRs, high-lighting their ligands, functions and new insights into the underlying mechanisms of immunity and homeostasis has been reviewed by Huysamen and Brown (2009) and given in Table 34.1. The arrangement of genes within the primate cluster differs from the order and orientation of the corresponding genes in the rodent complex which can be explained by evolutionary duplication and inversion events. Analysis of individual genes revealed a high sequence conservation supporting the prime importance of the encoded proteins (Sattler et al. 2010; Sobanov et al. 2001).

34.2 β-Glucan Receptor (Dectin 1) (CLEC7A or CLECSF12)

β-1—>3-D-Glucans are biological response modifiers with potent effects on the immune system. A number of receptors are thought to play a role in mediating these responses, including murine Dectin-1, which was identified as a β-glucan receptor in humans. The receptor possessed a single C-type lectin-like CRD connected to the transmembrane region by a stalk and a cytoplasmic tail possessing an immunoreceptor tyrosine-based activation motif (ITAM). Dectin-1 is widely expressed in mouse tissues and acts as a pattern recognition receptor, recognizing a variety of carbohydrates containing β-1—>3- and/or β-1—>6-glucan linkages and intact *Saccharomyces cerevisiae* and *Candida albicans* (Brown and Gordon 2001). Dectin-2 and its isoforms, together with dectin-1, represent a unique subfamily of DC-associated C-type lectins (Ariizumi et al. 2000a).

34.2.1 Characterization of β-Glucan Receptor (Dectin 1)

Dectin-1 gene encodes a type II membrane-integrated polypeptide of 244 amino acids containing a single carbohydrate recognition domain motif at the COOH-terminal end. This molecule was expressed abundantly at both mRNA and

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protein levels by the XS52 DC line, but not by non-DC lines (Taylor et al. 2002). Dectin-1 mRNA was detected predominantly in spleen and thymus and in skin-resident DC, i.e. Langerhans cells. Dectin-1 was identified a 43-kDa glycoprotein in membrane fractions of XS52 DC line and from dectin-1 cDNA-transfected COS-1 cells. In vitro results suggest that dectin-1 on DC may bind to a ligand(s) on T cells, thereby delivering T cell co-stimulatory signals (Ariizumi et al. 2000b). Mouse Dectin 1 has a CRD consisting of six cysteine residues, which are highly conserved in C-type lectins. The human homologue of Dectin 1 is structurally and functionally similar to the mouse receptor. The human β-glucan receptor is a type II transmembrane receptor with a single extracellular CRD and an ITAM in its cytoplasmic tail.

Dectin-1 is regulated by different immune stimuli; GM-CSF and IL-4 up-regulate surface expression, whereas IL-10 and LPS down-regulate the expression (Willment et al. 2003). Murine dectin-1 has been suggested to have an alternative splice form that lacks the stalk region (Yokota et al. 2001). In humans, eight isoforms of the homologue of dectin-1 have been described (Willment et al. 2003). The most common isoforms, BGR-A and BGR-B (β-glucan receptor/dectin-1), represent full-length and stalkless isoforms, respectively, and both mediate the recognition of yeast particles in a β-glucan-dependent manner. The human homologue is expressed by cells similar to those in the mouse as well as by peripheral B cells and eosinophils (Willment et al. 2005). Human dectin-1 undergoes cell-specific isoform expression during monocyte maturation. Monocytes express both BGR-A and BGR-B, but during maturation to Mφ, the expression levels of BGR-A decrease with time. Immature DCs express high levels of both isoforms; however, DCs lose the expression of both isoforms when matured with LPS (Willment et al. 2005). RT-PCR analysis revealed that mice have at least two splice forms of dectin-1, generated by differential usage of exon 3, encoding the full-length dectin-1A and a stalkless MΦ dectin-1B. MΦ from BALB/c mice and genetically related mice expressed both isoforms in similar amounts, whereas MΦ from C57BL/6 and related mice mainly expressed the smaller isoform. Evidence suggests that dectin-1 isoforms are functionally distinct and indicate that differential isoform usage may represent a mechanism of regulating cellular responses to β-glucans (Heinsbroek et al. 2006). The Dectin-1 gene was

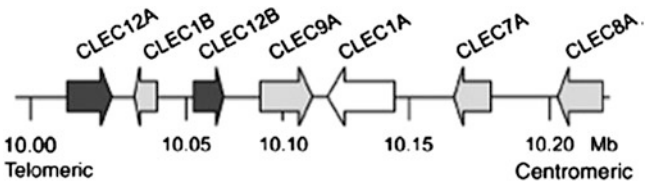

Fig. 34.1 Genomic organization of 'Dectin-1 cluster' in NKC on human chromosome 12. Activation receptors are shown in *light grey*, inhibitory receptors in *dark grey*, and those whose function is unclear are shown in *white* (Adapted with permission from Huysamen and Brown 2009 © John Wiley and Sons)

Table 34.1 Selected ligands and expression profiles of the Dectin-1 cluster of C-type lectin receptors (Adapted and modified from Huysamen and Brown 2009 © John Wiley and Sons)

Official name	Alternative name	Cell expression	Exogenous ligands	Endogenous ligands	Functions
CLEC7A	Dectin 1	Myeloid cells, B cells, Mast cells, T cell subsets, eosinophils	β-Glucon, mycobacterial ligands	T cells	Activation, apoptoic cells
CLEC12A	MICL, DCAL2, CLL1, KLRL1	Myeloid cells	?	Yes, identity unknown	Inhibition
CLEC18	CLEC2	Platelets, myeloid cells, B cells, $CD8^+$ T cells, NK cells in BM[a]	Rhodocytin, HIV	Podoplanin RACK1[b]	Activation, modulation of platelet activity
CLEC12B	Macrophage Antigen H	Macrophages	?	?	Inhibition
CLEC9A	DNGR1	$BDCA3^+$ DC, monocyte subsets, B-cells	?	?	Activation
CLEC1A	CLEC-1	DC	?	?	?
CLEC8A	Lox-1	Endothelium, smooth muscle, platelets, fibroblasts, macrophgages	Gram positive and Gram negative bacteria	ox-LDL, modified lipoproteins aged/apoptotic cells, AGE-products, HSP70	Activation

'?' refers to unknown
BM bone marrow, *RACK1* the receptor for activated C-kinase-1, *AGE-products* advanced glycation end products

localized in NKC on human Chromosome 12p12.3-p13.2, between OLR1 and CD94 (position 21.8 cM on genetic map). The Dectin-1 gene is highly expressed at the mRNA level in DCs and is not further up-regulated during the maturation of DCs (Hermanz-Falcón et al. 2001). The amino acid sequence Try^{221}-Ile^{222}-His^{223} seemed critical for formation of a β-glucan binding site in CRD of dectin 1 (Adachi et al. 2004).

34.2.2 Crystal Structure of Dectin-1

Brown et al. (2007) reported dectin-1 crystal structures, including a short soaked natural β-glucan, trapped in the crystal lattice. In vitro characterization of dectin-1 in presence of its natural ligand indicates higher-order complex between dectin-1 and β-glucans. These combined structural and biophysical results considerably extend the current knowledge of dectin-1 structure and function, and suggest potential mechanisms of defense against fungal pathogens. Amino acid analysis indicates that dectin-1 is a 28-kDa type II membrane protein. An extracellular C-type lectin-like domain (CTLD) is connected by a stalk to a transmembrane region, followed by a cytoplasmic tail containing an ITAM-like motif (Ariizumi et al. 2000b). First recognized as a calcium-dependent carbohydrate-binding domain, the CTLD fold is also seen in non-calcium dependent protein recognition interactions. Few of the residues required for calcium coordination in classical CTLDs are conserved in dectin-1. In classical CTLDs, the long loop region (LLR) contains residues responsible for calcium binding, but these residues are absent in dectin-1 and no metal ions stabilize the LLR. As expected from sequence analysis, the dectin-1 fold is similar to that of other long-form CTLDs (Brown et al. 2007) (Fig. 34.2), comprising two antiparallel β-sheets and two α-helices.

The N and C termini are close together with domain integrity maintained by three disulphide bridges—Cys^{119}-Cys^{130}, Cys^{147}-Cys^{240}, and Cys^{219}-Cys^{232}—stabilizing the LLR. For their CTLDs alone, the residue identity between murine dectin-1 and its human homolog β-glucon receptor is 59.5%, implying that the structure of murine dectin-1 provides a reasonably good model for the structure of the human protein. Among several hydrophobic side chains exposed to solvent, Trp^{221} and His^{223} deserve attention and are in agreement with results of Adachi et al. (2004), who suggested that side groups of these amino acids participate in ligand binding and are conserved in all Dectin-1 homologues. Xray analysis of Dectin-1 structure revealed a shallow surface groove running between the Trp^{221} and His^{223} side chains (Fig. 34.2).

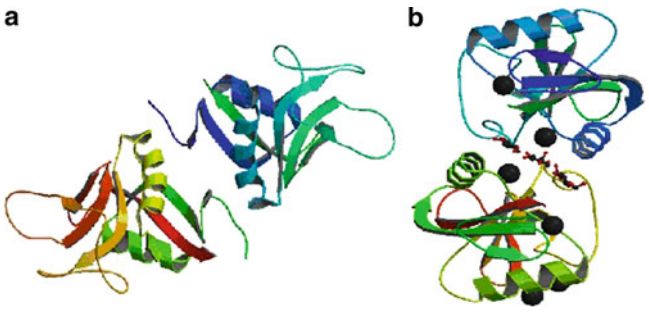

Fig. 34.2 Crystal structure of murine Dectin-1 (known as β–glucon receptor in humans). Two dectin-1 monomers (**a**) form a dimer into which a short β-glucan binds (**b**). (**a**) A cartoon diagram of the dectin-1 dimer (PDB: 2BPD), with each monomer from N terminus to C terminus. (**b**) Dectin-1 in complex with β-glucan (PDB: 2CL8). Disulphide linkages, the metal ion, and the bound β-glucan are shown

34.2.3 Interactions of Dectin-1 with Natural or Synthetic Glucans

Dectin-1 (CLEC7A) specifically recognizes β-D-(1—>3)-glucan, a polysaccharide and a component of fungal cell wall. As a result, dectin-1 is involved in recognition of fungi such as *C. albicans* and *Aspergillus fumigatus*. Due to the presence of (1—>3)-β-D-glucans on fungal cell walls, dectin 1 is considered important for recognizing fungal invasion. Although Dectin-1 is highly specific for β-D-(1—>3)-glucans, it does not recognize all glucans equally and interacts with β-D-(1—>3)-glucan over a very wide range of binding affinities (2.6 mM-2.2 pM). Among different polysachharides, Dectin-1 binding was detected exclusively to (1—>3)-linked glucose oligomers, the minimum length required for detectable binding being a 10- or 11-mer (Palma et al. 2006; Ujita et al. 2009). Dectin-1 can differentiate between glucan ligands based on structural determinants and can interact with both natural product and synthetic glucan ligands (Adams et al. 2008). Dectin-1 lacks residues involved in calcium ligation that mediates carbohydrate-binding by classical C-type lectins; nevertheless, it binds zymosan, a particulate β-glucan-rich extract of *Saccharomyces cerevisiae*, and binding is inhibited by polysaccharides rich in (1—>3)-β or both (1—>3)-β/(1—>6)-linked glucose (Palma et al. 2006). Dectin-1 is responsible for β-glucan-dependent, nonopsonic recognition of zymosan by primary macrophages and is an important target for examining the immunomodulatory properties of β-glucans for therapeutic drug design (Brown et al. 2002).

34.2.4 Regulation of Dectin-1

In mice, the expression of Dectin-1 can be influenced by various cytokines, steroids and microbial stimuli.

Interleukin-4 and IL-13, for example, which are associated with the alternative activation of macrophages, markedly increase the expression of Dectin-1 at the cell surface, whereas LPS and dexamethasone repress Dectin-1 expression (Willment et al. 2003). However, in *Pneumocystis*-infected mice, the mRNA levels of Dectin-1 gene decreased in AMΦs. This was associated with the decreased expression of Dectin-1 on the surface of these cells and reduced expression of mRNA of transcription factor PU.1 in AMΦs from *Pneumocystis*-infected mice. Down-regulation of PU.1 during *Pneumocystis* pneumonia appears to decrease the expression of Dectin-1 in AMΦs (Zhang et al. 2010). Leukocyte Dectin-1 levels are modulated in response to infections of fungal and nonfungal origin (Ozment-Skelton et al. 2009). Glucan phosphate (GP) resulted in a significant reduction in peripheral leukocyte membrane-associated Dectin-1 positivity. The systemic administration of GP has a specific and prolonged effect on loss of leukocyte membrane Dectin-1 positivity (Ozment-Skelton et al. 2006).

34.2.5 Signaling Pathways by Dectin-1

34.2.5.1 Raf-1 and CARD9 Dependent Pathways

Dectin-1-induced-signaling leads to the production of cytokines and non-opsonic phagocytosis of yeast by murine macrophages (LeibundGut-Landmann et al. 2007; Herre et al. 2004). Studies indicate that the alveolar macrophage inflammatory response, specifically the production of TNF-α, IL-1α, IL-1β, IL-6, CXCL2, CCL3-CSF, and GM-CSF, to live *A. fumigatus* is dependent on recognition via Dectin-1. The Dectin-1 is involved in calcium-independent recognition of β-(1—>3)-glucans exposed on particles such as zymosan, or many fungal species, including *Saccharomyces, Pneumocystis, Aspergillus* and *Candida* (Brown 2006; Brown et al. 2003; Heinsbroek et al. 2008). Signaling pathway activation of Dectin-1 depends on its ITAM the phosphorylation of which by Src kinase leads to the recruitment of spleen tyrosine kinase Syk in macrophages. Studies suggest that the increase in Dectin-1 expression by IL-4 involved the STAT signaling pathway (Murray 2007). Moreover, genetic models of macrophage specific peroxisome proliferator-activated receptor γ (PPARγ) or STAT-6 knockout mice showed that IL-4/IL-13/STAT-6/PPARγ axis is required for the maturation of alternatively activated macrophages (Odegaard et al. 2007; Ricote et al. 1998; Galès et al. 2010). In vitro and in vivo Dectin-1 is essential both to trigger the phagocytosis of non-opsonized *C. albicans* and the respiratory burst after yeast challenge and to control fungal gastrointestinal infection (Galès et al. 2010); the ManR alone is not sufficient to trigger antifungal functions during macrophage alternative activation. Interestingly, Dectin-1 and ManR were increased by IL-13 through the activation of the nuclear receptor PPARγ, suggesting that PPARγ could be a therapeutic target to eliminate fungal infection.

The intracellular signaling of Dectin-1 has been demonstrated to be mediated mainly by Raf-1 and Syk-adaptor molecule *C*aspase *A*ctivating *R*ecruitment *D*omain 9 (CARD9) dependent pathways to induce production of pro-inflammatory cytokines and reactive oxygen species (Underhill et al. 2005; Gringhuis et al. 2009; Rogers et al. 2005; Gross et al. 2006; Hara et al. 2007). Upon activation, dectin-1 recruites spleen Syk which in turn activates NF-κB, requiring the CARD9, a key adaptor for non-TLR signal transduction (Gross et al. 2006) and also has a critical function in NOD2-mediated activation of kinases p38 and Jnk, required for the production of pro-inflammatory cytokines in innate immune responses to intracellular pathogens (Hsu et al. 2007). LeibundGut-Landmann et al. (2007) showed that Dectin-1-Syk-CARD9 signaling induces DC maturation and secretion of pro-inflammatory cytokines like IL-6, TNF-α, IL-17 and IL-23 (Fig. 34.3). Several studies suggest that dectin-1 converges with Toll Like Receptor (TLR) signaling (Ferwerda et al. 2008; Dennehy et al. 2008) for the induction of cytokine responses and is able to promote Th17 and cytotoxic T-cell responses through activation of DCs (Gerosa et al. 2008; Leibundgut-Landmann et al. 2007; Gow et al. 2007). There is also evidence of Syk-dependent, but CARD9-independent, pathways, such as those leading to the induction of ERK, a MAP kinase regulating the Dectin-1-mediated production of cytokines, particularly IL-10 and IL-2 (Slack et al. 2007; Dillon et al. 2006).

Dectin-1 can also induce intracellular signaling through Syk-independent pathways. Phagocytosis in macrophages, for example, does not require Syk, although this response still involves the ITAM-like motif of the receptor (Brown 2006). These pathways are still largely uncharacterised, but Dectin-1 was found to induce a Syk-independent pathway involving the serine–threonine kinase Raf-1 (Gringhuis et al. 2009). This pathway was shown to integrate with the Syk pathway, at the level of NF-κB, and to be involved in controlling Dectin-1 mediated cytokine production. Thus, dectin-1 activates two independent signaling pathways, one through Syk and one through Raf-1, to induce immune responses.

Dectin-1 signaling can also directly modulate gene expression via activation of NFAT. Dectin-1-triggered NFAT activation plays a role in the induction of early growth response transcription factors, and cyclooxygenase-2. Furthermore, NFAT activation regulates IL-2, IL-10 and IL-12 p70 production by zymosan-stimulated DCs. This study establishes NFAT activation in myeloid cells as a novel mechanism of regulation of innate antimicrobial

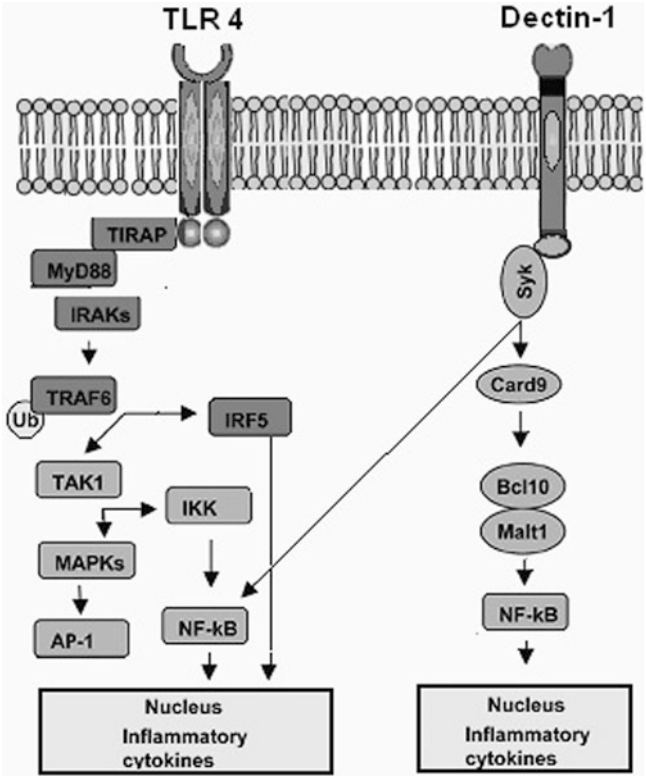

Fig. 34.3 Signaling pathway downstream of TLR4 and Dectin-1. Antigen presenting cells (APCs), including monocytes, macrophages and DCs, engage pathogens (fungi) and activate host responses via several PRRs including the Toll-like receptors (*TLR-4*) and Dectin-1. Dectin-1 is alternatively spliced into two functional isoforms, which differ by the presence or absence of a stalk region (shown in *dark black*). Dectin-1 recognises linear or branched 1—>3-linked β-glucan, which triggers intracellular signaling through at least two pathways, involving Syk kinase (shown in Figure) and Raf-1(not shown in Figure). Upon zymosan recognition, Dectin-1 can recruit Syk, and subsequently, Card9 relays the signal to the Bcl10-Malt1 complex to activate NF-κB, thus leading to the expression of inflammatory cytokines (IL-6, IL-10, IL-12, IL-17, IL-23, TNF). TLRs, on the contrary, which recognise various mannosylated and other fungal cell wall structures, signal through the MyD88-Mal mediated NF-κB pathway and induce the production of both pro and anti-inflammatory cytokines, including TNF, IL-10, IL-12 and TGFβ. Costimulation of both receptors can amplify the production of cytokines, including TNF, IL-23, IL-10 and IL-6 while downregulating the production IL-12, influencing the resultant generation of adaptive immunity (Lee and Kim 2007; Reid et al. 2009)

response (Goodridge et al. 2007). DCs stimulated through Dectin-1 can generate efficient Th, CTL and B cell responses and can therefore be used as effective mucosal and systemic adjuvants in humans (Agrawal et al. 2010). Engagement of Dectin-1 with β-glucan on the surface of murine primary microglia results also in an increase in tyrosine phosphorylation of Syk, a feature of Dectin-1 signaling pathway. Dectin-1 pathway may play an important role in antifungal immunity in the CNS (Shah et al. 2008).

34.2.5.2 PLCγ2 Is Critical for Dectin-1-Mediated Ca^{2+} Flux and Cytokine Production in DCs

Stimulation of DCs with zymosan triggers an intracellular Ca^{2+} flux that can be attenuated by a blocking anti-Dectin-1 antibody or by pre-treatment of cells with PLCγ-inhibitor U73122. This suggests that Dectin-1 could elicit Ca^{2+} signaling through PLCγ2 in Dectin-1 signal transduction pathway (Xu et al. 2009b). Lipid rafts are plasma membrane microdomains that are enriched in cholesterol, glycosphingolipids, and glycosylphosphatidylinositol-anchored proteins and play an important role in the signaling of ITAM-bearing lymphocyte antigen receptors. It was demonstrated that Dectin-1 translocates to lipid rafts upon stimulation of DCs with β-glucan. In addition, two key signaling molecules, Syk and PLCγ2 are also recruited to lipid rafts upon the activation of Dectin-1, suggesting that lipid raft microdomains facilitate Dectin-1 signaling. Xu et al. (2009a) indicated that Dectin-1 and perhaps also other CTLDs are recruited to lipid rafts upon activation and that the integrity of lipid rafts is important for the signaling and cellular functions initiated by this class of innate receptors.

34.2.5.3 Activation and Regulation of Phospholipase A2

Dectin-1 also mediates respiratory burst (Gantner et al. 2003) and its involvement has been suggested in the activation and regulation of phospholipase A2 (PLA2) and cyclooxygenase-2 (COX-2) (Suram et al. 2006). Secretory PLA_2 ($sPLA_2$) translocates from Golgi and recycling endosomes of mouse peritoneal macrophages to newly formed phagosomes and regulates the phagocytosis of zymosan, suggesting a role in innate immunity. The $sPLA_2$ regulates phagocytosis and contributes to the innate immune response against *C. albicans* through a mechanism that is likely dependent on phagolysosome fusion (Balestrieri et al. 2009). Co-stimulation of dectin-1 and DC-SIGN triggers the arachidonic acid cascade in human monocyte-derived DCs through both opsonic and nonopsonic receptors. The FcγR route depends on ITAM/Syk/cytosolic phospholipase A_2 axis, whereas the response to zymosan involves the interaction with dectin-1 and DC-SIGN (Valera et al. 2008).

34.2.6 Functions of Dectin-1

34.2.6.1 Recognition of Pathogens and Antifungal Defense

Dectin-1 has been shown to be involved in AMΦ recognition, nonopsonic phagocytosis, and killing of *Pneumocystis* organisms both in vitro and in vivo. Dectin-1 is a major β-glucan receptor on the surface of macrophages, DCs, neutrophils and it is also expressed on certain lymphocytes

(Taylor et al. 2002), subpopulations of MΦs in splenic red and white pulp, Kupffer cells, and MΦs and DCs in the lamina propria of gut villi (Reid et al. 2004). This is consistent with its role in pathogen surveillance. Tissue localization thus revealed potential roles of Dectin-1 in leukocyte interactions during innate immune responses and T cell development. Dectin-1 functionally interacts with leukocyte-specific tetraspanin CD37. Dectin-1 and CD37 colocalize on the surface of human APCs. Tetraspanin CD37 is important for dectin-1 stabilization in APC membranes and controls dectin-1-mediated IL-6 production (Meyer-Wentrup et al. 2007).

RAW 264.7 macrophages overexpressing Dectin-1 bind *Pneumocystis* organisms (Steele et al. 2003) and increases macrophage-dependent killing of *Pneumocystis* (Gross et al. 2006; Rapaka et al. 2007). Dectin-1-deficient mice have compromised clearance of *Pneumocystis* (Saijo et al. 2007) and *Candida albicans* (Taylor et al. 2007), as well as attenuated macrophage inflammatory responses to these organisms. Dectin-1 is centrally required for the generation of alveolar macrophage proinflammatory responses to *A. fumigatus* and provides in vivo evidence for the role of dectin-1 in fungal innate defense (Leal et al. 2010; Steele et al. 2005). Naive mice lacking Dectin-1 (Dectin-$1^{-/-}$) are more sensitive to intratracheal challenge with *A. fumigatus* than control mice, exhibiting >80% mortality within 5 days, ultimately attributed to a compromise in respiratory mechanics (Werner et al. 2009). A fusion protein consisting of Dectin-1 extracellular domain linked to the Fc portion of murine IgG1 augmented alveolar macrophage killing of *A. fumigatus* and shifted mortality associated with IPA via attenuation of *A. fumigatus* growth in the lung (Mattila et al. 2008). A role for Dectin-1 in promoting M. tuberculosis-induced IL-12p40 production by DC has been suggested by Rothfuchs et al. (2007) in which the receptor augments bacterial-host cell interaction and enhances the subsequent cytokine response through an unknown mechanism involving Syk signaling. Non-typeable *Haemophilus influenzae* (NTHi) can induce an innate inflammatory response in eosinophils that is mainly mediated via β-glucan receptors (Ahren et al. 2003; Weck et al. 2008). Dectin-1 is a potential targeting molecule for immunization and has implications for the specialization of DC subpopulations (Carter et al. 2006).

34.2.6.2 Collaborative Responses Mediated by Dectin-1 and TLR2

During fungal infection, a variety of receptors initiates immune responses, including TLR and the Dectin-1. TLR recognition of fungal ligands and subsequent signaling through myeloid differentiation factor 88 (MyD88) pathway (Fig. 34.3) was thought to be the most important interactions required for the control of fungal infection. However, recent studies have highlighted the role of Dectin-1 in induction of cytokine responses and the respiratory burst. Mitogen-activated protein kinase (MAPK) activation and TNF-α production in macrophage infected with *Mycobacterium avium* or *M smegmatis* is dependent on MyD88 and TLR2 but not TLR4, ManR, or CR3. Interestingly, the TLR2-mediated production of TNF-α by macrophages infected with *M smegmatis* requires dectin-1. A similar requirement for Dectin-1 in TNF-α production was observed for macrophages infected with *M bovis Bacillus Calmette-Guerin* (BCG), *M. phlei, M avium 2151*-rough, and *M tuberculosis H37Ra*. Studies established a significant role for Dectin-1, in cooperation with TLR2, to activate a macrophage's proinflammatory response to a mycobacterial infection (Yadav and Schorey 2006)

Saijo et al. (2007) argue that Dectin-1 plays a minor role in control of *Pneumocystis carinii* by direct killing and that TLR-mediated cytokine production controls *P. carinii* and *C. albicans*. By contrast, Taylor et al. (2007) argue that Dectin-1-mediated cytokine and chemokine production, leading to efficient recruitment of inflammatory cells, is required for control of fungal infection. Dennehy and Brown (2007) argued that collaborative responses induced during infection may partially explain these apparently contradictory results. It appears that Dectin-1 can mediate their own signaling, as well as synergize with TLR to initiate specific responses to infectious agents (Dennehy et al. 2009).

The DCs activated via Dectin can convert T_{reg} to IL17 producing cells (Osorio et al. 2008). Furthermore, they also prime cytotoxic T-lymphocyte (CTL) and mount potent CTL responses (LeibundGut-Landmann et al. 2008). Dectin-1 also induces antibody production in rodents (Kumar et al. 2009). Thus, DCs stimulated through Dectin-1 can generate efficient Th, CTL and B cell responses and can therefore be used as effective mucosal and systemic adjuvants in humans (Agrawal et al. 2010).

34.2.7 Genetic Polymorphism in Relation to Pathology

CARD9 is a susceptibility locus for inflammatory bowel disease (IBD) (Zhernakova et al. 2008). Dectin-1 polymorphism c.714T>G on chromosome 12p13 has been described with a transition from a tyrosine to an early stop codon on amino acid position 238 (p.Y238X) (Ferwerda et al. 2009). The functional consequence of this polymorphism is a complete loss of function, and immune cells expressing this truncated protein produce significantly less cytokines, including TNF-α, IL-1β and IL-17, upon in vitro stimulation with β-glucan or *C. albicans* (Plantinga et al. 2009). Th17 responses are considered to be involved in the pathogenesis

of auto-immune diseases. Interestingly, both NOD2 [Nucleotide-binding oligomerization domain-containing protein 2 (also known as caspase recruitment domain-containing protein 15 (CARD15)] and Dectin-1 are shown to be capable of inducing Th17 responses after activation (LeibundGut-Landmann et al. 2007; van Beelen et al. 2007). In this respect, the Dectin-1 c.714T>G polymorphism could influence the Th17 response towards fungi such as *C. albicans* in gastrointestinal tract. de Vries et al. (2009) demonstrated that Dectin-1 expression is elevated on macrophages, neutrophils, and other immune cells involved in the inflammatory reaction in IBD. The Dectin-1 c.714T>G polymorphism however, is not a major susceptibility factor for developing IBD.

It has been well established that fungal particles, either intact yeast or fungal cell wall components that can be recognized by dectin-1, such as zymosan, can act as adjuvants in several experimental models of RA (Leibundgut-Landmann et al.. 2007; Frasnelli et al. 2005; Hida et al. 2007). In addition, Yoshitomi et al. (2005) revealed that β-glucan induced autoimmune arthritis in genetically susceptible SKG mice could be prevented by blocking the dectin-1 receptor. These studies imply that dectin-1 plays a pivotal role in the innate immune system and is able to modulate adaptive immune responses, of which, especially Th17 responses are implicated in immunopathology. Furthermore, Dectin-1 is involved in the induction of arthritis in mouse models through induction of intracellular signaling on recognition of fungal components. As a consequence, dectin-1 mediated inflammatory responses could contribute to the aetiology or disease severity of RA. An early stop codon polymorphism Y238X (c.714T>G, rs16910526) in Dectin-1 (Veerdonk et al. 2009) has resulted in a complete loss of function of the protein. Cytokine production capacity of peripheral blood mononuclear cells (PBMCs) from individuals homozygous for the Dectin-1 Y238X polymorphism on β-glucan or *C. albicans* exposure are impaired, including TNF-α, interleukin (IL-)1β, IL-6, and IL-17 responses. In the same assays, individuals heterozygous for Dectin-1 Y238X polymorphism exhibited intermediate cytokine responses compared with wild-type individuals (Ferwerda et al. 2009). Considering both the involvement of Dectin-1 in pro-inflammatory responses and the significant consequences of the Y238X polymorphism for Dectin-1 function, Plantinga et al. (2010) suggest that Dectin-1 Y238X polymorphism does not play a role in the pathogenesis of RA. Although expression of dectin-1 was high in synovial tissue of RA patients, and reduced cytokine production was observed in macrophages of individuals bearing Dectin-1 Y238X polymorphism, loss of one functional allele of Dectin-1 was not associated with either susceptibility to or severity of RA (Plantinga et al. 2010).

34.3 The C-Type Lectin-Like Protein-1 (CLEC-1) or CLEC-1A

34.3.1 *CLEC-1* Gene

The region telomeric of CD94 contains, in addition to the LOX-1 and DECTIN-1, genes: the CLEC-1 and CLEC-2 genes within about 100 kb. Sequence similarities and chromosomal arrangement suggest that these genes form a separate subfamily of lectin-like genes within the NK gene complex. Human CLEC-1 displays a single CRD and a cytoplasmic tyrosine-based motif. It is homologous to the NK cell receptors NKG2s and CD94 and also to LOX-1and preferentially transcribes in DCs (Colonna et al. 2000; Sobanov et al. 2001). CLEC-1 is over-expressed in a model of rat allograft tolerance. CLEC-1 is expressed by myeloid cells and specifically by endothelial cells in tolerated allografts. CLEC-1 expression can be induced in endothelial cells by alloantigen-specific regulatory $CD4^{+}CD25^{+}$T cells. Expression of CLEC-1 is down-regulated by inflammatory stimuli but increased by the immunoregulators IL-10 or TGFβ. Interestingly, inhibition of CLEC-1 expression in rat DCs increases the subsequent differentiation of allogeneic Th17 T cells and decreases the regulatory $Foxp3^{+}$ T cell pool in vitro. In chronically rejected allograft, the decreased expression of CLEC-1 is associated with a higher production of IL-17. It is suggested that CLEC-1, expressed by myeloid cells and endothelial cells, is enhanced by regulatory mediators and moderates Th17 differentiation (Thebault et al. 2009).

34.4 CLEC-18 or the C-Type Lectin-Like Protein-2 (CLEC-2) or CLEC1B

34.4.1 Characterization

CLEC-1 and CLEC-18 (CLEC-2) possess a single CRD and a cytoplasmic tyrosine-based motif. Both are homologous to the NK cell receptors NKG2s and CD94 and also to LOX-1. CLEC-2 is expressed on the surface of transfected cells as a protein of approximately 33 kDa. The CLEC-2 is expressed on platelets and signaling through CLEC-2 is sufficient to mediate platelet aggregation (Suzuki-Inoue et al. 2006). The gene encoding CLEC-2 is located in the human NK complex on chromosome 12, along with the C-type lectin-like receptors NKG2D, LOX-1, and Dectin-1 (Sobanov et al. 2001). CLEC-2 is a type II transmembrane receptor, and its transcripts have been identified in immune cells of myeloid origin, including monocytes, DCs, and granulocytes, and in liver (Colonna et al. 2000). Human CLEC-2 has been reported to facilitate the capture of HIV-1. Xie et al.

(2008) identified two novel splicing variants of mCLEC-2 derived from omission of exon 2 and 2/4, respectively. These two variants had different expression profiles and subcellular localization from full-length mCLEC-2. Moreover, the full-length mCLEC-2 could be cleaved probably by proteases sensitive to aprotinin and PMSF into a soluble form that partially existed as a disulfide-linked homodimer. The interacting partner of the cytoplasmic region of CLEC-2 is RACK1, the receptor for activated C-kinase 1. Moreover, over-expression of RACK1 decreased the stability of CLEC-2 through promoting its ubiquitin-proteasome degradation, suggesting that RACK1 as a novel modulator of CLEC-2 expression (Ruan et al. 2009).

34.4.2 Ligands for CLEC-2

CLEC-2 has been shown to be a receptor on the surface of platelets for the snake venom toxin rhodocytin, which is produced by the Malayan pit viper *Calloselasma rhodostoma* (Shin and Morita 1998; Suzuki-Inoue et al. 2006) and the endogenous sialoglycoprotein podoplanin and functions as stimulator in platelet activation.

34.4.2.1 Rhodocytin/Aggretin

Aggretin, also known as rhodocytin, is a C-type lectin from *Calloselasma rhodostoma* snake venom. It is a potent activator of platelets, resulting in a collagen-like response by binding and clustering platelet receptor CLEC-2 (Suzuki-Inoue et al. 2006). The rhodocytin has been reported to bind to integrin α2β1 and glycoprotein (GP) Ibα on platelets, but it is also able to induce activation independent of two receptors and of GPVI. CLEC-2 in platelets confers signaling responses to rhodocytin when expressed in a cell line. CLEC-2 has a single tyrosine residue in a YXXL motif in its cytosolic tail, which undergoes tyrosine phosphorylation upon platelet activation by rhodocytin or an antibody to CLEC-2. The pathway in platelets included activation of CLEC-2 by rhodocytin, binding of Syk, initiation of downstream tyrosine phosphorylation, and the activation of PLCγ2. CLEC-2 is the first C-type lectin receptor on platelets which signals through this novel pathway (Suzuki-Inoue et al. 2006). Rhodocytin may induce cytokine TNF-α/IL-6 release after interaction with CLEC-2 and the subsequent MAPK and NF-kB activation in monocytes/macrophages (Chang et al. 2010).

The Rhodocytin/Aggretin at 1.7 A is an unique tetrameric quaternary structure. The two αβ heterodimers are arranged through twofold rotational symmetry, resulting in an antiparallel side-by-side arrangement. The Rhodocytin thus presents two ligand binding sites on one surface and can therefore cluster ligands in a manner reminiscent of convulxin and flavocetin. Molecular interaction of the rhodocytin with CLEC-2 was studied using molecular modeling after docking the aggretin αβ structure with the CLEC-2 N-terminal domain (CLEC-2N). This model positions the CLEC-2N structure face down in the "saddle"-shaped binding site which lies between the rhodocytin α and β lectin-like domains. A twofold rotation of this complex to generate the rhodocytin tetramer reveals dimer contacts for CLEC-2N which bring the N- and C-termini into the proximity of each other, and a series of contacts involving two interlocking β-strands close to N-terminus. A comparison with homologous lectin-like domains from the immunoreceptor family reveals a similar but not identical dimerization mode, suggesting this structure may represent the clustered form of CLEC-2 capable of signaling across the platelet membrane (Hooley et al. 2008). Rhodocytin displays a concave binding surface, which is highly complementary to the experimentally determined binding interface on CLEC-2. Using computational dynamic methods, surface electrostatic charge and hydrophobicity analyses, and protein-protein docking predictions, it was proposed that the $(\alpha\beta)_2$ rhodocytin tetramer induces clustering of CLEC-2 receptors on the platelet surface, which will trigger major signaling events resulting in platelet activation and aggregation (Watson et al. 2008).

34.4.2.2 Podoplanin as Ligand

Podoplanin (aggrus), a transmembrane mucin-like sialoglycoprotein, is involved in tumor cell-induced platelet aggregation, tumor metastasis, and lymphatic vessel formation. Podoplanin is an endogenous ligand for CLEC-2 and facilitates tumor metastasis by inducing platelet aggregation. Podoplanin induces platelet aggregation with a long lag phase, which is dependent upon Src and phospholipase Cγ2 activation. However, it does not bind to glycoprotein VI. This mode of platelet activation was reminiscent of the snake rhodocytin, the receptor of CLEC-2 (Suzuki-Inoue et al. 2006). Results suggest that CLEC-2 is a physiological target protein of podoplanin and imply that it is involved in podoplanin-induced platelet aggregation, tumor metastasis, and other cellular responses related to podoplanin (Kato et al. 2008; Suzuki-Inoue et al. 2007). In addition, indirect evidence indicates for an endogenous ligand for CLEC-2 in renal cells expressing HIV-1. Podoplanin is expressed on renal cells (podocytes) and acts as a ligand for CLEC-2 on renal cells (Christou et al. 2008). Incorporation of podoplanin into HIV released from HEK-293T cells is required for efficient binding to the attachment factor CLEC-2 (Chaipan et al. 2010). Mice, deficient in podoplanin, which is also expressed on the surface of lymphatic endothelial cells, show abnormal patterns of lymphatic vessel formation.

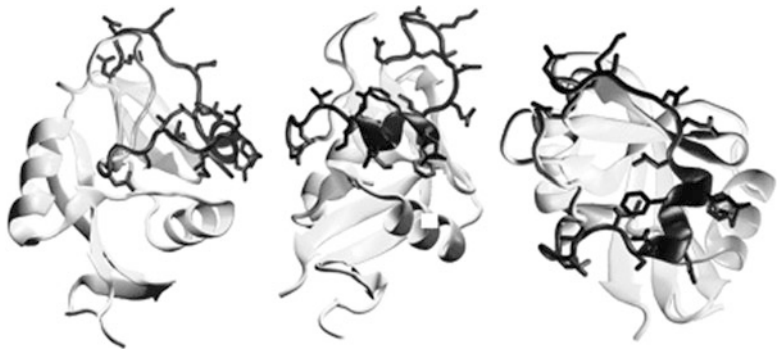

Fig. 34.4 Extracellular domain of the platelet-activating receptor CLEC-2: the semi-helical long loop region. Semi-helical long loop region dominates the upper surface of CLEC-2. The loop region is shown in *black* with the side chains illustrated. With respect to the *left panel*, the central panel is rotated 270° anticlockwise about the *y*-axis, and the *right panel* 90° anticlockwise about the *x*-axis. *B*, CLEC-2 is the only structurally characterized C-type lectin-like molecule with a formal helix in the long loop region (Adapted with permission from Watson et al. 2007 © American Society for Biochemistry and Molecular Biology)

Laminin VWF bridges exposed collagen, at damaged vessels, to GPIb. Subsequently, GPVI binds to collagen, leading to integrin α2β1 activation. Platelets also adhere to laminin, another major ECM component, through integrin α6β1, and are activated through GPVI. Laminin also interacts with VWF, leading to platelet adhesion via GPIb under sheer stress (Ozaki et al. 2009).

34.4.3 Crystal Structure of CLEC-2

The crystals of CLEC-2 belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 35.407, b = 55.143, c = 56.078 A. The presence of one molecule per asymmetric unit is consistent with a crystal volume per unit weight (V_M) of 1.82 A^3 Da^{-1} and a solvent content of 32.6% (Watson and O'Callaghan 2005). Watson et al. (2007) solved the crystal structure of the extracellular domain of CLEC-2 and identified the key structural features involved in ligand binding. A semi-helical loop region and flanking residues (Fig. 34.4) dominate the surface that is available for ligand binding. The precise distribution of hydrophobic and electrostatic features in this loop will determine the nature of any endogenous ligand with which it can interact. Major ligand-induced conformational change in CLEC-2 is unlikely as its overall fold is compact and robust. However, ligand binding could induce a tilt of 3–10 helical portion of long loop region in CLEC-2. Mutational analysis and surface plasmon resonance binding studies support these observations (Watson et al. 2007).

34.4.4 Functions of CLEC-2

34.4.4.1 Platelet Activation

CLEC-2 has been described to play crucial roles in thrombosis/hemostasis, tumor metastasis, and lymphangiogenesis. Platelets play an essential role in wound healing by forming thrombi that plug holes in the walls of damaged blood vessels. To achieve this, platelets express a diverse array of cell surface receptors and signaling proteins that induce rapid platelet activation. Platelet glycoprotein receptors that signal via an ITAM or an ITAM-like domain, namely collagen receptor complex GPVI-FcR γ-chain and CLEC-2, respectively, support constitutive (i.e. agonist-independent) signaling. The CLEC-2 mediates powerful platelet activation through Src and Syk kinases, but regulates Syk through a novel dimerization mechanism via a single YXXL motif known as a hemITAM. Inhibition of constitutive signaling through Src and Syk tyrosine kinases by G6b-B may help to prevent unwanted platelet activation (Mori et al. 2008; O'Callaghan et al. 2009). CLEC-2 is a receptor for podoplanin, which is expressed at high levels in several tissues (Section 34.4.2.2) but is absent from vascular endothelial cells and platelets. Platelet activation at sites of vascular injury is critical for primary hemostasis, but can also trigger arterial thrombosis in vascular disease. The ability of rhodocytin or CLEC-2-specific antibodies to trigger platelet aggregation in the absence of other stimuli indicates the potency with which CLEC-2 can modulate platelet activity (Suzuki-Inoue et al. 2006). Therefore, CLEC-2 is a potential therapeutic target in thrombotic cardiovascular disease. In addition, a molecular understanding of the effects of rhodocytin could lead to more effective therapy of snake envenomation (Chaipan et al. 2006). Phenotypes in CLEC-2-deficient mice are lethal at the embryonic/neonatal stages associated with disorganized and blood-filled lymphatic vessels and severe edema. Transplantation of fetal liver cells from Clec-$2^{-/-}$ demonstrated that CLEC-2 is involved in thrombus stabilization in vitro and in vivo, possibly through homophilic interactions without apparent increase in bleeding tendency. These results reveal an essential function of CLEC-2 in hemostasis and thrombosis (May et al. 2009;

Kerrigan et al. 2009; Watson et al. 2009). CLEC-2 could be an ideal novel target protein for an anti-platelet drug, which inhibits pathological thrombus formation but not physiological hemostasis (Suzuki-Inoue et al. 2010; Spalton et al. 2009).

34.4.4.2 Lymphatic Vascular Development Through CLEC-2-SLP-76 Signaling

Although platelets appear by embryonic day 10.5 in the developing mouse, an embryonic role for these cells has not been identified. The SYK-SLP-76 signaling pathway is required in blood cells to regulate embryonic blood-lymphatic vascular separation, but the cell type and molecular mechanism underlying this regulatory pathway are not known. Bertozzi et al. (2010) demonstrated that platelets regulate lymphatic vascular development by directly interacting with lymphatic endothelial cells through CLEC-2 receptors. Podoplanin, expressed on surface of lymphatic endothelial cells, is required in nonhematopoietic cells for blood-lymphatic separation. These studies identify a nonhemostatic pathway in which platelet CLEC-2 receptors bind lymphatic endothelial podoplanin and activate SLP-76 signaling to regulate embryonic vascular development.

CLEC-2, originally thought to be restricted to platelets, is also expressed by peripheral blood neutrophils, and only weakly by bone marrow or elicited inflammatory neutrophils. On circulating neutrophils, CLEC-2 can mediate phagocytosis of Ab-coated beads and the production of proinflammatory cytokines, including TNF-α, in response to CLEC-2 ligand, rhodocytin. Like dectin-1, CLEC-2 can recruit the signaling kinase Syk in myeloid cells, however, stimulation of this pathway does not induce the respiratory burst. Thus, CLEC-2 also functions as an activation receptor on neutrophils (Kerrigan et al. 2009).

34.4.5 CLEC-2 Signaling

The CLEC-2 and Dectin-1 have been shown to signal through a Syk-dependent pathway, despite the presence of only a single YXXL in their cytosolic tails. Stimulation of CLEC-2 in platelets and in two mutant cell lines is dependent on YXXL motif and on proteins that participate in signaling by ITAM receptors, including Src, Syk, and Tec family kinases, and on phospholipase Cγ. Results demonstrate that CLEC-2 signals through a single YXXL motif that requires the tandem SH2 domains of Syk but is only partially dependent on the SLP-76/BLNK family of adapters (Fuller et al. 2007). Ligand binding by CLEC-2 promotes phosphorylation of a tyrosine in the cytoplasmic domain YXXL motif of CLEC-2 by Src kinases and further downstream signaling events trigger platelet activation and aggregation. The snake venom protein rhodocytin and the endogenous protein podoplanin have been identified as ligands. The structures of CLEC-2 and rhodocytin suggest that ligand binding could cluster CLEC-2 molecules at the platelet surface and initiate signaling.

The signaling pathway used by CLEC-2 shares many similarities with that used by receptors that have 1 or more copies of an ITAM, defined by the sequence YXX(L/I)X(6–12)YXX(L/I), in their cytosolic tails or associated receptor chains. Evidence suggests that Syk activation by CLEC-2 is mediated by the cross-linking through the tandem SH2 domains with a stoichiometry of 2:1. Cross-linking and electron microscopy demonstrated that CLEC-2 is present as a dimer in resting platelets and converted into larger complexes on activation. This is a unique mode of activation of Syk by a single YXXL-containing receptor (Hughes et al. 2010a, b). Pollitt et al. (2010) demonstrated that CLEC-2 translocates to lipid rafts upon ligand engagement and that translocation is essential for hemITAM phosphorylation and signal initiation. HemITAM phosphorylation, but not translocation, is also critically dependent on actin polymerization, Rac1 activation, and release of ADP and thromboxane A_2 (TxA_2). This reveals a unique series of proximal events in CLEC-2 phosphorylation involving actin polymerization, secondary mediators, and Rac activation (Pollitt et al. 2010).

34.5 CLEC9A (DNGR-1)

CLEC9A is a group V C-type lectin-like receptor located in the "Dectin-1 cluster" of related receptors, which are encoded within the NK-gene complex. Expression of human CLEC9A is highly restricted in peripheral blood, being detected only on $BDCA3^+$ dendritic cells and on a small subset of $CD14^+CD16^-$ monocytes. CLEC9A is expressed at the cell surface as a glycosylated dimer and can mediate endocytosis, but not phagocytosis. CLEC9A possesses a cytoplasmic ITAM that can recruit Syk kinase. This receptor can induce proinflammatory cytokine production and can function as an activating receptor (Huysamen et al. 2008). Mouse *Clec9A* encodes a type II membrane protein with a single extracellular C-type lectin domain. Surface staining of mAbs against mouse and humans revealed that *Clec9A* was selective for mouse DCs and was restricted to the $CD8^+$ conventional DC and plasmacytoid DC subtypes. A subset of human blood DCs also expressed CLEC9A (Caminschi et al. 2008).

Sancho et al. (2008) characterized a C-type lectin of the NK cell receptor group that was named DC, NK lectin group receptor-1 (DNGR-1). DNGR-1 is expressed in mice at high levels by $CD8^+$ DCs and at low levels by plasmacytoid DCs but not by other hematopoietic cells. Human DNGR-1 was also restricted in expression to a small subset of blood DCs that bear similarities to mouse $CD8\alpha^+$ DCs. The selective

expression pattern and observed endocytic activity of DNGR-1 suggested that it could be used for antigen targeting to DCs. DNGR-1 is a highly specific marker of mouse and human DC subsets that can be exploited for CTL cross-priming and tumor therapy.

In the mouse, the CD8α$^+$ subset of dendritic cells phagocytose dead cell remnants and cross-primes CD8$^+$ T cells against cell-associated antigens. Sancho et al. (2009) showed that CD8α$^+$ DCs use CLEC9A (DNGR-1), to recognize a preformed signal that is exposed on necrotic cells. Loss or blockade of CLEC9A does not impair the uptake of necrotic cell material by CD8$^+$ DCs, but specifically reduces cross-presentation of dead-cell-associated antigens in vitro and decreases the immunogenicity of necrotic cells in vivo. The function of CLEC9A requires a key tyrosine residue in its intracellular tail that allows the recruitment and activation of the tyrosine kinase SYK, which is also essential for cross-presentation of dead-cell-associated antigens. Thus, CLEC9A functions as a SYK-coupled C-type lectin receptor to mediate sensing of necrosis by the principal DC subset involved in regulating cross-priming to cell-associated antigens. In addition, DNGR-1 is a target for selective in vivo delivery of antigens to DC and the induction of CD8$^+$ T-cell and Ab responses (Joffre et al. 2010; Poulin et al. 2010).

34.6 Myeloid Inhibitory C-Type Lectin-Like Receptor (MICL/CLEC12A)

Marshall et al. (2004) reported the characterization of a human myeloid inhibitory C-type lectin-like receptor (MICL) which is primarily restricted to myeloid cells, including granulocytes, monocytes, macrophages, and dendritic cells. MICL contains a single C-type lectin-like domain and a cytoplasmic ITIM, and is variably spliced and highly N-glycosylated. It preferentially associates with the signaling phosphatases SHP-1 and SHP-2, but not with SHIP (Huysamen and Brown 2009). Chimeric construct combining MICL and the β-glucan receptor showed that MICL can inhibit cellular activation through its cytoplasmic ITIM. MICL is a negative regulator of granulocyte and monocyte function (Marshall et al. 2004). Although MICL was highly N-glycosylated in primary cells, the level of glycosylation varied between cell types. MICL surface expression was down-regulated during inflammatory/activation conditions in vitro, as well as during an in vivo model of acute inflammation. This suggests that human MICL may be involved in the control of myeloid cell activation during inflammation (Marshall et al. 2006). The murine homolog of MICL (mMICL) is structurally and functionally similar to the human orthologue (hMICL), although there are some notable differences. mMICL is expressed as a dimer and is not heavily glycosylated; however, like hMICL, the receptor can recruit inhibitory phosphatases upon activation, and is down-regulated on leukocytes following stimulation with selected TLR agonists. Like the human receptor, mMICL is predominantly expressed by myeloid cells. However, mMICL is also expressed by B cells and CD8$^+$ T cells in peripheral blood, and NK cells in the bone marrow. The mMICL recognises an endogenous ligand in a variety of murine tissues, suggesting that the receptor plays a role in homeostasis (Pyz et al. 2008).

34.7 CLEC12B or Macrophage Antigen H

Hoffmann et al. (2007) described a new member of CTLD family, CLEC12B. The extracellular domain of CLEC12B shows considerable homology to the activating natural killer cell receptor NKG2D, but unlike NKG2D, CLEC12B contains an ITIM (VxYxxL) within its cytoplasmic tail in its intracellular domain. Human CLEC12B is widely expressed at low levels in various human tissues, except the brain, and that the receptor is alternatively spliced, generating at least two isoforms, one of which lacks part of CRD and predicted to be nonfunctional (Hoffmann et al. 2007). Despite the homology of CLEC12B to NKG2D, CLEC12B does not appear to bind NKG2D ligands and therefore does not represent the inhibitory counterpart of NKG2D. However, CLEC12B has the ability to counteract NKG2D-mediated signaling, and that this function is dependent on the ITIM and the recruitment of the phosphatases SHP-1 and SHP-2. This receptor plays a role in myeloid cell function.

34.8 CLECSF7 (CD303)

C-type lectin domain superfamily member 7 was previously called CLECSF11 (C-type lectin domain superfamily member 11). The new designation is CLEC4C (C-type lectin domain family 4 member C). It is also named as blood dendritic cell antigen 2 (BDCA-2) or dendritic lectin, CD303 and contains one C-type lectin domain. It functions in antigen-capturing and targets ligand into antigen processing and peptide-loading compartments for presentation to T-cells. It may mediate potent inhibition of induction of IFN- α/β. Its expression in plasmacytoid dendritic cells may act as a signaling receptor that activates protein-tyrosine kinases and mobilizes intracellular Ca^{+2}. CLECSF7 does not seem to bind mannose (Fernandes et al. 2000). *HECL* (HGMW-approved symbol *CLECSF7*) gene maps close to the NK gene complex on human chromosome 12p47. Sequence analysis revealed a complete ORF of

549 bp comprising several putative glycosylation and phosphorylation sites as well as a C-terminal C-type CRD. *HECL* exhibits a significant degree of divergence from the NKCs that comprise the NK gene complex. The NKC receptors all belong to group V of the C-type lectin superfamily, where as HECL, is most closely related to the sole group II C-type lectins reported to map near this region of the genome, the murine *Nkcl* and *Mpcl* genes. Like Nkcl, HECL is expressed in a variety of hematopoietic cell types and has a complete Ca^{2+}-binding site 2. Despite the presence of critical amino acids for sugar binding in Ca^{2+} binding site 2, HECL does not seem to bind carbohydrate. Moreover, HECL is the first non-receptor-like C-type lectin to map near the NK gene complex (Fernandes et al. 2000).

34.9 Lectin-Like Oxidized LDL Receptor (LOX-1) (CLEC8A)

Endothelial activation, accumulation of oxidatively modified low density lipoprotein (Ox-LDL) and intense inflammation characterize atherosclerotic plaque in acute myocardial ischemia. Endothelial dysfunction, or activation, elicited by Ox-LDL and its lipid constituents has been shown to play a key role in the pathogenesis of atherosclerosis. Ox-LDL induces expression of lectin-like receptor (LOX-1) on endothelial cells and leads to the expression of matrix metalloproteinases (MMPs), which destabilize atherosclerotic plaque. LOX-1 and scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX) are type II and I membrane glycoprotoeins, respectively, both of which can act as cell-surface endocytosis receptors for Ox-LDL. In vivo, endothelial cells that cover early atherosclerotic lesions, and intimal macrophages and activated vascular smooth muscle cells (VSMC) in advanced atherosclerotic plaques dominantly express LOX-1 (Adachi and Tsujimoto 2006). In association with Ox-LDL, LOX-1 is a marker of atherosclerosis that induces vascular endothelial cell activation and dysfunction, resulting in pro-inflammatory responses, pro-oxidative conditions and apoptosis. In addition to binding Ox-LDL, it acts as a receptor for HSP70 protein involved in antigen cross-presentation to naive T-cells in DCs, thereby participating in cell-mediated antigen cross-presentation. Soluble LOX-1 concentration in human blood also has been shown to be elevated in coronary heart diseases especially in acute coronary syndrome (Kume and Kita 2004). In addition to endothelial cells, LOX-1 is expressed in macrophages and smooth muscle cells accumulated in the intima of advanced atherosclerotic plaques in vivo. LOX-1 is critical in foam cell formation of macrophages (Mφ) and smooth muscle cells (SMC). Inhibition of LOX-1 expression reduces foam cell formation and might influence lipid core formation in atherosclerotic lesions. In contrast to LOX-1 expressed by a variety of cell types, SR-PSOX expression appeared relatively confined to macrophages in atherogenesis.

34.9.1 General Features of LOX-1

LOX-1, the lectin-like oxidized LDL receptor is the receptor for Ox-LDL and is abundantly expressed in endothelial cells; it might mediate some of the actions of Ox-LDL in the endothelium. LOX-1 is initially synthesized as a 40-kDa precursor protein with N-linked high mannose-type carbohydrate, which is further glycosylated and processed into a mature form of 52-kD. The mature form of LOX-1 is a lectin-like receptor and is a homodimer linked through disulfide bond. It may form a hexamer composed of three homodimers. LOX-1 belongs to the same family as NKR and functionally undergoes dimerization. The LOX-1 is a type II membrane protein, and acts as a cell-surface endocytosis receptor for atherogenic Ox-LDL, though secretory form of LOX-1 also exists. Its mRNA was shown to be expressed in human atheromatous lesions. LOX-1 can support binding, internalization and proteolytic degradation of Ox-LDL, but not of significant amounts of acetylated LDL, which is a well-known high-affinity ligand for class A scavenger receptors and scavenger receptor expressed by endothelial cells (Sawamura et al. 1997). Cell-surface LOX-1 can be cleaved through some protease activities that are associated with the plasma membrane, and released into the culture media. The N-terminal amino-acid sequencing identified two cleavage sites (Arg86-Ser87 and Lys89-Ser90), both of which are located in the membrane proximal extracellular domain of LOX-1. LOX-1 is often co-localized with apoptotic cells and implicated in vascular inflammation and atherosclerotic plaque initiation, progression, and destabilization (Kume and Kita 2001).

Mouse LOX-1 is composed of 363 amino acids and has a C-type lectin domain type II membrane protein structure. Mouse LOX-1 has triple repeats of the sequence in extracellular "Neck domain," which is unlike human and bovine LOX-1. The LOX-1 binds Ox-LDL with two affinity constants in the presence of serum. The binding component with higher affinity showed the lowest value of K_D among the known receptors for Ox-LDL (Hoshikawa et al. 1998). Measurement of soluble LOX-1 in vivo may provide a novel diagnostic tool for the evaluation and prediction of atherosclerosis and vascular disease (Kume and Kita 2001). Soluble forms of LOX-1 are present in conditioned media of cultured bovine aortic endothelial cells (BAECs) and CHO-K1 cells transfected with LOX-1 cDNA. In TNF-α-activated BAECs, cell-surface expression of LOX-1 precedes soluble LOX-1 production. The soluble LOX-1 in cell-conditioned media is derived from LOX-1 expressed on the cell surface (Murase et al. 2000).

LOX-1 expression is not constitutive, but can be induced by proinflammatory stimuli, such as TNF-α, TGF-β and bacterial endotoxin, and fluid shear stress. It is up-regulated in atherosclerotic lesions, by Ox-LDL, reactive oxygen species, suggesting that it may participate in amplification of Ox-LDL-induced vascular dysfunction. This receptor is upregulated by angiotensin II, endothelin, cytokines and all participants in atherosclerosis and is up-regulated in the arteries of hypertensive, dyslipidemic, and diabetic animals. Defects in LOX-1 gene (HGMW-approved symbol *OLR1*) may be a cause of susceptibility to myocardial infarction. Defects in *OLR1* may also be associated with susceptibility to Alzheimer disease (AD). Upregulation of LOX-1 has been identified in atherosclerotic arteries of several animal species and humans, not only on the endothelial lining, but also in choroidal neovasculature of the atherosclerotic plaque (Mehta et al. 2006; Mukai et al. 2004; Vohra et al. 2006).

The extracellular domains of LOX-1 are post-translationally modified by N-linked glycosylation. LOX-1 is synthesized as a 40-kDa precursor protein with N-linked high mannose carbohydrate chains (pre-LOX-1), which is subsequently further glycosylated and processed into the 48-kDa mature form. Both TNF-α-activated bovine aortic endothelial cells and CHO-K1 cells stably expressing bovine LOX-1 (BLOX-1-CHO) exclusively produced a 32-kDa deglycosylated form of LOX-, follwing treatment with an N-glycosylation inhibitor, tunicamycin. The deglycosylated form of LOX-1 is not efficiently transported to the cell surface, but is retained in the ER or Golgi apparatus in TNF-α-activated bovine aortic endothelial cells, but not in BLOX-1-CHO cells. It shows that N-linked glycosylation plays key roles in the cell-surface expression and ligand binding of LOX-1 (Kataoka et al. 2000).

34.9.2 LOX-1 Gene in Human

The natural killer (NK) gene complex is a genomic region containing lectin-type receptor genes located on human chromosome 12 that contains several families of lectin-like genes including the CD94 and NKG2 NK receptor genes (Fig. 34.1). Bull et al. (2000) established a contig of PAC and BAC clones comprising about 1 Mb of the centromeric part of the NK gene complex. This region extending from LOX-1 gene and found within 100 kb telomeric of STS marker D12S77, contains CD94 and NKG2 NK receptor genes and reaches beyond D12S852 on the proximal side.

The human LOX-1 has the highest homology with C-type lectin receptors expressed on NK cells. The human LOX-1 gene is a single-copy gene and assigned to p12.3-p13.2 region of chromosome 12. In contrast, the cellular expression pattern of LOX-1 is different from that of NK cell receptors. A 1,753-bp fragment of 5′ flanking region of LOX-1 gene had a functional promoter activity and contains binding sites for several transcription factors, including STAT family and NF-IL6. The 5′-regulatory region contained several potential cis-regulatory elements, such as GATA-2 binding element, c-ets-1 binding element, 12-O-tetradecanoylphorbol 13-acetate-responsive element and shear-stress-responsive elements, which may mediate the endothelium-specific and inducible expression of LOX-1. The major transcription-initiation site was found to be located 29 nt downstream of the TATA box and 61 nt upstream from the translation-initiation codon. The minor initiation site was found to be 5 bp downstream from the major site. Most of the promoter activity of the LOX-1 gene was ascribed to the region (−150 to −90) containing the GC and CAAT boxes. The coding sequence was divided into six exons by five introns. The first three exons corresponded to the different functional domains of the protein (cytoplasmic, transmembrane and neck domains), and the residual three exons encoded the carbohydrate-recognition domain similar to other C-type lectin genes. Since the locus for a familial hypertension has been mapped to the overlapping region, LOX-1 might be the gene responsible for the hypertension. The expression of LOX-1 was upregulated by several cytokines (Aoyama et al. 1999; Yamanaka et al. (1998)).

34.9.2.1 Rat LOX-1 Gene

The genomic organization of rat LOX-1 shows several consensus sequences in 59-flanking region. Rat LOX-1 cDNA clone encodes a single-transmembrane protein with its N terminus in the cytoplasm (Nagase et al. 1997). The extracellular region consists of a spacer, 46-amino acid triple repeats, and C-type lectin-like domains. The rat LOX-1 gene, encoded by a single copy gene spanning over 19 kb, consists of eight exons (Fig. 34.5). The promoter region contained putative TATA and CAAT boxes and multiple cis-elements such as NF-kB, AP-1 and AP-2 sites, and a shear stress response element. Exon boundaries correlated well with the functional domain boundaries of the receptor protein (Nagase et al. 1998b). The rat endothelial receptor LOX-1 cDNA encodes a protein of 364 amino acids that showed approximately 60% similarity to its bovine and human counterparts. The protein consisted of intracellular N-terminal, transmembrane and extracellular lectin-like domains. Rat LOX-1 is unique in having three repeats of a 46-amino-acid motif between the transmembrane and lectin-like regions. Two isoforms of mRNA were found to be generated by alternative use of two polyadenylation signals in a tissue-specific manner. The 3′-untranslated region contained multiple A + U-rich elements for rapid degradation of mRNA. Northern-blot analysis revealed that LOX-1 mRNA was expressed predominantly in the lung. Nagase et al. (1998b) reported an unexpected blood-pressure-

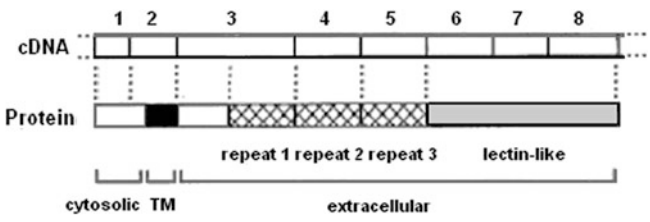

Fig. 34.5 Genomic organization of rat LOX-1. Schematic representation of the cDNA and protein. Exons in the cDNA and functional domains in the protein are indicated by *boxes*. The *solid box* represents the transmembrane (*TM*) domain. *Hatched boxes* represent the 46-amino acid repeat unit (*repeats 1–3*). The *dotted box* represents the C-type lectin-like domain (Adapted from Nagase et al. 1998; © American Society for Biochemistry and Molecular Biology)

associated regulation of LOX-1 expression, a new relation between lipid metabolism and blood-pressure control. The expression of LOX-1 was dramatically up-regulated in the aorta in hypertensive SHR-SP/Izm rats compared with very low levels in control WKY/Izm rats, suggesting a potential role for LOX-1 in the pathogenesis of hypertension as well as atherosclerosis.

34.9.3 Ligands for LOX-1

As a scavenger receptor, LOX-1 is capable of binding to a variety of structurally unrelated ligands. Ox-LDLs appear to play key roles in atherosclerotic progression and plaque rupture (Li et al. 2002b). LOX-1 is a major receptor for Ox-LDL in activated vascular smooth muscle cells (VSMC) as in endothelial cells. Human recombinant LOX-1 protein showed that the protein had specific Ox-LDL-binding activity (Huang et al. 2005). LOX-1 binds multiple classes of ligands that are implicated in the pathogenesis of atherosclerosis. Besides Ox-LDL, LOX-1 can recognize apoptotic/aged cells, activated platelets, and bacteria, implying versatile physiological functions (Chen et al. 2002; Shimaoka et al. 2001b). LOX- from bovine aortic endothelial cells (BAE) is the major binding protein for Ox-LDL on the surface of BAE and is expressed in atheromatous intima of human carotid artery as well as in intima of normal bovine aorta. In addition, human LOX-1 recognizes a key cellular phospholipid, PS (phosphatidylserine), in a Ca^{2+}-dependent manner, both in vitro and in cultured cells. A recombinant, folded and glycosylated LOX-1 molecule binds PS, but not other phospholipids. LOX-1 recognition of PS was maximal in presence of mM Ca^{2+} levels (Murphy et al. 2006). C-reactive protein (CRP), a risk factor for cardiovascular events and an amplifier of vascular inflammation through promoting endothelial dysfunction, can act as a novel ligand for LOX-1. This interaction could be disrupted with known LOX-1 ligands, such as Ox-LDL and carrageenan. In vivo assays revealed that in LOX-1 the basic spine arginine residues are important for binding, which is lost upon mutation of Trp^{150} with alanine. Simulations of the wild-type LOX-1 and of the Trp^{150}Ala mutant C-type lectin-like domains revealed that the mutation does not alter the dimer stability, but a different dynamical behaviour differentiates the two proteins. The symmetrical motion of monomers is completely damped by the structural rearrangement caused by the Trp^{150}Ala mutation. An improper dynamical coupling of the monomers and different fluctuations of the basic spine residues are observed, with a consequent altered binding affinity (Falconi et al. 2007). Mutagenesis studies demonstrated that the arginine residues forming the basic spine structure on LOX-1 ligand-binding interface were dispensable for CRP binding, suggesting a novel ligand-binding mechanism for LOX-1, distinct from that used for Ox-LDL binding. Study suggests that CRP- LOX-1 interaction may mediate CRP-induced endothelial dysfunction (Shih et al. 2009). Advanced glycation end product (AGE) is recognized by LOX-1. Cellular binding experiments revealed that AGE-bovine serum albumin (AGE-BSA) specifically binds CHO cells over-expressing bovine LOX-1 (bLOX-1). Cultured bovine aortic endothelial cells also showed specific binding for AGE-BSA (Jono et al. 2002). Interaction of AGE with AGE receptors induces several cellular phenomena potentially relating to diabetic complications. Study indicates that CD36-mediated interaction of AGE-modified proteins with adipocytes might play a pathological role in obesity or insulin-resistance (Kuniyasu et al. 2003).

Adhesion of bacteria to vascular endothelial cells as well as mucosal cells and epithelial cells appears to be one of the initial steps in the process of bacterial infection, including infective endocarditis. LOX-1 with C-type lectin-like structure, can support adhesion of bacteria and work as a cell surface receptor for Gram-positive and Gram-negative bacteria, such as *S. aureus* and *E. coli*, however, other unknown molecules may also be involved in the adhesion of *E. coli* to cultured bovine aortic endothelial cells, which is enhanced by poly(I) (Shimaoka et al. 2001a). Development of antagonists for LOX-1 might be a good therapeutic approach to vascular diseases (Chen et al. 2002)

34.9.4 Structural Analysis

34.9.4.1 Carbohydrate Recognition Domain

LOX-1 from human aortic ECs has a sequence identical to that from human lung. Human LOX-1 can recognize modified LDL, apoptotic cells and bacteria. The CRD is the ligand-binding domain of human LOX-1. LOX-1s carrying a mutation on each of six Cys in CRD resulted in a variety of N-glycosylation and failed to be transported to cell surface. This gave an evidence for the involvement of all six Cys in the intrachain disulfide bonds required for proper folding, processing and transport of LOX-1. The C-terminal sequence

(KANLRAQ) was also essential for protein folding and transport, while the final residues (LRAQ) were involved in maintaining receptor function. In addition, positively charged (R^{208}, R^{209}, H^{226}, R^{229} and R^{231}) and uncharged hydrophilic (Q^{193}, S^{198}, S^{199} and N^{210}) residues are involved in ligand binding, suggesting that ligand recognition of LOX-1 is not merely dependent on the interaction of positively charged residues with negatively charged ligands (Shi et al. 2001). Truncation of the lectin domain of LOX-1 abrogated oxLDL-binding activity (Chen et al. 2001). Deletion of the utmost C-terminal ten amino acid residues (261–270) was enough to disrupt the Ox-LDL-binding activity. Substitutions of Lys-262 and/or Lys-263 with Ala additively attenuated the activity. Serial-deletion analysis showed that residues up to 265 are required for the expression of minimal binding, although deletion of C-terminal three residues (268–270) still retained full binding activity. These results demonstrate distinct role of lectin domain as functional domain recognizing LOX-1 ligand.

To understand the interaction between hLOX-1 and its ligand Ox-LDL, Xie et al. (2003) reconstituted the functional C-type lectin-like domain (CTLD) of LOX-1 from inactive aggregates in *E. coli*. The CD spectra of the domain suggested that the domain has α-helical structure where as the blue shift of Trp residues indicated refolding of the domain. Like wild-type hLOX-1, the refolded CTLD was able to bind modified LDL. Thus, even though CTLD contains six Cys residues that form disulfide bonds, it recovered its specific binding ability on refolding. This suggests that the correct disulfide bonds in CTLD were formed by the artificial chaperone technique. Although the domain lacked N-glycosylation, it showed high affinity for its ligand in surface plasmon resonance experiments. Thus, unglycosylated CTLD is sufficient for binding modified LDL (Xie et al. 2003).

Though, the lectin-like domain of LOX-1 is essential for ligand binding, the neck domain is not. In particular, the large loop between the third and fourth cysteine of lectin-like domain plays a critical role for Ox-LDL binding as well as C-terminal end residues. Alanine-directed mutagenesis of the basic amino acid residues around this region revealed that all the basic residues are involved in Ox-LDL binding. Therefore, electrostatic interaction between basic residues in lectin-like domain of LOX-1 and negatively charged Ox-LDL is critical for the binding activity of LOX-1 (Chen et al. 2001). Chen et al. (2005) suggested that Ox-LDL/LOX-1 plays a role in signaling pathway in rat cardiac fibroblasts, which naturally express low levels of LOX-1. It was suggested that in cardiac fibroblasts, Ox-LDL binds to LOX-1 and activates p38 MAPK, followed by the expression of ICAM-1, VCAM-1, and MMP-1. Thus, fibroblasts transform into an endothelial phenotype on transfection with CMV-LOX-1wild-type (WT) and subsequent exposure to Ox-LDL.

Role of Cytoplasmic Sequences in Cell-Surface Sorting of LOX-1: The ability of LOX-1 binding to Ox-LDL can be distinguished from other NK receptors. Domain swapping of the lectin-like domain between LOX-1 and the NK cell receptors CD94, NKG2D, and LY-49A demonstrated the crucial role of this domain for recognition of Ox-LDL by LOX-1, but not for the correct cell-surface sorting of LOX-1. Using N-terminal deletions Chen and Sawamura (2005) found that the correct cell-surface localization is dependent on a positively charged motif present in the cytosolic juxtamembrane region of LOX-1. Furthermore, the extracellular localization of LOX-1 C-terminus is disrupted when cytoplasmic basic amino acids, Lys-22, Lys-23 and Lys-25 were mutated to Glu. These results indicated that the N-terminal cytoplasmic domain of LOX-1 determines the correct expression of lectin domain on the cell-surface.

Dominant-Negative LOX-1 Blocks Homodimerization of Wild-Type LOX-1-Induced Cell Proliferation: LOX-1 is thought to function as a monomer. Yet, Xie et al. (2004) suggest that human LOX-1 (hLOX-1) forms constitutive homo-interactions in vivo. Site-directed mutagenesis studies indicated that Cys^{140} has a key role in the formation of disulfide-linked hLOX-1 dimers. Eliminating this intermolecular disulfide bond markedly impairs the recognition of *E. coli* by hLOX-1. These dimers can act as a "structural unit" to form noncovalently associated oligomers. These results provided evidence for the existence of hLOX-1 dimers/oligomers (Xie et al. 2004).

Although Lys^{262} and Lys^{263} in C-terminus of bovine bLOX-1 play important roles in the uptake of Ox-LDL, mutation of these residues did not suggest as potential source of the dominant-negative property of bLOX-1. It is possible that dominant-negative human hLOX-1 forms a heterodimer with LOX-1-wild-type (WT) and blocks LOX-1-WT-induced cell signaling. Homodimerization of hLOX-1-WT was localized in cell membrane, and Ox-LDL activated ERK-1/2 without the translocation of hLOX-1-WT. Tanigawa et al. (2006a) gave an evidence that blocking cell-proliferative pathways at the receptor level could be useful for impairing LOX-1-induced cell proliferation.

34.9.4.2 Crystal Structure

The X-ray crystallography of 136–270 subunit revealed the presence of disulfide bonds. The 1.4 Å crystal structure of the extracellular C-type lectin-like domain of human Lox-1 reveals a heart-shaped homodimer with a ridge of six basic amino acids extending diagonally across the apolar top of Lox-1, a central hydrophobic tunnel that extends through the entire molecule, and an electrostatically neutral patch of 12 charged residues that resides next to the tunnel at each opening. Based on the arrangement of critical binding residues on the LOX-1 structure, a binding mode for the

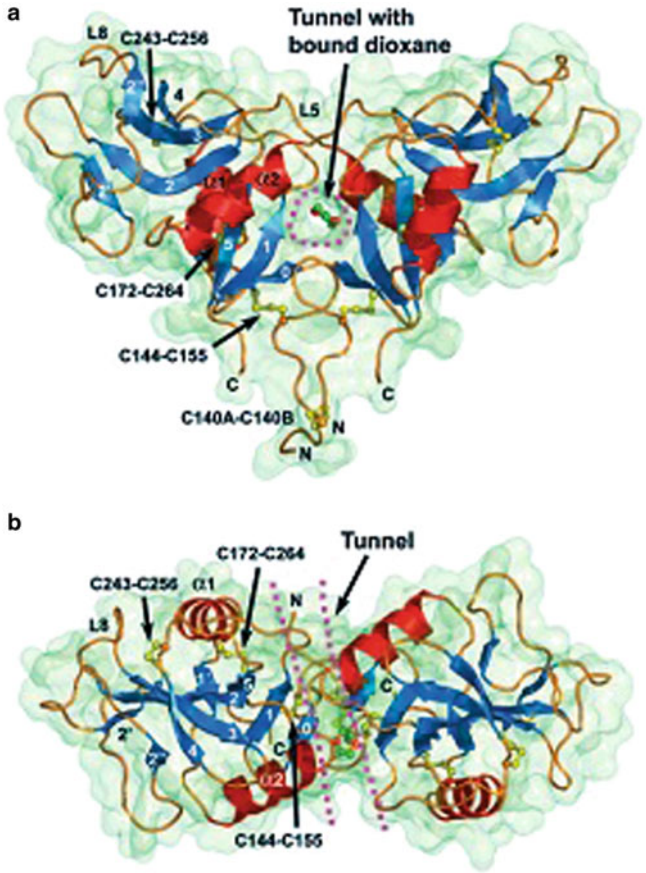

Fig. 34.6 Ribbon structure and transparent surface of human Lox-1 CTLD homodimer. *Side view* (**a**) and *top view* (**b**) (opposite the N and C termini) of Lox-1 from the high resolution monoclinic crystal. The central tunnel containing bound dioxane is outlined by a *dotted line*. α-Helices are in *red*; β-strands are *blue*. Secondary structures are *numbered* as proposed for CTLDs by Zelensky and Gready (2003). Loops are *numbered sequentially*. A semitransparent molecular surface representation is shown in *light green*. The disulfide bonds and dioxane are represented by *yellow* and *green ball-and-stick models*, respectively (Adapted with permission from Park et al. 2005a © American Society for Biochemistry and Molecular Biology)

recognition of modified LDL and other Lox-1 ligands has been proposed (Fig. 34.6) (Park et al. 2005a). The central hydrophobic tunnel that extends through the entire LOX-1 molecule is a key functional domain of the receptor and is critical for the recognition of modified LDL (Francone et al. 2009).

Ohki et al. (2005) determined the crystal structure of the ligand binding domain of 143–271 subunit (and mutagenesis of Trp^{150}; Arg^{208}; Arg^{209}; His^{226}; Arg^{229}; Arg^{231} and Arg^{248}) of LOX-1 at 2.4 Å and revealed a short stalk region connecting the domain to the membrane-spanning region, as a homodimer linked by an interchain disulfide bond. In vivo assays with LOX-1 mutants revealed that the "basic spine," consisting of linearly aligned arginine residues spanning over the dimer surface, is responsible for ligand binding. Single amino acid substitution in the dimer interface caused a severe reduction in LOX-1 binding activity, suggesting that the correct dimer arrangement is crucial for binding to Ox-LDL. Based on the LDL model structure, possible binding modes of LOX-1 to Ox-LDL are proposed (Ohki et al. 2005).

The crystal of the monomeric ligand-binding domain of LOX-1 belongs to space group $P2_12_12_1$, with unit-cell parameters a = 56.79, b = 67.57, c = 79.02 A. The crystal of the dimeric form belongs to space group C2, with unit-cell parameters a = 70.86, b = 49.56, c = 76.73 A, beta = 98.59°. Data for the dimeric form of the LOX-1 ligand-binding domain have been collected to 2.4 A. For the monomeric form of the ligand-binding domain, native, heavy-atom derivative and SeMet-derivative crystals have been obtained; their diffraction data have been measured to 3.0, 2.4 and 1.8 A resolution, respectively (Ishigaki et al. 2005).

The NECK domain of LOX-1 displays sequence similarity to the coiled-coil region of myosin, having been suggested it adopts a rod-like structure. The structural analysis of human LOX-1 reveals a unique structural feature of LOX-1 NECK. Despite significant sequence similarity with the myosin coiled-coil, LOX-1 NECK does not form a uniform rod-like structure. Although not random, one-third of the N-terminal NECK is less structured than the remainder of the protein and is highly sensitive to cleavage by a variety of proteases. The coiled-coil structure is localized at the C-terminal part of the NECK, but is in dynamic equilibrium among multiple conformational states on a mus-ms time scale. This chimeric structural property of the NECK region may enable clustered LOX-1 on the cell surface to recognize Ox-LDL (Ishigaki et al. 2007).

34.9.5 Functions of LOX-1

34.9.5.1 In Support of Cell Adhesion

LOX-1 can support adhesion of mononuclear leukocytes. Under a static condition, CHO-K1 cells stably expressing LOX-1 showed more prominent adhesion of human peripheral blood mononuclear leukocytes and human monocytic cell line than untransfected CHO-K1 cells. Mononuclear leukocytes also adhered to plastic plates precoated with recombinant soluble LOX-1 extracellular domain (Hayashida et al. 2002). Kakutani et al. (2000) showed the possibility that LOX-1 is involved in the platelet-endothelium interaction and hence directly in endothelial dysfunction. More importantly, the binding of platelets to LOX-1 enhanced the release of endothelin-1 from endothelial cells, supporting the induction of endothelial dysfunction, which would, in turn, promote the atherogenic process. LOX-1 may initiate and promote atherosclerosis, binding not only Ox-LDL but also platelets. LOX-1 can support

cell adhesion to fibronectin (FN) in a divalent cation-independent fashion. CHO-K1 cells expressing bovine LOX-1, but not untransfected CHO-K1 cells, can adhere to FN-coated plates, but not to collagen-coated plates, in presence of EDTA (Shimaoka et al. 2001a).

LOX-1 on Endothelial Cells Binds with Bacterial Ligands: Adhesion of bacteria to vascular endothelial cells as well as mucosal cells and epithelial cells appears to be one of the initial steps in the process of bacterial infection, including infective endocarditis. LOX-1 with C-type lectin-like structure, can support adhesion of bacteria and works as a cell surface receptor for Gram-positive and Gram-negative bacteria, such as *S. aureus* and *E. coli*, in a mechanism similar to that of class A scavenger receptors; however, other unknown molecules may also be involved in the adhesion of *E. coli* to cultured bovine aortic endothelial cells (BAEC), which is enhanced by poly-I (Palaniyar et al. 2002; Shimaoka et al. 2001b). Thus, LOX-1 is an adhesion molecule involved in leukocyte recruitment and represents an attractive target for modulation of endotoxin-induced inflammation (Honjo et al. 2003).

34.9.5.2 Role in Apoptosis

LOX-1 mediates Ox-LDL-induced apoptosis of endothelial cells, monocyte adhesion to endothelium, smooth muscle cells (SMCs), and phagocytosis of aged cells. LOX-1 activation by Ox-LDL causes endothelial changes that are characterized by activation of NF-kB through an increased ROSs, subsequent induction of adhesion molecules, and endothelial apoptosis. Ox-LDL also induced apoptosis of HCAECs and LOX-1 mediates in this function. Findings suggest that Ox-LDL through its receptor LOX-1 decreases the expression of anti-apoptotic proteins Bcl-2 and c-IAP-1. This is followed by activation of apoptotic signaling pathway, involving release of cytochrome c and Smac and activation of caspase-9 and then caspase-3 (Chen et al. 2004). Duerrschmidt et al. (2006) reported the presence of LOX-1 in granulosa cells from patients under in vitro fertilization therapy after Ox-LDL induced expression of LOX-1 mRNA. The Ox-LDL treatment caused autophagy form of programmed cell death in association with reorganization of the actin cytoskeleton. It suggested that follicular atresia is not under the exclusive control of apoptosis. The LOX-1-dependent autophagy represents an alternate form of programmed cell death. Obese women with high blood levels of Ox-LDL may display an increased rate of autophagic granulosa cell death.

34.9.5.3 In Organ Transplantation

The pathogenesis of posttransplant CAD, which is a major form of chronic rejection after cardiac transplantation, is not fully understood. Suga et al. (2004) investigated the expression of LOX-1 mRNA in murine allografted hearts that develop diffuse coronary obstruction. Results indicate that alloimmune responses induce up-regulation of LOX-1 mRNA in transplanted hearts and hence increased LOX-1 may be involved in the progression of obstructive vascular changes (Suga et al. 2004). In human atherosclerotic lesions, the expression of LOX-1 is significantly increased in atherosclerotic grafted vein and carotid artery specimens compared with that in normal arteries. LOX-1 expression was colocalized with apoptotic cells. The apoptotic cells were present mostly in the rupture-prone regions of the atherosclerotic plaque. These observations indicated that LOX-1 is extensively expressed in the proliferated intima of grafted veins and in advanced atherosclerotic carotid arteries (Li et al. 2002a, b; Jin et al. 2004).

Older-age renal allografts are associated with inferior survival, though the mechanisms are not clear. Since, ROS participate in aging and in chronic vascular disease, Brasen et al. (2005) reported that induction of LOX-1-related oxidation pathways and increased susceptibility to oxidative stress plays significant role in promoting vascular injury in old transplants independent of age of recipient (Brasen et al. 2005).

34.9.6 LOX-1 and Pathophysiology

LOX-1 in Atherogenesis: The expression of LOX-1 receptor activates a variety of intracellular processes that leads to expression of adhesion molecules and endothelial activation. The experimental evidence suggested that LOX-1 may contribute to the development of vascular injury. For example, LOX-1 is up-regulated in aorta from hypertensive, diabetic, and hyperlipidemic animal models. Also, LOX-1 over-expression is observed in atherosclerotic regions and damaged kidneys. In vivo, endothelial cells covering early atherosclerotic lesions and macrophages and smooth muscle cells accumulated in the intima of advanced atherosclerotic plaques express LOX-1. LOX-1 is cleaved at membrane proximal extracellular domain by some protease activities and released from the cell surface. Measurement of soluble LOX-1 in vivo may provide novel diagnostic strategy for the evaluation and prediction of atherosclerosis and vascular diseases (Kume and Kita 2001).

LOX-1 in Hypertensive Rats: LOX-1 is expressed in hypertensive state. LOX-1 expression was low in the aorta of salt-resistant (DR: 0.3% salt) and of Dahl salt-sensitive (DS: 8% salt) rats on a control diet, whereas it was elevated in salt-loaded DS rats. Results indicated that LOX-1 expression in aorta and vein was upregulated in hypertensive rats, which may be involved in impaired endothelium-dependent vasodilation in these rats (Nagase et al. 1997). In kidneys and glomeruli of hypertensive DS rats, LOX-1 gene

expression was markedly elevated compared with those of normotensive DR and DS rats. Prolonged salt loading further increased the renal LOX-1 expression in DS rats. Nagase et al. (2000) suggested a possible pathogenetic role for renal LOX-1 in the progression to hypertensive glomerulosclerosis.

LOX-1 and monocyte chemoattractant protein-1 (MCP-1) are involved in the initiation and progression of atherosclerosis. In order to examine differential role of LOX-1 and MCP-1 on the severity of early stage of atherosclerosis, Hamakawa et al. (2004) investigated atherosclerotic changes by exposure to hypertension and hyperlipidemia in common carotid arteries (CCAs) of stroke-prone spontaneously hypertensive rat (SHR-SP). Three groups were examined as: control [Wistar Kyoto rat (WKY) group], hypertension (SHR-SP group) and hypertension + hyperlipidemia [SHR-SP + high fat and cholesterol (HFC) group]. LOX-1 and MCP-1 expressions were coordinately up-regulated at mRNA and protein levels in an early stage of sclerosis depending on the severity of atherosclerotic stress. Thus, activations of LOX-1 and MCP-1 were collectively involved in the early stage of atherosclerosis (Hamakawa et al. 2004).

LOX-1 is critical in foam cell formation of macrophages (Mφ) and SMC. Inhibition of LOX-1 expression reduces foam cell formation and might influence lipid core formation in atherosclerotic lesions. Balloon-injury model of rabbit aorta revealed that LOX-1 mRNA is expressed 2 days after injury, and remained elevated until 24 weeks after injury. However, LOX-1 was not detected in media of non-injured aorta but expressed in both medial and neointimal SMC weeks after injury. Thus, LOX-1 mediates Ox-LDL-induced SMC proliferation and plays a role in neointimal formation after vascular injury (Eto et al. 2006) and confirmed in a rat model of balloon injury (Hinagata et al. 2006).

Platelet-Endothelium Interaction Mediated by LOX-1 Reduces NO in ECs: The role of LOX-1 in the thrombotic system was addressed by Cominacini et al. (2003b) following platelet interaction with LOX-1. The incubation of Bovine aortic endothelial cells (BAECs) and CHO-K1 cells stably expressing bovine LOX-1 (BLOX-1-CHO) cells with human platelets induced a sharp and dose-dependent increase in intracellular concentration of ROS and O_2^-. The increase in intracellular concentration of O_2^- was followed by a dose-dependent reduction in basal and bradykinin-induced intracellular NO concentration. The increase in O_2^- and the reduction of NO were inhibited by the presence of vitamin C and anti-LOX-1 mAb. The results show that one of the pathophysiologic consequences of platelet binding to LOX-1 may be the inactivation of NO through an increased cellular production of O_2^-.

Remnant lipoprotein particles (RLPs), products of lipolytic degradation of triglyceride-rich lipoprotein derived from VLDL, exert atherogenesis. RLPs stimulated superoxide formation and induction of cytokines in HUVECs via activation of LOX-1, consequently leading to reduction in cell viability with DNA fragmentation. The cilostazol exerts a cell-protective effect by suppressing these variables. Upon incubation of HUVECs with RLPs, adherent monocytes increased along with increased cell surface expression of adhesion molecules and increased expression of LOX-1 receptor protein. Cilostazol repressed these variables and hence it was suggested that cilostazol suppresses RLP-stimulated increased monocyte adhesion to HUVECs by suppression of LOX-1 receptor-coupled NF-kB-dependent nuclear transcription (Park et al. 2005b; Shin et al. 2004).

LOX-1 Contributes to Vein Graft Atherosclerosis: Ge et al. (2004) designed a study to examine the expression of LOX-1 in vein grafts atherosclerosis and the modulating effect of losartan (an AT-1 receptor antagonist) on it in male rabbits. After surgery, rabbits were fed with high cholesterol diet (HC), high cholesterol diet plus losartan (LHC) or regular chow (control) for 12 weeks. Study revealed that LOX-1 was expressed in endothelium and neointima of autologous vein grafts of rabbits. Increased LOX-1 expression was associated with vein grafts atherosclerosis development. Downregulation of LOX-1 by losartan might contribute to its attenuating effect on vein grafts atherosclerosis.

34.9.7 Macrophage Differentiation to Foam Cells

Foam cell formation from macrophages with subsequent fatty streak formation plays a key role in early atherogenesis. LOX-1 may play an important role in Ox-LDL uptake by macrophages and subsequent foam cell formation in this cell type (Yoshida et al. 1998). This role of LOX-1 was substantiated by studies on monocyte adhesion to human coronary artery endothelial cells (HCAECs) and endothelial injury in response to Ox-LDL in presence of antisense oligodeoxynucleotides to 5′-coding sequence of human LOX-1 gene. Studies indicated that LOX-1 is a key factor in Ox-LDL-mediated monocyte adhesion to HCAECs (Li and Mehta 2000). Depression of scavenger receptor function in monocytes by TGF-β1 at low concentrations reduced foam cell formation (Draude and Lorenz 2000). The expression of many genes is likely modulated during macrophage transformation into a foam cell. Functional consequences of modulation of three groups of genes: Scavenger Receptors including LOX-1, the PPAR family of nuclear receptors, and a number of genes involved in eicosanoid biosynthesis have been described (Shashkin et al. 2005). Scavenger receptors appear to play a key role in

uptake of Ox-LDL, while minimally-modified LDL appears to interact with CD14/TLR4. The regulation of scavenger receptors is, in part, mediated by the PPAR family of nuclear receptors (Shashkin et al. 2005).

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Dendritic Cell Lectin Receptors (Dectin-2 Receptors Family)

35

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35.1 Dendritic Cells

Dendritic cells (DCs) are special subsets of antigen presenting cells characterized by their potent capacity to activate immunologically naive T cells to induce initial immune responses. DCs are far more potent than macrophages and B cells in their capacity to activate immunologically naive T cells. Infact, DCs are responsible for initiating T cell-mediated immune responses to a variety of antigens. Members of the DC family are distributed to virtually all the organs (except the brain), where they serve as tissue resident APCs, playing critical roles in presenting environmental, microbial, and tumor-associated antigens to the immune system. DCs operate at the interface of innate and acquired immunity by recognizing pathogens and presenting pathogen-derived peptides to T lymphocytes. As a component of the innate immune system, DCs organize and transfer information from the outside world to the cells of the adaptive immune system. DCs can induce such contrasting states as active immune responsiveness or immunological tolerance. Recent years have brought a wealth of information regarding DC biology and pathophysiology that shows the complexity of this cell system. Presentation of an antigen by immature (nonactivated) DC leads to tolerance, whereas mature, antigen-loaded DCs are geared towards the launching of antigen-specific immunity.

DCs Form a Major Resident Leukocyte Population in Human Skin Two main types of DCs are found in noninflamed skin: epidermal Langerhans cells (LCs) and dermal DCs. LCs express Langerin/CD207 that localizes to and forms Birbeck granules, as well as the CD1a class I–like molecule that presents glycolipids. Plasmacytoid dendritic cells (PDCs), which are also known as plasmacytoid T cells, plasmacytoid monocytes, natural IFN-α/ß–producing cells (natural IPCs), and type 2 predendritic cells (pDC2), constitute a subset of immature DCs, which is capable of differentiating in vitro into mature DCs with typical dendritic cell morphology and potent T cell stimulatory function when exposed to IL-3 alone, IL-3 and CD40L, viruses, and bacterial DNA containing unmethylated CpG motifs (CpG-DNA) (Dzionek et al. 2001; Ebner et al. 2004). Dzionek et al. (2000) identified two novel markers of PDCs, blood dendritic cell antigen-2 (BDCA-2) and BDCA-4, which enable direct identification of PDCs in human blood. BDCA-2 is presumably involved in ligand internalization, processing and presentation, as well as in inhibition of IFN-α/ß synthesis in PDCs.

Langerhans Cells Langerhans cells (LCs) are a unique population of dendritic cells found in the epidermis, where they capture and process antigens and subsequently migrate to the draining lymph nodes to activate naive T cells. About 70% of all dermal cells represent $CD45^+$ leukocytes. The vast majority of these cells (60% of total) express the mononuclear phagocyte markers murine macrophage galactose/*N*-acetylgalactosamine-specific C-type lectin (mMGL), F4/80 and CD11b. Studies suggest that mononuclear phagocyte populations form the majority of dermal cells underestimated earlier (Guironnet et al. 2002; Dupasquier et al. 2004). Evidences suggest that fresh human LCs are the only cells in the epidermis to express a fucose-mannose receptor on their surface (Condaminet et al. 1998). Freshly isolated LCs in epidermal cell suspensions phagocytose the yeast cell wall derivative zymosan, intact *S. cerevisiae*, *Corynebacterium parvum and S. aureus*, as well as 0.5–3.5 μ latex microspheres. Zymosan uptake by LCs is mediated by a mannose/β-glucan receptor(s) that is differentially expressed in mice and that is down-regulated during maturation of LCs in culture. In epidermis, they can be identified by expression of E-cadherin and cytoplasmic Birbeck granules (BGs) as their hallmark; the presence of Birbeck granules is the unique feature of LCs. BGs are disks

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of two limiting membranes, separated by leaflets with periodic "zipperlike" striations. Birbeck granules are unusual rod-shaped structures specific to epidermal LCs, whose origin and function remain undetermined. The normal dermis contains typical immunostimulatory myeloid DCs identified by CD11c and BDCA-1, as well as an additional population of poorly stimulatory macrophages marked by CD163 and FXIIIA (Zaba et al. 2007). Using Langerin as marker of LCs revealed the heterogeneity in the phenotype of gingival LC population (Seguier et al. 2003).

35.2 Dendritic Cell-Associated Lectins

35.2.1 Type-I and Type-II Surface Lectins on DC

The identification of DC-associated lectins is of particular interest, because one of the characteristic features of DC is the expression of many C-type lectins. DCs have a number of receptors for adsorptive uptake of antigens. Some are shared with other cells, such as Fcγ receptors, DEC-205, a type I membrane-integrated glycoprotein and the macrophage mannose receptor (MMR). Other receptors are more DC restricted, e.g., Langerin/CD207, DC-SIGN/CD209, asialoglycoprotein receptor or hepatic lectins (HL), and dendritic cell lectin (DLEC; also referred to as BDCA-2) (Abner et al. 2004; Dzionek et al. 2001) and C-type lectin receptor 1 (CLEC-1). Of the type II surface lectins, DCs express CD23, CD69, DCIR, dectin-1, and dectin-2 α, β, and γ isoforms) that form the list of DC-associated C-type lectin family. It was proposed that dectin-2 and its isoforms, together with dectin-1, represent a unique subfamily of DC-associated C-type lectins (Graham and Brown 2009). The cytoplasmic domains of the C-type lectins are diverse and contain several conserved motifs that are important for antigen uptake: a tyrosine-containing coated-pit intracellular targeting motif, a triad of acidic amino acids and a dileucine motif. Some type II C-type lectins contain potential signaling motifs (ITIM, ITAM, and proline-rich regions) (Table 35.1) (Fig. 35.1). Immature human dendritic cells express asialoglycoprotein receptor isoforms for efficient receptor-mediated endocytosis (Valladeau et al. 2001).

35.3 Macrophage Mannose Receptor (CD206) on DC

35.3.1 Expression and Characteristics

Humans express two types of mannose receptors, each encoded by its own gene: Macrophage mannose receptor 1 (CD206, MMR) and Macrophage mannose receptor 2 (C-type mannose receptor 2, Urokinase-type plasminogen activator receptor-associated protein, CD280). The macrophage mannose receptor (CD206) is a 175-kDa transmembrane glycoprotein that appears to be expressed on the surface of terminally differentiated macrophages and endothelial cell subsets whose natural ligands include both self glycoproteins and microbial glycans. The expression of the mannose receptor CD206 is regarded a differentiation hallmark of immature DCs, whereas monocytes, mature DCs, and epidermal LCs do not express CD206. In immature cultured DC, MMR mediates high efficiency uptake of glycosylated antigens. The expression of MMR by MHC class II positive APC in non-lymphoid organs of the mouse is also described. The MMR positive APC have been identified in peripheral organs: skin, liver, cardiac and skeletal muscle and tongue. The mannose receptor positive cells in salivary gland, thyroid and pancreas co-express MHC class II and the myeloid markers macrosialin and sialoadhesin, but not the DC markers CD11c or DEC-205 (Linehan 2005). Being an R-type lectin, structural details of mannose receptors have been discussed in Chap 14 and Chap 15 and hence readers are advised to consult these Chapters for more details.

The mannose receptor family consists of four members [the mannose receptor, the M-type phospholipase A_2 receptor, DEC-205 and Endo180 (or urokinase plasminogen activator receptor-associated protein)] all of which share a common extracellular arrangement of an amino-terminal cysteine-rich domain related to R-type domain, followed by a fibronectin type II domain and 8–10 C-type lectin-like CRD domains within a single polypeptide. In addition, all have a short cytoplasmic domain, which mediates their constitutive recycling between the plasma membrane and the endosomal apparatus, suggesting that these receptors function to internalize ligands for intracellular delivery. However, despite the common presence of multiple lectin-like domains, these four endocytic receptors have divergent ligand binding activities, and it is clear that the majority of these domains do not bind sugars. All of the MR family members except DEC-205 recycle back to the cell surface from early endosomes, but DEC-205 recycles from late endosomes. However, each receptor has evolved to have distinct functions and distributions.

Understanding the molecular basis of cell surface ligand recognition and endosomal release by the MR requires information about how individual domains interact with sugars as well as the structural arrangement of the multiple domains. The NH2-terminal cysteine-rich domain and the fibronectin type II repeat are not necessary for endocytosis of mannose-terminated glycoproteins. The CRDs 1–3 have at most very weak affinity for carbohydrate, where as, of the eight C-type CRDs, CRDs 4–8 are required for binding and endocytosis of mannose/GlcNAc/fucose-terminated ligands, but only CRD-4 has demonstrable sugar binding activity in isolation. CRD 4 shows the highest affinity binding and has multi-specificity for a variety of monosaccharides. As the main mannose-recognition domain of MR (CRD4) is the central ligand binding domain of the receptor, analysis of this domain suggests ways in which multiple CRDs in whole

Table 35.1 Characteristics of C-type lectins produced by dendritic cells (DCs) and Langerhans cells (LCs) (Adapted and modified from Figdor et al. 2002; Feinberg et al. 2011; Graham and Brown 2009; Shrimpton et al. 2009; Tateno et al. 2010)

C-type lectin	Type	Amino acids (human/ mouse)	Chromosome localization (human)	Types of cells produce	Ligands	Functions
MMR (CD206)	I	1,456	10p13	DCs, LCs, Mo, MΦs, DMECs	Mannose, fucose, sLe^x	Antigen uptake
DEC-205 (CD205)	I	1,722	2q24	DCs, LCs, Act DCs, Thymic ECs	?	Antigen uptake
Dectin 1	II	247	12p13	DCs, LCs	β-Glucan	T cell interaction
Dectin 2	II	209	2p13	DCs, LCs	High mannose structures; ligands on $CD4^+CD25^+$ T cells ?	Antigen uptake
Langerin (CD207)	II	328	2p13	LCs	Galactose-6- SO4 oligosaccharides; oligomannose; Bd Gr-B antigen; Glcβ1-3Glcβ13Glc: A fr of β-glucan	BGs formation
DC-SIGN (CD209)	II	404	19p13	DCs	Mannan, ICAM2, ICAM3, HIV-1 (gp120), simion virus	T cell interaction, antigen uptake, migration, HIV-1 pathology
BDCA-2	II	?	12	pDCs	?	Antigen uptake
DCIR (LLIR)	II	237	12p13	DCs, Mo, MΦ, B, PMN	HIV-1	Autoimmunity
DLEC	?	231	12p13			
CLEC-1	II	280/229	12	DCs	?	?
CLEC9A	II	241/238	12	DCs in mouse and pDCs in humans	Necrotic cells	Activation
DCAR		?/209	?	DCs in mouse	?	Activation

The CLEC-1 and CLEC9A have been discussed with dectin-1 family in Chap. 34
? indicates unknown data
acDCs activated DCs, *pDCs* plasmacytoid DCs, *B* B cells, *Bd* blood, *BG* Birbeck granules, *DMECs* dermal microvascular endothelial cells, *Mo* monocytes, *MΦ* macrophage, *PMN* polymorphic nuclear cells, *sLeX* sialyl Lewis X, *Thymic ECs* thymic endothelial cells

receptor might interact with each other (Feinberg et al. 2000; Mullin et al. 1997). However, CRD 4 alone cannot account for the binding of the receptor to glycoproteins. At least 3 CRDs (4, 5, and 7) are required for high affinity binding and endocytosis of multivalent glycoconjugates. In this respect, the MR is like other carbohydrate-binding proteins, in which several CRDs, each with weak affinity for single sugars, are clustered to achieve high affinity binding to oligosaccharides (Taylor et al. 1992). The overall structure of CRD-4 (Fig. 15.1a) is similar to other C-type CRDs, containing two α helices and two small antiparallel β sheets. MBP (Fig. 15.1b). The core region of the CRD-4 domain, consisting of β strands 1–5 and the two α helices, superimposes on the equivalent residues of the rat MBP-A CRD. The principal difference resides in the position of helix α2, which is the most variable element of secondary structure among the C-type lectin-like folds.

The ectodomain of mannose receptor recognizes the patterns of sugars that adorn a wide array of bacteria, parasites, yeast, fungi, and mannosylated ligands. The capability to take up mannosylated protein antigens is important for the biologic function of DCs, as many glycoproteins derived from bacteria and fungi are mannosylated. The mannose receptor is also expressed by immature cultured DCs, where it mediates high efficiency uptake of glycosylated antigens, though its role in antigen handling in vivo is not clear. Endocytosis of mannose receptor -antigen complexes takes place via small coated vesicles, while non-mannosylated antigens were mainly present in larger vesicles. Shortly after internalization the mannose receptor and its ligand appear in the larger vesicles. Within 10 min, the mannosylated and non-mannosylated antigens co-localize with typical markers for MHC class II-enriched compartments and lysosomes (Tan et al. 1997). Peripheral blood DCs produce IFN-α in response to challenge by many enveloped viruses including herpes simplex virus (HSV) and HIV. The mannose receptor is an important receptor for the nonspecific recognition of enveloped viruses by DCs and stimulates the production of IFN-α by these viruses (Milone and Fitzgerald-Bocarsly 1998). The inflammatory dendritic-epidermal cells expressing MMR/CD206 in situ use CD206

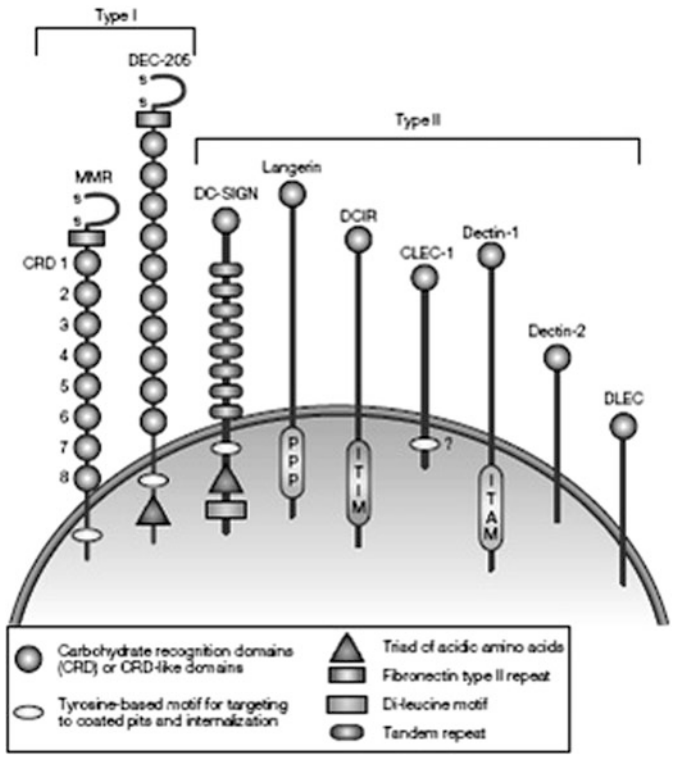

Fig. 35.1 Two types of C-type lectins or lectin-like molecules are produced by dendritic cells and Langerhans cells. Type I C-type lectins (MMR and DEC-205) contain an amino-terminal cysteine-rich repeat (S–S), a fibronectin type II repeat (FN) and 8–10 carbohydrate recognition domains (CRDs), which bind ligand in a Ca^{2+}-dependent manner. MMR binds ligand through CRD4 and CRD5. Type II C-type lectins contain only one CRD at their carboxy-terminal extracellular domain. The cytoplasmic domains of the C-type lectins are diverse and contain several conserved motifs that are important for antigen uptake: a tyrosine-containing coated-pit intracellular targeting motif, a triad of acidic amino acids and a dileucine motif. Other type II Ctype lectins contain other potential signaling motifs (ITIM, ITAM, proline-rich regions (P)). CLEC-1, C-type lectin receptor 1; DCIR, dendritic cell immunoreceptor; DC-SIGN, dendritic-cell specific ICAM-3 grabbing non-integrin; DLEC, dendritic cell lectin; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MMR, macrophage Mannose receptor (Reprinted by permission from Macmillan Publishers Ltd: Nature Rev Immunol., Figdor et al. © 2002)

for receptor-mediated endocytosis (Wollenberg et al. 2002). The MMR and DEC-205 belonging to R type lectins, which are characterized by the presence of trefoil group. Hence these lectins have been discussed along with R type lectins (Chap. 14 and Chap. 15).

35.3.2 Functions of Mannose Receptor in DC

Endocytosis Through Mannose Receptors: One of the major functions proposed for mannose receptor found on DCs as well as on macrophages and hepatic endothelial cells is in enhancing the uptake and processing of glycoprotein antigens for presentation by MHC class II molecules. Targeting recycling endocytic receptors with specific Abs provides a means for introducing a variety of tumor-associated Ags into human DCs, culminating in their efficient presentation to T cells. The specific targeting of soluble exogenous tumor Ag to the DC MR (CD206) directly contributes to the generation of multiple HLA-restricted Ag-specific T cell responses (Ramakrishna et al. 2004). Cell surface-bound receptors are suitable sites for gene delivery into cells by receptor-mediated endocytosis. Presence of mannose receptor on DCs can be exploited for targeted gene transfer by employing mannosylated conjugates (Bonifaz et al. 2002). It was demonstrated that DCs transfected with ManPEI/DNA complexes containing adenovirus particles are effective in activating T cells of T cell receptor transgenic mice in an antigen-specific fashion (Diebold et al. 1999).

Langerin Mediates Efficient Antigen Presentation: The LCs show poor endocytic capacity and do not exploit MR-mediated endocytosis pathways. This may serve to avoid hyper-responsiveness to harmless protein antigens that are likely to be frequently encountered in the skin due to skin damage (Mommaas et al. 1999). This seems to be related to low level of expression of the MR (CD206) by epidermal LCs. However, some epidermal DCs may express CD206 under inflammatory skin conditions such as atopic dermatitis and psoriasis. The inflammatory dendritic epidermal cells expressing CD206 in situ can be used for receptor-mediated endocytosis (Wollenberg et al. 2002; Idoyaga et al. 2008).

Neonatal LCs preferentially utilize a wortmannin-sensitive, fluid-phase pathway, rather than receptor-mediated endocytosis, to internalize antigen (Bellette et al. 2003).

The mannose receptor mediates induction of IFN-α in peripheral blood DCs. Suppressed stimulation of IFN-α synthesis by herpes simplex virus (HSV) by monosaccharides as well as the yeast polysaccharide mannan, supported a role for lectin(s) in the IFN-α stimulation. Blood DC and IFN-α-producing cells responding to HSV stimulation also express mannose receptor. The mannose receptor is an important receptor for the nonspecific recognition of enveloped viruses by DCs and the subsequent stimulation of IFN-α production by these viruses (Milone et al. 1998).

35.4 DEC-205

DEC-205 is an endocytic receptor with 10 membrane-external, contiguous C-type lectin domains. DEC-205 is expressed at high levels on DCs in the T cell areas of lymphoid organs (Witmer-Pack et al. 1995). αDEC-205 antibodies selectively target these DCs in mice (Hawiger et al. 2001). Small amounts of injected antigen, targeted to DCs by the DEC-205 adsorptive pathway, are able to induce solid peripheral $CD8^+$ T cell tolerance. The antigen-presentation function of DCs is associated with the high-level expression of DEC-205, which mediates the efficient processing and presentation of antigens in vivo (Hawiger et al. 2001; Jiang et al. 1995; Mahnke et al. 2000; Maruyam et al. 2002). The DEC-205 is rapidly taken up by means of coated pits and vesicles, and is delivered to a multi-vesicular endosomal compartment that resembles the MHC class II-containing vesicles implicated in antigen presentation (refer Chap. 15). Results of Shrimpton et al. (2009) demonstrate that two areas of the CD205 molecule, within C-type lectin-like domains (CTLDs) 3 + 4 and 9 + 10, recognise ligands expressed during apoptosis and necrosis of multiple cell types. Thus, CD205 acts as a recognition receptor for dying cells, potentially providing an important pathway for the uptake of self-antigen in intrathymic and peripheral tolerance.

35.5 Langerin: A C-Type Lectin on Langerhans Cells

35.5.1 Human Langerin (CD207)

Use of mAbs restricted to human DCs has enabled the identification of Langerin (CD207), a Ca^{2+}-dependent type II the mannose-type lectin receptor. Langerin reactivity was strongly stimulated by LCs differentiation factor TGF-β and down-regulated by CD40 ligation. The monoclonal antibody, DCGM4, selectively stained Langerhans Cells; hence the antigen was termed Langerin. Langerin neither co-localized with MHC class II rich compartments nor with lysosomal LAMP-1 markers. The DCGM4 was rapidly internalized at 37°C. The Langerin was found as a 40-kDa protein with a pI of 5.2–5.5 (Valladeau et al. 1999) and only expressed by LCs. Langerin is responsible for Birbeck granule (BG) formation by membrane superimposition and zippering. Langerin is a mannose specific lectin expressed by LCs in epidermis and epithelia. Remarkably, transfection of Langerin cDNA into fibroblasts created a compact network of membrane structures with typical features of BG. Langerin is thus a potent inducer of membrane superimposition and zippering leading to BG formation. The induction of BG is a consequence of the antigen-capture function of Langerin, allowing routing into these organelles and providing access to a non-classical antigen-processing pathway (Valladeau et al. 2000, 2003).

The gene encoding Langerin is localized on chromosome 2p13, a region that does not contain any known genetic complex. The human Langerin gene spans over 5.6 kbp and is organized in six exons. Cloning of a cDNA for langerin shows that langerin gene has an open reading frame 987 bp predicting a polypeptide of 328 amino acids with a molecular weight of 37.5 kDa. The characteristics of different types of lectins and organization of various domains are schematically shown in Fig 35.1. Langerin is a type II transmembrane cell surface receptor with an intracytoplasmic portion of 43 amino acids with proline rich motif (WPREPPP) and an extracellular lectin domain. The extracellular domain of langerin consists of a neck region containing a series of heptad repeats and a C-terminal C-type carbohydrate-recognition domain (CRD). The CRD of Langerin contains a EPN (Glu-Pro-Asn) motif characteristic of lectins with mannose specificity. As in other lectins having a single CRD, Langerin features a particular domain located between the CRD and the transmembrane domain that is rich in leucine zipper sequences. The extracellular region of Langerin exists as a stable trimmer held together by a coiled-coil of α-helices formed by the neck region, a structure found in mannose binding proteins. Finally Langerin has two potential sites of N-glycosylation at positions 87–89 and 180-18 amino acids. Trimer formation is essential for binding to oligosaccharide ligands because, as is typical for C-type CRDs, the CRD of langerin has only low affinity for monosaccharides. Oligomerization of C-type lectins is also important for determining selectivity for particular oligosaccharide structures.

35.5.1.1 The CRD of Human Langerin

Langerin CRD crystals at 1.5 Å resolution belonged to the tetragonal space group $P4_2$, with unit-cell parameters a = b = 79.55, c = 90.14 Å (Thépaut et al. 2008). The CRD of human Langerin, expressed in the periplasm of *E. coli*, was crystallized and analysed for Xray crystallography for

apo-Langerin and for the complexes with mannose and maltose, respectively. The Langerin CRD (dubbed LangA) fold resembles that of DC-SIGN (Chap. 36). However, especially in the long loop region (LLR), which is responsible for carbohydrate-binding, two additional secondary structure elements were present: a 3_{10} helix and a small β-sheet arising from the extended β-strand 2, which enters into a hairpin and a new strand β2′. Unexpectedly, the crystal structures in the presence of maltose and mannose revealed two sugar-binding sites. One was calcium-dependent and structurally conserved in the C-type lectin family whereas the second one represented a calcium-independent type. Based on these data, a model for the binding of mannan was proposed and the differences in binding behavior between Langerin and DC-SIGN with respect to the Lewis X carbohydrate antigen and its derivatives were explained (Chatwell et al. 2008).

Crystal structures of the CRD of human langerin in complex with mannose or maltose show that it binds monosaccharides by ligation to a bound Ca^{2+} at a site that is conserved in all C-type CRDs (Chatwell et al. 2008). Interestingly, the co-crystals also show the presence of a second sugar-binding site that has not been seen in many C-type lectins. Both monosaccharide residues of maltose, or the monosaccharide mannose, are bound in this second site largely via polar interactions with backbone residues in a cleft formed between two of the large loop regions in the top half of the domain (Chatwell et al. 2008). This cleft is wider in langerin than in other C-type CRDs, most likely due to the absence of auxiliary Ca^{2+} sites present in many other CRDs, including those of mannose-binding protein and DC-SIGN (Feinberg et al. 2001). Like the full-length protein, truncated langerin exists as a stable trimer in solution. Glycan array screening with the trimeric fragment shows that high mannose oligosaccharides are the best ligands for langerin. Structural analysis of the trimeric fragment of langerin confirms that the neck region forms a coiled-coil of α-helices. Multiple interactions between the neck region and the CRDs make the trimer a rigid unit with the three CRDs in fixed positions and the primary sugar-binding sites separated by a distance of 42 Å. The fixed orientation of the sugar-binding sites in the trimer is likely to place constraints on the ligands that can be bound by langerin (Feinberg et al. 2010).

35.5.1.2 Polymorphisms in Human Langerin

Analysis of human genome has identified three SNPs that result in amino acid changes in the CRD of langerin. Expression of full-length versions of the four common langerin haplotypes in fibroblasts revealed that all of these forms can mediate endocytosis of neoglycoprotein ligands. However, fragments from the extracellular domain showed that two of the amino acid changes reduced the affinity of the CRD for mannose and decreased the stability of the extracellular domain. In addition, analysis of sugar binding by langerin containing the rare W264R mutation, previously identified in an individual lacking Birbeck granules, showed that this mutation could abolish sugar binding activity. It suggests that certain langerin haplotypes may differ in their binding to pathogens and thus might be associated with susceptibility to infection (Ward et al. 2006).

35.5.2 Mouse Langerin: Homology to Human Langerin

Mouse Langerin (m-Langerin) displays 65% and 74% homologies in total amino acid and lectin domains with those of human (h)-Langerin. The cognate mouse and rat genes were assigned to chromosome 6D1-D2 and chromosome 4q33 distal-q34.1 proximal respectively, syntenic to the h-Langerin gene on chromosome 2p35. m-Langerin transcripts were as expected detected in MHC class II^+, but not MHC class II^-, cells from epidermis. However, m-Langerin transcripts were also expressed in spleen, lymph nodes (LN), thymus, liver, lung and even heart, but not gut-associated lymphoid tissues. In single-cell lymphoid suspensions, m-Langerin transcripts were mainly detected in the $CD11c^+$ DC, especially the $CD11^{blow}/CD8^{high}$ fraction of spleen and LN. Unexpectedly, significant amounts of m-Langerin transcripts were detected in skin and LN of TGF-β1-deficient mice, although in much lower amounts than littermate controls. It indicates that Langerin expression is regulated at several levels: by TGF-β1, DC subsets, DC maturation and the tissue environment (Takahara et al. 2002). The organization of human and mouse Langerin genes are similar, consisting of six exons, three of which encode the CRD. The m-Langerin, detected as a 48-kDa species, is abundant in epidermal LC in situ and is down-regulated upon culture (Valladeau et al. 2002).

Riedl et al (2004) isolated m-Langerin cDNA from murine fetal skin-derived DCs (FSDDC). In vitro-generated FSDDC and epidermal LC expressed both full-length and δE3Langerin mRNA, but tissue expression was not restricted to skin. Mouse Langerin protein isoforms were readily detected in fibroblasts transfected with cDNAs encoding epitope-tagged Langerin and δE3Langerin. Full-length m-Langerin bound mannan, whereas δE3Langerin and soluble bacterial recombinant Langerin protein lacking the neck domain did not. Fibroblasts transfected with m-Langerin cDNA contained typical BG and cored tubules, whereas δE3Langerin cDNA did not induce BG or cored tubule formation in transfected fibroblasts. Developmentally regulated expression of Langerin isoforms provides a mechanism by which Langerin involvement in antigen uptake and processing could be regulated (Riedl et al. 2004).

35.5.3 Ligands of Langarin

Mannoside-binding capacity was detected in normal epithelial cells. The Langerin CRD shows specificity for mannose, GlcNAc, and fucose, but only the trimeric extracellular domain fragment binds to glycoprotein ligands. Langerin extracellular domain binds mammalian high mannose oligosaccharides, as well mannose-containing structures on yeast invertase but does not bind complex glycan structures. Full-length Langerin, stably expressed in rat fibroblast transfectants, mediates efficient uptake and degradation of a mannosylated neoglycoprotein ligand. The pH-dependent ligand release appears to involve interactions between the CRDs or between the CRDs and the neck region in the trimer (Stambach and Taylor 2003). A role for langerin in processing of glycoprotein antigens has been proposed. Langerin binds HIV through its CRD and plays a protective role against its propagation by the internalization of virions in Birbeck granules. Access to the CRD of Langerin appeared to be impaired in proliferatively active environments (malignancies, hair follicles), indicating presence of an endogenous ligand with high affinity to saturate the C-type lectin under these conditions (Plzák et al. 2002).

Using glycoconjugate microarray and other analyses, Langerin showed outstanding affinity to galactose-6-sulfated oligosaccharides, including keratan sulfate, while it preserved binding activity to mannose, as a common feature of the C-type lectins with an EPN motif. Mutagenesis study showed that Lys^{299} and Lys^{313} form extended binding sites for sulfated glycans. Consistent with the former observation, the sulfated Langerin ligands were found to be expressed in brain and spleen, where the transcript of keratan sulfate 6-*O*-sulfotransferase is expressed. Langerin also recognized pathogenic fungi, such as *Candida* and *Malassezia*, expressing heavily mannosylated glycans. Observations provide strong evidence that Langerin mediates diverse functions on Langerhans cells through dual recognition of sulfated as well as mannosylated glycans by its uniquely evolved C-type CRD (Tateno et al. 2010). For endogenous ligands, fibroblast membrane fractions seemed to contain 140 and 240-kDa proteins, which bind Langerin. Though mass spectrometry suggested types I and III procollagen and fibronectin as candidate ligands, results indicated that Langerin selectively interacts with at least one ligand in extracellular matrix (type I procollagen) and may have an unanticipated role in cell-matrix interactions to modulate LC development (Tada et al. 2006). Crystal structures of the carbohydrate-recognition domain from human langerin bound to a series of oligomannose compounds, the blood group B antigen, and a fragment of β-glucan reveal binding to mannose, fucose, and glucose residues by Ca^{2+} coordination of vicinal hydroxyl groups with similar stereochemistry. Likewise, a β-glucan fragment, Glcβ1-3Glcβ1-3Glc, binds to langerin through the interaction of a single glucose residue with the Ca^{2+} site. The fucose moiety of the blood group B trisaccharide Galα1-3(Fucα1-2)Gal also binds to the Ca^{2+} site (Feinberg et al. 2011). Langerin binds to an unusually diverse number of endogenous and pathogenic cell surface carbohydrates, including mannose-containing O-specific polysaccharides derived from bacterial lipopolysaccharides.

35.5.4 Functions of Langerin

From absence of classical macrophage mannose receptor (MMR) from LCs, it could be thought that Langerin might be involved in the endocytosis of microorganisms through mannosylated components. Experiments showed that Langerin is implicated in recognition and internalization of *Myobacteria, HIV, Leishmania* or *Candida* components (Valladeau et al. 2003).

Birbeck Granule (BG) Formation: Birbeck granules are unusual rod-shaped structures specific to epidermal LCs, whose origin and function remain undetermined (Fig. 35.2). The presence of Birbeck granules is the unique feature of LCs. The BGs are disks of two limiting membranes, separated by leaflets with periodic "zipperlike" striations. The identification of Langerin/CD207 has allowed researchers to decipher the mechanism of BG formation and approach an understanding of their function. Remarkably, transfection of Langerin cDNA into fibroblasts creates a dense network of membrane structures with features typical of BGs. Furthermore, mutated and deleted forms of Langerin have been engineered to map the functional domains essential for BG formation. Langerin is a potent LC-specific regulator of membrane superimposition and zippering, representing a key molecule to trace LCs and to probe BG function (McDermott et al. 2002; Valladeau et al. 2003).

In the steady state, Langerin is predominantly found in the endosomal recycling compartment and in BGs. Langerin internalizes by classical receptor-mediated endocytosis and the first BGs accessible to endocytosed Langerin are those connected to recycling endosomes in the pericentriolar area, where Langerin accumulates. Drug-induced inhibition of endocytosis results in the appearance of abundant open-ended Birbeck granule-like structures appended to the plasma membrane, whereas inhibition of recycling induces BGs to merge with a tubular endosomal network. In mature Langerhans cells, Langerin traffic is abolished and the loss of internal Langerin is associated with a concomitant depletion of BGs. An exchange of Langerin occurs between early endosomal compartments and the plasma membrane, with dynamic retention in the endosomal recycling compartment. Mc Dermott et al. (2002) suggested that BGs are not endocytotic structures, rather they

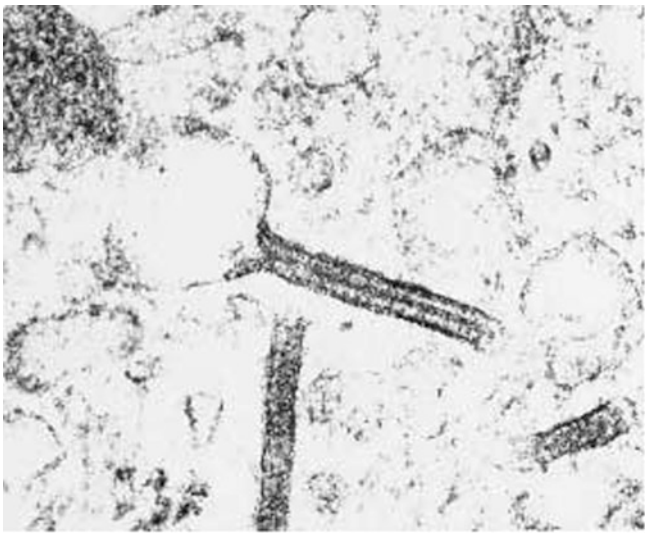

Fig. 35.2 Birbeck granules (BGs) are "tennis-racket" or rod shaped cytoplasmic organelles with a central linear density and a striated appearance. Birbeck granules were discovered by Michael Stanley Clive Birbeck (1925–2005), a British scientist and electron microscopist. Figure shows Birbeck granules, EM—Tennis racket shaped structures found in Langerhan's cells in histiocytosis X (Langerhan's cell histiocytosis) which includes eosinophilic granuloma, Letterer-Siwe disease and Hand-Schuller-Christian disease. The origin and function of BGs remain under question, but one theory is that they migrate to the periphery of the Langerhans cells and release its contents into the extracellular matrix. Another theory is that the Birbeck granule functions in receptor-mediated endocytosis, similar to clathrin-coated pits

are subdomains of the endosomal recycling compartment that form where Langerin accumulates. ADP-ribosylation factor proteins are implicated in Langerin trafficking and the exchange between BGs and other endosomal membranes (Mc Dermott et al. 2002).

Rab11A in Biogenesis of BG by Regulating Langerin Recycling and Stability: The extent to which Rab GTPases, Rab-interacting proteins, and cargo molecules cooperate in the dynamic organization of membrane architecture remains to be clarified. Langerin, accumulating in the Rab11-positive compartments of LCs, induces the formation of BGs, which are membrane subdomains of the endosomal recycling network. Results show that Rab11A and Langerin are required for BG biogenesis, and they illustrate the role played by a Rab GTPase in the formation of a specialized subcompartment within the endocytic-recycling system (Uzan-Gafsou et al. 2007).

Contact Hypersensitivity and LCs: The general role of LCs in skin immune responses is not clear because distinct models of LC depletion resulted in opposite conclusions about their role in contact hypersensitivity (CHS) responses. For example, LCs not only dispensable for CHS, but they regulate the response as well (Kaplan et al. 2005). While comparing various models, Bursch et al. (2007) suggested that dermal LCs could mediate CHS and provided an explanation for previous differences observed in the two-model systems (Bursch et al. 2007). Bennett and Clausen (2007) reviewed the impact of CD11c-DTR and Langerin-DTR mice on DC immunobiology, and highlighted the problems while interpreting data from these models. In another study Wang et al. (2008) found that acute depletion of mouse LC reduced CHS, but the timing of toxin administration was critical: toxin administration 3 days before priming did not impair CHS, whereas toxin administration 1 day before priming did. Moreover, LC elimination reduced the T cell response to epicutaneous immunization with OVA Ag. However, this reduction was only observed when OVA was applied on the flank skin, and not on the ear. Additionally, peptide immunization was not blocked by depletion, regardless of the site. Finally it was shown that conditions which eliminate epidermal LC but spare other Langerin$^+$ DC do not impair the epicutaneous immunization response to OVA. Overall, these results reconciled with previous conflicting data, and suggested that Langerin$^+$ cells do promote T cell responses to skin Ags, but only under defined conditions (Wang et al. 2008).

Langerin: A Natural Barrier to HIV-1 Transmission: The protective function of LCs is dependent on the function of Langerin and is thought to mediate HIV-1 transmission sexually. In the genital tissues, two different DC subsets are present: the LCs and the DC-SIGN$^+$-DCs. Although DC-SIGN$^+$-DCs mediate HIV-1 transmission, studies demonstrate that LCs prevent HIV-1 transmission by clearing invading HIV-1 particles. This protective function of LCs is dependent on the function of Langerin. Thus Langerin is a natural barrier to HIV-1 infection, and strategies to combat infection must enhance, preserve or, at the very least, not interfere with Langerin expression and function (de Witte et al. 2007, 2008).

35.6 DC-SIGN and DC-SIGNR on DCs

Additional C-type lectins with specificity for mannose, which have been characterized, are DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin where ICAM is intercellular adhesion molecule) and DC-SIGNR (DC-SIGN-related; L-SIGN, Liver-SIGN) (Geijtenbeek et al. 2004). DC-SIGN or CD209 is a mannose-specific C-type lectin expressed by DCs and plays an important role in the activation of T-lymphocytes. Evidences suggest that DC-SIGN can function both as an adhesion receptor and as a phagocytic

pathogen-recognition receptor, similar to the Toll-like receptors. Although major differences in the cytoplasmic domains of these receptors might predict their function, findings show that differences in glycosylation of ligands can dramatically alter C-type lectin-like receptor usage (Cambi and Figdor 2003).

DC-SIGN and DC-SIGNR may function in DC migration and DC/T-cell synapse formation through interaction with ICAM-2 and ICAM-3, respectively, as well as playing roles in pattern recognition by binding carbohydrates on the surface of viruses and other microbes in a manner similar to other C-type lectin receptors (Figdor et al. 2002). The DC-SIGN can also bind HIV gp120 in a calcium-dependent manner. DC-SIGN may deliver bound HIV to permissive cell types, mediating infection with high efficiency. Determination of the crystal structure of DC-SIGN/DC-SIGNR has revealed high-affinity binding to an internal feature of high-mannose oligosaccharides (Feinberg et al. 2001). This is in contrast to MBL and the MR, which have preferred specificity for terminal mannose residues. This, taken with evidence that DC-SIGN forms tetramers at the cell surface (Mitchell et al. 2001), provides a mechanism for lectins to exhibit different specificity, whilst all being loosely classified as 'mannose specific'. Recent experiments with model oligosaccharide ligands demonstrated distinct ligand preferences for both MR and DC-SIGN (Frison et al. 2003). The macrophage-expressed murine homolog of DC-SIGNR (SIGNR1) appears to function analogously to its human counterpart (Geijtenbeek et al. 2002; Kang et al. 2003) but has distinct activities compared to other murine DC-SIGN homologs.

35.7 Dectin-2 Cluster in Natural Killer Gene Complex (NKC)

35.7.1 Natural Killer Gene Complex

A number of genes encoding C-type lectin molecules have been mapped to the natural killer gene complex (NKC) at the distal region of mouse chromosome 6 and to a syntenic region on human chromosome 12p12-p13. In addition to those receptors which regulate NK cell function, related structures expressed on other cells types have also been localized to this chromosomal region. Although many receptors of the NKC are expressed primarily by NK and T cells, a growing number have been found to be expressed on myeloid cells (Pyz et al. 2006). In contrast to the NK and T cell specific receptors which function mostly in detection of tumorous or virally infected cells, largely by means of MHC class I recognition, the myeloid expressed receptors seem to have a more diverse repertoire of ligands and cellular functions, including pathogen recognition and maintenance of homeostasis (Yokoyama and Plougastel 2003). The Dectin-1 cluster of receptors is one such example, which includes Dectin-1, LOX-1, C-type lectin-like receptor-1 (CLEC-1), CLEC-2, CLEC12B, CLEC9A and myeloid inhibitory C-type lectin-like receptor (MICL) and form part of the Group V C-type lectin-like receptors (Huysamen and Brown 2009) and discussed in Chap. 34. Dectin-1 specifically recognizes fungal (1,3)-linked β-glucans, while CLEC9A recognizes a ligand in necrotic cells (Brown and Gordon 2001; Brown 2006). Another cluster of receptors of interest is the Dectin-2 family of C-type lectins. These receptors are clustered in the telomeric region of the NKC, in close proximity to the Dectin-1 family (Figs. 35.3 and 35.4) and also appear to have diverse functions in both immunity and homeostasis (Graham and Brown 2009). Dectin-1 is selectively expressed in DCs and to a lower extent in monocytes and macrophages. mRNA forms with and without a stalk exon are observed. This family of lectin-like genes encodes receptors with important immune and/or scavenger functions in monocytic, dendritic and endothelial cells (Sobanov et al. 2001).

The Dectin-2 cluster comprises Dectin-2, DCIR, DCAR, BDCA-2, MCL, Mincle and Clecsf8, which are members of the Group II C-type lectin family. These type II receptors are expressed on myeloid and non-myeloid cells and contain a single extracellular CRD and have diverse functions in both immunity and homeostasis. DCIR is the only member of the family which contains a cytoplasmic signaling motif (ITIM) and has been shown to act as an inhibitory receptor, while BDCA-2, Dectin-2, DCAR and Mincle all associate with FcRγ chain to induce cellular activation, including phagocytosis and cytokine production. Dectin-2 and Mincle have been shown to act as pattern recognition receptors for fungi, while DCIR acts as an attachment factor for HIV. In addition to pathogen recognition, DCIR has been shown to be pivotal in preventing autoimmune disease by controlling dendritic cell proliferation, whereas Mincle recognizes a nuclear protein released by necrotic cells (Graham and Brown 2009).

The amino acid sequences comprising the single C-type lectin domains of MCL, Mincle, DCIR and Dectin-2 are closely related to each other. These molecules show overall similarity to groups of animal C-type lectins, which demonstrate type II transmembrane topology. Sequence analysis suggests that MCL, Mincle, DCIR and Dectin-2 represent a subset of group II-related C-type lectins which may participate in analogous recognition events on macrophages and DCs. The genomic organization of the MCL gene and the sequence of the promoter region, with putative regulatory elements, have been described (Balch et al. 2002). Flornes et al (2004) reported the cDNA cloning and positional arrangement of C-type lectin superfamily (CLSF) receptor genes, which represent rat orthologues to human Mincle and

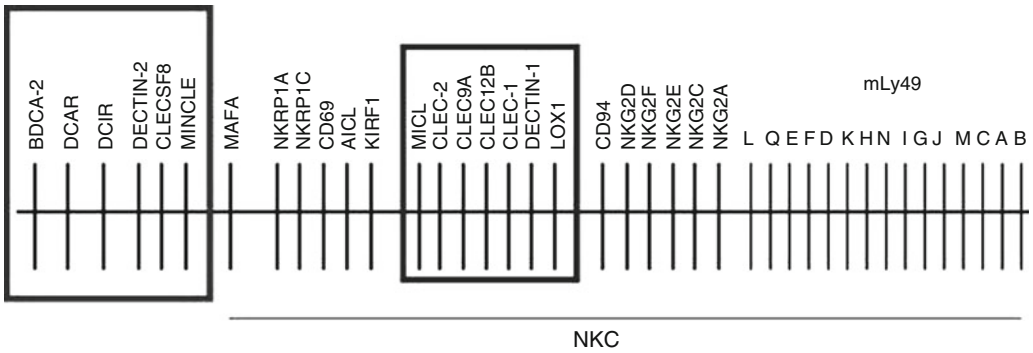

Fig. 35.3 The dectin-2 family genes form a cluster in the telomeric region of the NKC. The dectin-2 gene family includes BDCA-2, DCAR, DCIR, dectin-2, Clecsf8 and mincle, and form a cluster (*red square*) in the telomeric region of the NKC, close to the Dectin-1 cluster (*blue square*), on mouse chromosome 6 and human chromosome 12 (Adapted with permission from Graham and Brown 2009 © Elsevier)

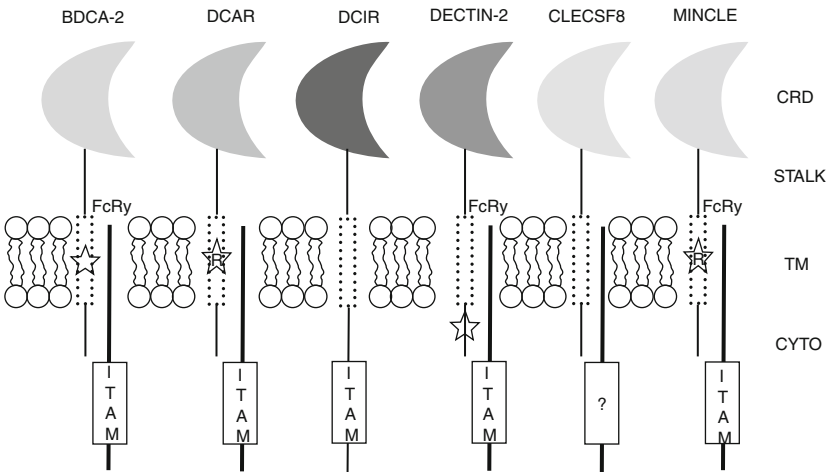

Fig. 35.4 Cartoon representation of the dectin-2 family of C-type lectins. Members of the dectin-2 family of C-type lectins are type II proteins with a single C-terminal extracellular carbohydrate recognition domain (CRD), a stalk region, a transmembrane region (TM), and a cytoplasmic domain (cyto). DCIR contains an immunoreceptor tyrosine-based signalling motif (ITIM) in its cytoplasmic domain, while BDCA-2, DCAR, dectin-2 and mincle associate with FcRγ chain which contains an ITAM. It is not yet known whether Clecsf8 associates with an adaptor molecule (?). The *star* represents the region responsible for association with the adaptor. R, arginine (Reprinted with permission from Graham and Brown 2009 © Elsevier)

DCIR and to mouse MCL and Dectin-2, as well as four other receptors DCIR2, DCIR3, DCIR4 and DCAR1, not reported in other species. Flornes et al (2004) also reported the cDNA cloning of human Dectin-2 and MCL, and of the mouse orthologues to the rat receptors. Similar to KLR some of these receptors exhibit structural features, which suggest that they regulate leukocyte reactivity; e.g., human DCIR and rodent DCIR1 and DCIR2 carry an ITIM, predicting inhibitory function. Conversely, Mincle has a positively charged amino acid in the transmembrane region, which suggests activating function in all three species. Sequence comparisons show that the receptors form a discrete family, more closely related to group II CLSF receptors than to the group V KLR. Most of the genes are preferentially expressed by professional APCs (DCs, macrophages and B cells) and neutrophils. In all three species, the genes map together, forming an evolutionary conserved gene complex, which is called as the antigen presenting lectin-like receptor complex (APLEC) (Flornes et al. 2004). Arce et al. (2001) identified a protein designated DLEC (dendritic cell lectin), which is a type II membrane glycoprotein of 213 amino acids and belongs to the human C-type lectin family. The cytoplasmic tail of DLEC lacks consensus signaling motifs and its extracellular region shows a single CRD, closest in homology to DCIR CRD. The DLEC gene has been localized linked to DCIR on the telomeric region of the NK gene complex.

DLEC mRNA is preferentially expressed in monocyte-derived DCs.

35.7.2 Antigen Presenting Lectin-Like Receptor Complex (APLEC)

In an experimental autoimmune rat model, a quantitative trait locus (QTL) conferring susceptibility to experimentally induced arthritis, a region on distal part of Chromosome 4 (Ribbhammar et al. 2003) was predicted to contain genes that encode proteins related to the human and mouse C-type lectin superfamily (CLSF) receptors: dendritic cell immunoreceptor (DCIR) (Bates et al. 1999), dendritic cell immunoactivating receptor (DCAR) (Kanazawa et al. 2003), dendritic cell lectin (DLEC; also referred to as BDCA-2) (Arce et al. 2001; Dzionek et al. 2001), dendritic cell associated C-type lectin 2 (Dectin-2) (Ariizumi et al. 2000; Fernandes et al. 1999), macrophage C-type lectin (MCL) (Balch et al. 1998) and macrophage inducible C-type lectin (Mincle) (Matsumoto et al. 1999). An important subgroup of CLSF receptors belongs to the opposing regulatory leukocyte receptors that come in two functional forms: activating and inhibitory, mediating their effects via protein tyrosine kinases and phosphatases, respectively. As a rule, the inhibitory variants have cytoplasmic tails containing immunoreceptor tyrosinebased inhibitory motifs (ITIMs) (Ravetch and Lanier 2000). The activating receptors lack such motifs, but contain instead a positively charged residue in the transmembrane (TM) region, mediating non-covalent association with chains containing immunoreceptor tyrosine- based activating motifs (ITAMs) (Lanier 2001). In the human and the mouse, regulatory roles for DCIR are suggested by the presence of an ITIM in the cytoplasmic tail, and for Mincle by a positively charged amino acid in the TM region. Functional studies of DCIR showed ITIM-dependent inhibition of B-cell receptor-mediated Ca^{2+} mobilization and protein tyrosine phosphorylation (Kanazawa et al. 2002), whereas antibody crosslinking of DLEC/BDCA-2 (Dzionek et al. 2001) and DCAR (Kanazawa et al. 2003) induced Ca^{2+} mobilization and protein tyrosine phosphorylation in dendritic cells. DCIR, DCAR and DLEC were reported to be preferentially expressed by professional antigen-presenting cells (APC), in particular by dendritic cells (Bates et al. 1999; Dzionek et al. 2001). Their suggested role as quantitative regulators of APC reactivity makes these receptors interesting candidates for being QTL associated with autoimmune diseases. Future positional cloning of QTL associated genes relies on exact definition of gene content and arrangement. Flornes et al. (2004) described cDNA cloning of the corresponding receptors in rat and for related receptors in rat, mouse and human. Flornes et al. (2004) described the cDNA cloning and positional arrangement of these receptor genes, which represent rat orthologues to human Mincle and DCIR and to mouse MCL and Dectin-2, as well as four novel receptors DCIR2, DCIR3, DCIR4 and DCAR1, not reported in other species. These genes are expressed mainly by professional APC (DCs, macrophages and B cells) and neutrophils and. map together, close to, but separate from NKC (Yokoyama and Plougastel 2003), with positions and orientations largely conserved between the human, the mouse and the rat.

35.7.2.1 Impact of APLEC on Arthritis and Autoimmunity

Congenic DA rats were resistant to oil-induced arthritis (OIA) when they carried PVG alleles for APLEC, which encodes immunoregulatory C-type lectin-like receptors. Five corresponding human APLEC genes were identified and targeted. The SNP rs1133104 in *Dcir* and a haplotype including that marker and 4 other SNPs in *Dcir* and its vicinity gave an indication of allelic association with susceptibility to RA in patients who were negative for antibodies to cyclic citrullinated peptide. Results supported that rat APLEC is associated with susceptibility to polyarthritis, and human APLEC and *Dcir* may be associated with susceptibility to anti-CCP-negative RA (Lorentzen et al. 2007). The APLEC locus is a major locus regulating the severity of experimentally induced arthritis in rats (Rintisch et al. 2010). Rat APLEC regulates autoimmunity and multiple phenotypes in several types of arthritis. However, delineating the genetic impact may require stratification for sex or mode of arthritis induction. This pathogenetic complexity should be considered when evaluating APLEC in inflammatory and autoimmune diseases, including RA (Guo et al. 2008a; Bäckdahl et al. 2009). In vivo and in vitro phenotypes in DA rats and APLEC-congenic rats suggested that both strains had a notably dichotomous expression of genes, with general down-regulation of all four *Dcir* genes and upregulation of *Mincle* and *Mcl*. It is suggested that human APLEC genes may similarly regulate infectious diseases, differential delayed type hypersensitivity (DTH) and status of general macrophage activation (Guo et al. 2009).

35.8 Dectin-2 (CLECF4N)

35.8.1 Characteristics

Dendritic cell-associated lectin-2 (Dectin-2) was discovered in an experimental murine model of acute myeloid leukemia as a receptor over-expressed in the spleen (Fernandes et al. 1999). It is the best characterized member of this receptor family and was identified as an over-expressed transcript in a myeloid leukaemia mouse model and in macrophages, neutrophils and pleuripotent myeloid

precursors (Fernandes et al. 1999). Tissue distribution of Dectin-2 is tightly restricted to myeloid cells of the macrophage and DC lineages. Dectin-2 surface expression on these cells was also found to be low but exhibited enhanced expression on inflammatory monocytes during the acute phase of an inflammatory reaction (Taylor et al. 2005). Dectin-2 is a C-type lectin-like receptor, which is associated to DC and involved in the initiation and maintenance of UV-induced tolerance. In naïve mice Dectin-2 is predominantly expressed by a wide variety of tissue macrophages and has a novel distribution pattern compared with other myeloid markers. Its expression is limited to DCs and notably absent from brain microglia and choroid plexus or meningeal macrophages. On peripheral blood monocytes, Dectin-2 expression is very low on the surface but is transiently and markedly up-regulated on induction of inflammation in vivo using a variety of stimuli. This change in Dectin-2 expression occurs on 'inflammatory' monocytes after arrival at the inflammatory lesion as demonstrated by adoptive cell-transfer studies, and is independent of whether the macrophages elicited by the stimuli ultimately expressed Dectin-2. Observations suggest Dectin-2 expression to be characteristic of monocyte activation/maturation at an inflammatory lesion and provide a new interpretation of Dectin-2 function in vivo (Taylor et al. 2005). Dectin-2 is encoded in the NK cell complex of C-type lectin-like genes and shares features with other classical C-type lectins in that it has conserved motifs for the recognition of mannose in a Ca^{2+}-dependent manner (Fernandes et al. 1999). Despite this, reports of a lectin activity of Dectin-2 have been contradictory (Fernandes et al. 1999; Ariizumi et al. 2000).

Clone 1B12 gene is expressed in a DC-specific manner as a type II membrane-integrated polypeptide of 209 amino acids containing a single CRD motif in the COOH terminus. The expression pattern of dectin-2 was almost indistinguishable from that of dectin-1; that is, both were expressed abundantly at mRNA and protein levels by the XS52 DC line, but not by non-DC lines, and both were detected in spleen and thymus, as well as in skin resident DC (i.e. Langerhans cells). One of the striking findings was the identification of two truncated isoforms of dectin-2, i.e. the β isoform with 34 aa deletions in the neck domain and the γ isoform with 41 aa deletions within the CRD domain. Genomic analyses indicated that a full-length dectin-2 (α isoform) is encoded by 6 exons, whereas truncated isoforms (β and γ) are produced by alternative splicing (Ariizumi et al. 2000a). RT-PCR and immunoblotting of multiple bands of dectin-2 transcripts and proteins confirmed molecular heterogeneity.

The human Dectin-2 (hDectin-2) (Kanazawa et al. 2004a; Gavino et al. 2005) transcripts have been localized in lung, spleen, lymph node, leukocytes, bone marrow and tonsils, but unlike mouse, Dectin-2 was not expressed in the human thymus. In peripheral blood cells, hDectin-2- transcripts were preferentially expressed in plasmacytoid, rather than myeloid DCs and constitutively expressed in $CD14^+$ monocytes and B cells. The hDectin-2 could be induced in $CD4^+$ T cells upon activation with Con A (Kanazawa et al. 2004a; Gavino et al. 2005). Similar to mouse Dectin-2, hDectin-2 appears to be upregulated in inflammatory settings, as gene expression in $CD14^+$ monocytes could be upregulated by treatment with GM-CSF, TGF-β1 and TNF-α and downregulated with the addition of IL-4 and IL-10 (Gavino et al. 2005). The hDectin-2 was also expressed on Langerhans cells.

A truncated isoform of hDectin-2 has been identified by Gavino et al. (2005). Truncated isoform of hDectin-2 lacks part of the intracellular domain and most of the transmembrane domain of the receptor. The lack of transmembrane region has been proposed to encode a secreted protein which may act as an antagonist to full-length Dectin-2 (Gavino et al. 2005). Human Dectin-1 isoform E also lacks a transmembrane region. This isoform is retained intracellularly where it interacts with Ran-binding protein, which is presumed to act as a scaffold protein to coordinate signals from cell surface receptors with intracellular signaling pathways (Xie et al. 2006). The Dectin-2 promoter was also defined as a Langerhans cell specific regulatory element and, while numerous splice forms of Dectin-2 mRNA were shown to be highly expressed in a Langerhans cell-like skin-derived cell line compared to other cell lines, these transcript were also found to be abundant in spleen and thymus (Ariizumi et al. 2000a; Bonkobara et al. 2001).

35.8.2 Ligands of Dectin-2

35.8.2.1 Dectin-2 Shows Specificity for High Mannose and Fucose

The CRD of Dectin-2 functions as a C-type lectin with specificity for glycoconjugates bearing high-mannose sugars, which was predicted to be due to the presence of an EPN motif in the CRD (Fernandes et al. 1999). The CRD of Dectin-2 exhibited cation-dependent mannose/fucose-like lectin activity, with an IC_{50} for mannose of approximately 20 mM compared to an IC_{50} of 1.5 mM for the MMR when assayed by similar methodology. The extracellular domain of Dectin-2 exhibited binding to live *Candida albicans* and the *Saccharomyces*-derived particle zymosan. Binding of *C. albicans* hyphae by RAW cells transduced with Dectin-2 resulted in tyrosine phosphorylation of intracellular proteins (Sato et al. 2006). Though, both mannose receptor and SIGNR1 were able to bind bacterial capsular

polysaccharides derived from *Streptococcus pneumoniae*, the Dectin-2 CRD exhibited only weak interactions to these capsular polysaccharides, indicative of different structural or affinity requirements for binding, when compared with the other two lectins. Glycan array analysis of the carbohydrate recognition by Dectin-2 indicated specific recognition of high-mannose structures ($Man_9GlcNAc_2$). The differences in the specificity of these three mannose-specific lectins indicated that mannose recognition is mediated by distinct receptors, with unique specificity, and are expressed by discrete subpopulations of cells. This highlights the complex nature of carbohydrate recognition by immune cells (McGreal et al. 2006; Sato et al. 2006).

Use of a soluble form of the CRD of Dectin-2 as a probe, revealed that the receptor could recognize zymosan and numerous pathogens including *S. cerevisiae, C. albicans, M. tuberculosis, Microsporum audounii, Trichophyton rubrum Paracoccoides brasiliensis, Histoplasma capsulatum* and capsule-deficient *Cryptococcus neoformans*. Although the binding to these pathogens differed greatly, binding could be inhibited by chelation of Ca^{2+} or in presence of mannose (McGreal et al. 2006; Sato et al. 2006). A glycan microarray showed that the receptor had specificity for high-mannose structures (McGreal et al. 2006). Dectin-2 has been shown to play a role in response to allergens. Dectin-2 on bone-marrow-derived DCs (BMDCs) was able to bind to extracts from house dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) and *Aspergillus fumigatus* in a mannose-dependent manner. Stimulation of mast cells co-expressing Dectin-2 and FcRγ chain with these extracts resulted in production of proinflammatory lipid mediators which are not produced by untransfected cells (Barrett et al. 2009).

Experiments with a soluble recombinant form of Dectin-2 have suggested the presence of a ligand on $CD4^+CD25^+$ T cells, and in vivo administration of this protein in mice impaired the development and maintenance of ultraviolet (UV)-induced tolerance (Aragane et al. 2003). It has been speculated that prevention of the interaction between endogenous Dectin-2 and T cells causes this defect, although the nature of this ligand was yet uncharacterized. UV-B irradiation was shown to increase Dectin-2 expression in Langerhans cells of the skin at both mRNA and protein levels (Bonkobara et al. 2005). It is possible that Dectin-2 recognises an endogenous ligand that is not a carbohydrate, perhaps via an alternative binding site to that which recognises fungi, as has been reported for other C-type lectins, such as Dectin-1 (Willment et al. 2001). However, while the expression of Dectin-2 was upregulated upon UV-B radiation in mice, it was downregulated in human cells. The discrepency may be due to use of $CD14^+$ monocytes as a surrogate model for epidermal Langerhans cells in the human experiment (Gavino et al. 2005).

35.8.2.2 Dectin-1 and -2, Share Several Important Features

DC-associated, dectin-2, shared several important features with dectin-1. First, both dectin-2 and dectin-1 exhibited a common domain structure, consisting of a relatively short cytoplasmic domain, a transmembrane domain, an extracellular neck domain, and a single CRD in the COOH termini. Second, dectin-1 mRNA and dectin-2 mRNA expression profiles were indistinguishable, i.e. both were expressed: (a) at relatively high levels in the XS52 DC line but not in other tested non-DC lines, (b) most abundantly in spleen and thymus, and (c) constitutively in the Ia^+ epidermal cell population (i.e. Langerhans cells). Third, expression of dectin-2 and dectin-1 proteins also occurred exclusively in the XS52 DC line among the tested cell lines. Despite these similarities, the degree of sequence homology between dectin-2 and dectin-1 was relatively low (19.6% identity in the overall sequence and 24.8% within the CRD motif). Thus, dectin-2 and dectin-1 represent two structurally independent, DC-associated C-type lectins.

35.8.2.3 Dectin-1 and Dectin-2 Receptors in Control of Fungal Infection

Both, dectin-1 and dectin-2 are type II- transmembrane proteins with extracellular domains containing a carbohydrate recognition domain highly conserved among C-type lectins (Ariizumi et al. 2000a, b). Dectin-1 is expressed widely by APC (Ariizumi et al. 2000a) and is a PRR for β-glucan in yeasts (Brown et al. 2003). Dectin-2 is constitutively expressed at very high levels by mature DC and can be induced on macrophages after activation (Ariizumi et al. 2000b; Taylor et al. 2005). Dectin-2 is a PRR for fungi that employ Fc receptor (FcR) chain signaling to induce internalization, activate NF-κB, and up-regulate production of TNFα and IL-1ra (Sato et al. 2006).

During fungal infection, a variety of receptors, including TLR and Dectin-1 initiate immune responses. TLR recognition of fungal ligands and subsequent signaling through the MyD88 pathway were thought to be the most important interactions required for the control of fungal infection. However, Dectin-1-deficient mice, address the role of Dectin-1 in control of fungal infection. Saijo et al. (2007) argue that Dectin-1 plays a minor role in control of *Pneumocystis carinii* by direct killing and that TLR-mediated cytokine production controls *P. carinii* and *Candida albicans*. By contrast, Taylor et al. (2005) argue that Dectin-1-mediated cytokine and chemokine production, leading to efficient recruitment of inflammatory cells, is required for control of fungal infection.

Binding assays using soluble dectin-2 receptors showed the extracellular domain to bind preferentially to hyphal (rather than yeast/conidial) components of *Candida albicans*, *Microsporum audouinii*, and *Trichophyton rubrum*. Selective

binding for hyphae was also observed using RAW macrophages expressing dectin-2, the ligation of which by hyphae or cross-linking with dectin-2-specific antibody led to protein tyrosine phosphorylation. Because dectin-2 lacks an intracellular signaling motif, Sato et al. (2006) searched for a signal adaptor that permits it to transduce intracellular signals. First, it was found that the Fc receptor (FcR) chain can bind to dectin-2. Second, ligation of dectin-2 on RAW cells induced tyrosine phosphorylation of FcR, activation of NF-κB, internalization of a surrogate ligand, and up-regulated secretion of tumor necrosis factor α and interleukin-1 receptor antagonist. Finally, these dectin-2-induced events were blocked by PP2, an inhibitor of Src kinases that are mediators for FcR chain-dependent signaling. It is suggested that dectin-2 is a PRR for fungi that employs signaling through FcR to induce innate immune responses (Sato et al. 2006). Dennehy and Brown (2007) argue that collaborative responses induced during infection may partially explain some apparently contradictory results. It seems that Dectin-1 is the first of many pattern recognition receptors that can mediate their own signaling, as well as synergize with TLR to initiate specific responses to infectious agents (Dennehy and Brown 2007).

35.9 The DC Immunoreceptor and DC-Immunoactivating Receptor

The dendritic cell (DC) immunoreceptors (DCIR) and DC-immunoactivating receptors (DCAR) represent a subfamily of cell surface C-type lectin receptors, whose multifunctional capacities range from classical Ag uptake and immunoregulatory mechanisms to the involvement in DC ontogeny.

35.9.1 Dendritic Cell Immunoreceptor (DCIR) (CLECsF6): Characterization

DCIR was identified by screening a nucleotide database for molecules homologous to the Group II C-type lectin hepatic asialoglycoprotein receptors, which also contain a single CRD at the C-terminal end (Bates et al. 1999). The DCIR (official gene symbol *Clec4a2*, called *Dcir*) is a type II C-type lectin expressed mainly in DCs that has a CRD in its extracellular region of the protein. DCIR mRNA was found to be highly expressed in human peripheral blood leukocytes, and at lower levels in lymph node, spleen, bone marrow and thymus, while mouse DCIR was found to be expressed at highest levels in spleen and lymph node. The term "DC immunoreceptors" is applied to a distinct set of signaling pattern-recognition receptors (PRR) described by Kanazawa et al. (2004b) and Kanazawa (2007), who reviewed their signaling mechanisms, carbohydrate recognition, and other features that contribute to the function of DC to control immunity.

DCIR is a type II membrane glycoprotein of 237 aa with a single CRD, closest in homology to those of the macrophage lectin and hepatic asialoglycoprotein receptors. The intracellular domain of DCIR contains a consensus ITIM. The gene encoding human DCIR was localized to chromosome 12p13, in a region close to the NK gene complex. The DCIR mRNA is predominantly transcribed in hematopoietic tissues. A closer look at protein expression on cells, revealed that DCIR was expressed on APCs such as $CD14^+$ monocytes, $CD19^+$ B cells, macrophages, neutrophils as well as myeloid and plasmacytoid DCs (pDCs), but not on $CD3^+$ T cells nor on $CD56^+$ or $CD16^+$NK cells (Bates et al. 1999; Kanazawa et al. 2002; Richard et al. 2002). DCIR acts as an inhibitory receptor via an ITIM in its cytoplasmic tail that transduces negative signals into cells. In contrast, DCAR is a molecule that forms a putative pair with DCIR. While both molecules share the highly homologous extracellular lectin domain, DCAR lacks the ITIM in its short cytoplasmic tail and acts as an activating receptor through association with the Fc receptor γ chain. Dectin-2 and BDCA-2 are highly related to DCAR by similarities of their amino acid sequence, molecular structure and chromosomal localization.

The DCIR was strongly expressed by DC derived from blood monocytes cultured with GM-CSF and IL-4. DCIR was mostly expressed by monocyte-related rather than Langerhans cell related DCs obtained from $CD34^+$ progenitor cells. The DCIR expression was down-regulated by signals inducing DC maturation such as CD40 ligand, LPS, or TNF-α. Thus, DCIR is differentially expressed on DC depending on their origin and stage of maturation/activation. DCIR represents a novel surface molecule expressed by Ag presenting cells, and of potential importance in regulation of DC function (Bates et al. 1999). Expression of mouse DCIR mRNA was observed specifically in spleen and lymph node, slightly increased with DC maturation during in vitro culture of bone marrow cells, and was not detected in cultured NK cells. Surface expression of mouse DCIR protein is observed in splenic APCs including B cells, monocytes/macrophages, and DCs, but not in T cells. The dectin-2 α isoform showed the highest degree of homology (33.5% identity in the overall sequence and 44.8% within the CRD motif) to murine DCIR. Dectin-2 differs from DCIR in ITIM motif found in the intracellular domain of DICR. ITIM was absent from short intracellular domain (14 aa) of dectin-2. Interestingly, dectin-2 also lacked an ITIM, which was identified in the intracellular domain of dectin-1. Thus, a simplified scenario would be that dectin-1 and DCIR deliver counteracting signals into DC, whereas dectin-2 has no apparent signaling potential. Unfortunately, information with respect to the

natural ligands that are recognized by CD69, DCIR, dectin-1, and/or dectin-2 is not available.

Duck DCIR encodes an inhibitory receptor that features an ITIM in the cytoplasmic domain. While DCAR1 is a pseudogene, DCAR2 encodes an activating receptor with a positively charged residue in transmembrane region. Study suggests the presence of full-length and alternatively spliced forms of both DCIR and DCAR2. Duck DCIR and DCAR transcripts are preferentially expressed in immune and mucosal tissues including spleen, bursa of Fabricius, intestine and lung. Targeting these receptors on DCs holds promise for enhancing immune responses relevant for hepatitis B and vaccination against avian influenza (Guo et al. 2008b). Recombinant duck β-defensin, expressed in HEK293T cells significantly down-regulated DCIR mRNA, without changing the expression of TLR-7, DCAR, CD44, CD58 and cytokines (Soman et al. 2009).

DCIR as Inhibitory Receptor with an ITIM Motif: After coligation with a chimeric Fcγ receptor IIB containing the cytoplasmic portion of mouse DCIR, Kanazawa et al. (2002) detected two distinct inhibitory effects of cytoplasmic DCIR (-) inhibition of B-cell-receptor-mediated Ca^{2+} mobilization and protein tyrosine phosphorylation (-); both these effects required the tyrosine residue inside the ITIM. This report suggested that mouse DCIR expressed on APCs can exert two distinct inhibitory signals depending on its ITIM tyrosine residue (Kanazawa et al. 2002). This inhibition was completely abolished when the tyrosine of the DCIR ITIM motif was mutated to a phenylalanine (Kanazawa et al. 2002).

DCIR also called CLECSF6, is dominantly present in neutrophil membranes. The expression of this protein was down-regulated in neutrophils treated with GM-CSF, TNF-α, LPS, and IL-1α, where as anti-inflammatory stimuli, including IL-4, IL-10 and IL-13, did not affect expression, that suggests that DCIR may be down-regulated during inflammation (Richard et al. 2002). Interestingly, GM-CSF, IL-3, IL-4 and IL-13 stimulation of neutrophils resulted in accumulation of a short form of DCIR mRNA, which encodes a putative non-functional protein which has been proposed to act as an antagonist to the full-length receptor (Richard et al. 2002, 2003).

During neutrophil signaling, the peptide bearing the ITIM in its phosphorylated form associates with both SHP-1 and SHP-2. Phosphorylated SHP-1 binds the ITIM whereas phosphorylated SHP-2 does not. In addition, GM-CSF reduces the binding of SHP-2 to the ITIM of CLECSF6 while enhancing the phosphorylation level of SHP-2. GM-CSF is known to recruit SHP-2 to its receptor. Results suggest that the phosphorylation of SHP-2 by GM-CSF promotes the binding of SHP-2 to the GM-CSF receptor to the disadvantage of CLECSF6. Therefore, upon a treatment with GM-CSF, SHP-2 could move from a CLECSF6 associated signalosome with a repressor function to a GM-CSF receptor associated signalosome with an activator function. This work supports the hypothesis that CLECSF6 is involved in the control of inflammation in neutrophils (Richard et al. 2003, 2006).

DCIR Inhibits TLR8-Mediated Cytokine Production: Targeting DCIR on PDCs not only results in efficient antigen presentation but also affects TLR9-induced IFN-α production. It suggests that targeting of DCIR can modulate human PDC (Meyer-Wentrup et al. 2008). The endogenous DCIR is internalized efficiently into human moDC after triggering with DCIR-specific mAb. Collectively, DCIR acts as an APC receptor that is endocytosed efficiently in a clathrin-dependent manner and negatively affects TLR8-mediated cytokine production (Meyer-Wentrup et al. 2009). Cross-priming $CD8^+$ T cells by targeting antigens to human DCs through DCIR allows activation of specific $CD8^+$ T-cell immunity (Klechevsky et al. 2010).

DCIR As an Attachment Factor for HIV-1: DCIR also plays a role in the capture and transmission of HIV-1 by DCs (Lambert et al. 2008). Although DC-SIGN was responsible for the trans-infection function of the virus, subsequent studies demonstrated that trans-infection of $CD4^+$ T cells with HIV-1 can also occur through DC-SIGN-independent mechanisms as well (Lambert et al. 2008). It was proposed that DCIR interaction with HIV could allow the virus to gain access to nondegradative endosomal organelles and lead to fusion of viral and endosomal membranes, allowing productive infection of the cells (Lambert et al. 2008). Thus, DCIR acts as a ligand for HIV-1 and is involved in events leading to productive virus infection. In this process, the neck domain of DCIR is important for DCIR-mediated effect on virus binding and infection. Therefore, DCIR plays a role in HIV-1 pathogenesis by supporting the productive infection of DCs and promoting virus propagation (Lambert et al. 2008).

DCIR Deficiency and Autoimmune Disorders: In addition to acting as a PRR for HIV, DCIR has been shown to play a role in controlling autoimmune disease. Aged DCIR deficient mice were found to spontaneously develop joint abnormalities, had elevated levels of autoantibodies and showed higher levels of $CD11c^+$ DCs and a proportional expansion of T cell populations in their lymph nodes (Fujikado et al. 2008). The DCIR deficiency was related to development of autoimmune disorders and DCIR gene polymorphisms were associated with rheumatoid arthritis (RA). When studying DCIR expression in patients with rheumatoid arthritis, the receptor was found to be abundantly expressed in synovial fluid but was not found in

healthy controls (Eklöw et al. 2008). In these samples, the DCIR was expressed on numerous cell types and surprisingly also on $CD56^+$ NK cells and $CD4^+$ and $CD8^+$ T cells. $DCIR^+$ T cells in the synovial fluid were activated, as well as much more abundant, than those found in peripheral blood (Eklöw et al. 2008). *Dcir* expression was also high in joints of two mouse RA models. These observations suggested that DCIR has an essential role in maintaining homeostasis of the immune system by controlling DC expansion and the development of autoimmune disease. Ronninger et al. (2008) analyzed the mRNA expression from the four known transcripts of DCIR in IFN-γ-treated human leukocytes together with fine mapping across the locus. RA patients and healthy controls were genotyped for several single nucleotide polymorphisms (SNPs) in *Dcir* and flanking regions. Results revealed that IFN-γ significantly downregulates the average expression of transcripts DCIR_v1, DCIR_v2, DCIR_v3 and DCIR_v4. The expression of *Dcir* showed significant association with variations in the gene. Cells with the RA-associated allele rs2024301 exhibited a significant increase in the expression of DCIR_v4. A fifth isoform, lacking exons 2, 3 and 4, illustrated that common genetic variations might influence *Dcir* mRNA expression. It also showed that the expression is regulated by the inflammatory mediator IFN-γ, affecting all four transcripts and that this was independent of genotype (Ronninger et al. 2008).

Since the *Dcir* may have an immune regulatory role, and since autoimmune-related genes are mapped to the DCIR locus in humans, Fujikado et al. (2008) found that aged $Dcir^{-/-}$ mice spontaneously develop sialadenitis and enthesitis associated with elevated serum autoantibodies. $Dcir^{-/-}$ mice showed a markedly exacerbated response to collagen-induced arthritis. The DC population was expanded excessively in aged and type II collagen-immunized $Dcir^{-/-}$ mice. Upon treatment with GMC-SF, $Dcir^{-/-}$ mouse-derived bone marrow cells (BMCs) differentiated into DCs more efficiently than did wild-type BMCs, owing to enhanced signal transducer and activator of transcription-5 phosphorylation. These observations indicated that Dcir is a negative regulator of DC expansion and has a crucial role in maintaining the homeostasis of the immune system.

35.9.2 Dendritic Cell Immunoactivating Receptor (DCAR)

In contrast to DCIR that induces negative signals through an ITIM in its cytoplasmic tail, Kanazawa et al. (2003) identified a C-type lectin receptor, dendritic cell immunoactivating receptor (DCAR), whose extracellular lectin domain is highly homologous to that of DCIR. The DCAR is expressed in tissues similar to DCIR, but its short cytoplasmic portion lacks signaling motifs like ITIM. However, presence of a positively charged arginine residue in the transmembrane region of the DCAR may explain its association with FcRγ chain and its stable expression on the cell surface. In A20 cells co-transfected with the FcRγ chain, cross-linking a chimeric receptor consisting of the extracellular region of FcγRIIB demonstrated that signaling from DCAR takes place via ITAM motif of the adaptor. The FcRγ chain was also required for surface expression of DCAR, as it enhanced receptor expression in transduced in 293T cells. Two isoforms of DCAR have been identified, one of which lacks the stalk region, but the ligands and biological functions of these isoforms still remain undefined. Thus, DCAR introduces activating signals into APCs through its physical and functional association with ITAM-bearing γ chain and provides an example of signaling via a DC-expressed C-type lectin receptor (Kanazawa et al. 2003). The gene of the DCAR has been identified next to the DCIR gene, and this acts as a putative activating pair of DCIR through association with an ITAM-bearing FcRγ chain. Kaden et al. (2009) functionally characterized mouse DCAR1 (mDCAR1) whose expression was strongly tissue dependent. The mDCAR1 expression was restricted to the $CD8^+$ DC subset in spleen and thymus and on subpopulations of $CD11b^+$ myeloid cells in bone marrow and spleen, and not detectable on both cell types in lymph nodes and peripheral blood. The Ag delivered via mDCAR1 was internalized, trafficked to early and late endosomes/lysosomes and, as a consequence, induced cellular and humoral responses in vivo even in absence of CD40 stimulation. Results indicated that mDCAR1 is a functional receptor on cells of the immune system and provides further insights into the regulation of immune responses by CLRs.

35.10 Macrophage-Inducible C-Type Lectin (Mincle)

Matsumoto et al. (1999) reported a macrophage-inducible C-type lectin (Mincle), as a downstream target of NF-IL6 in macrophages. NF-IL6 belongs to the CCAAT/enhancer binding protein (C/EBP) of transcription factors and plays a crucial role in activated macrophages. Mincle exhibits the highest homology to the members of group II C-type lectins. Mincle mRNA expression was strongly induced in response to several inflammatory stimuli, such as LPS, TNF-α, IL-6, and IFN-γ in wild-type macrophages. In contrast, NF-IL6-deficient macrophages displayed a much lower level of

Mincle mRNA induction following treatment with these inflammatory reagents. The mouse Mincle proximal promoter region contains an indispensable NF-IL6 binding element, demonstrating that Mincle is a direct target of NF-IL6. The Mincle gene locus was mapped at 0.6 centiMorgans proximal to CD4 on mouse chromosome 6. Mincle (also called as Clec4e and Clecsf9) is an FcRγ-associated activating receptor that senses damaged cells and involved in sensing necrosis (Brown 2008). Mincle selectively associates with the Fc receptor common γ-chain and activated macrophages to produce inflammatory cytokines and chemokines. Mincle-expressing cells are activated in the presence of dead cells. SAP130, a component of small nuclear ribonucloprotein, acts as a Mincle ligand that is released from dead cells. Thus, Mincle is a receptor that senses nonhomeostatic cell death and thereby induces the production of inflammatory cytokines to drive the infiltration of neutrophils into damaged tissue (Yamasaki et al. 2008).

35.10.1 Recognition of Pathogens

Yeast *Candida albicans* is a causative agent in mycoses of the skin, oral cavity, and gastrointestinal tract (Flores-Langarica et al. 2005). Identification of receptors, and their respective ligands, that are engaged by immune cells when in contact with *C. albicans* is crucial for understanding inflammatory responses leading to invasive candidiasis. Mincle, expressed predominantly on macrophages, was shown to play a role in macrophage responses to *Candida albicans*. The carbohydrate-recognition domain of human and mouse Mincle demonstrated the recognition of whole *C. albicans* yeast cells. After exposure to the yeast in vitro, Mincle localized to the phagocytic cup. However, it was not essential for phagocytosis. In absence of Mincle, production of TNF-α by macrophages was reduced, both in vivo and in vitro. In addition, mice lacking Mincle showed a significantly increased susceptibility to systemic candidiasis. Thus, Mincle plays a novel and non-redundant role in the induction of inflammatory signaling in response to *C. albicans* infection (Bugarcic et al. 2008; Wells et al. 2008). Mincle specifically recognizes *Malassezia* species among 50 different fungal species tested. *Malassezia* is a pathogenic fungus that causes skin diseases, such as tinea versicolor and atopic dermatitis, and fatal sepsis. Analyses of glycoconjugate revealed that Mincle selectively binds to α-mannose but not mannan. *Malassezia* activated macrophages to produce inflammatory cytokines/chemokines. *Malassezia*-induced cytokine/chemokine production by macrophages from Mincle$^{-/-}$ mice was significantly impaired. Results indicate that Mincle is the specific receptor for *Malassezia* species that plays a crucial role in immune responses to this fungus (Yamasaki et al. 2009).

35.10.2 Recognition of Mycobacterial Glycolipid, Trehalose Dimycolate

Trehalose-6,6′-dimycolate (TDM; also called cord factor) is a mycobacterial cell wall glycolipid that is the most studied immunostimulatory component of *M. tuberculosis*. However, its host receptor has not been clearly identified. Mincle is an essential receptor for TDM, which acts as a mincle ligand. TDM activated macrophages produce inflammatory cytokines and nitric oxide, which are completely suppressed in Mincle-deficient macrophages. The TDM and its synthetic analog trehalose-6,6-dibehenate (TDB) are potent adjuvants for Th1/Th17 vaccination that activate Syk-Card9 signaling in APCs. Further studies established that Mincle is a key receptor for the mycobacterial cord factor and controls the Th1/Th17 adjuvanticity of TDM and TDB (Geijtenbeek et al. 2003; Ishikawa et al. 2009; Matsunaga and Moody 2009; Schoenen et al. 2010).

35.11 Blood Dendritic Cell Antigen-2 (BDCA-2) (CLEC-4C)

35.11.1 BDCA-2: A Plasmacytoid DCs (PDCs)-Specific Lectin

Dzionek et al. (2000) identified three human blood DC Ags: BDCA-2, BDCA-3, and BDCA-4 which were expressed by PDCs. BDCA-2 and BDCA-4 were expressed on CD11c^{-} CD123^{bright} PDCs, whereas BDCA-3 was expressed on small population of CD11c^{+} CD123^{-} DCs. All three Ags were not detectable on a third blood DC population, which corresponded to CD1c^{+} CD11c^{bright} CD123^{dim}. Expression of all three Ags dramatically changes once blood DCs undergo in vitro maturation. For example, BDCA-2 was completely down-regulated on plasmacytoid CD11c^{-} CD123^{bright} DCs where as expression of BDCA-3 and BDCA-4 was up-regulated on sub-types of DCs on which they were localized. BDCA-2 was rapidly internalized at 37°C after mAb labeling. The three Ags serve as markers for respective subpopulations of blood DCs in fresh blood (Dzionek et al. 2000). Thus, BDCA-2 is a marker for human PDC.

35.11.2 Characterization

Molecular cloning of BDCA-2 (CD303) revealed that BDCA-2 is a type II C-type lectin, which shows 50.7% sequence identity at the amino acid level to its putative murine ortholog, the murine DC-associated C-type lectin 2. Anti-BDCA-2 mAbs are rapidly internalized and efficiently presented to T cells, indicating that BDCA-2 could play a role in ligand internalization and presentation. Furthermore,

ligation of BDCA-2 potently suppresses induction of interferon α/β production in plasmacytoid DCs, presumably by a mechanism dependent on protein-tyrosine phosphorylation by protein-tyrosine kinases (Dzionek et al. 2001). The-BDCA-2 receptor is involved in the down-regulation of virus triggered interferon-α/β production in PDC (Dzionek et al. 2001).

BDCA-2 transcripts were weakly detected in tonsils, bone marrow, pancreas, lymph nodes, peripheral blood leukocytes, testis and ovary, (Dzionek et al. 2001; Fernandes et al. 2000). While testes from patients with testicular cancer, neoplastic and normal epithelium were negative for BDCA-2, $CD123^+$ PDCs were associated with lymphoid aggregates in tumors. It appears that expression of BDCA-2 in tissues is restricted to PDCs (Dzionek et al. 2001). The murine homolog(s) of BDCA-2 are not yet known, whereas, at least five truncated BDCA-2 mRNA species have been detected in humans (Dzionek et al. 2001; Fernandes et al. 2000). BDCA-2 contains an EPN motif in the CRD but ligand(s) for this receptor have not been identified. BDCA-2 from cell lysates was shown not to recognize mannose as carbohydrate (Fernandes et al. 2000; Arce et al. 2001). However, transfection studies on 293T and Jurkat cells and freshly isolated PDCs revealed that BDCA-2 couples with FcRγ chain. This was substantiated in Jurkat cells co-transfected with BDCA-2 and FcRγ chain, resulting into stimulation of BDCA-2 induced intracellular protein phosphorylation and Ca^{2+} influx which was not possible when BDCA-2 was expressed alone (Dzionek et al. 2001; Cao et al. 2007).

35.11.3 BDCA-2 Signals in PDC via a BCR-Like Signalosome

Human BDCA2 protein lacks an identifiable signaling motif. Signaling through BDCA-2 is dependent on the ITAM motif in FcRγ chain and downstream pathways involve Syk, SH-2 domain-containing leukocyte protein of 65 kDa (Slp65), Vav1, phospholipase C-γ (PLCγ2) and Erk1/2. It forms a complex with the transmembrane adapter Fc εRI γ. The signaling machinery in human PDCs is similar to that which operates downstream of BCR, which is distinct from the system involved in TCR signaling. BDCA2 crosslinking resulted in the activation of the BCR-like cascade, which potently suppressed the ability of PDCs to produce type I interferon and other cytokines in response to Toll-like receptor ligands (Cao et al. 2007; Rock et al. 2007).

By associating with Fc εRI γ, BDCA2 activates a BCR-like signaling pathway to regulate the immune functions of PDCs. BDCA-2 signaling induces tyrosine phosphorylation and Src kinase dependent Ca^{2+} influx. Cross-linking BDCA2 results in the inhibition of IFN-I production in stimulated PDC. PDCs express a signalosome similar to the BCR signalosome, consisting of Lyn, Syk, Btk, Slp65 (Blnk) and PLCγ2. BDCA2 associates with the signaling adapter FcR γ-chain. Triggering BDCA2 leads to tyrosine phosphorylation of Syk, Slp65, PLCγ2 and cytoskeletal proteins. Analogous to BCR signaling, BDCA2 signaling is likely linked with its internalization by clathrin-mediated endocytosis. The inhibition of IFN-I production by stimulated PDC is at least partially regulated at the transcriptional level. These results support a possible therapeutic value of an anti- BDCA2 mAb strategy, since the production of IFN-I by PDCs is considered to be a major pathophysiological factor in SLE patients (Röck et al. 2007).

Engagement of BDCA-2 Blocks TRAIL-Mediated Cytotoxic Activity of PDC: PDCs express Toll-like receptor (TLR) 9, which mediates recognition of microbial DNA during infection or self-DNA in autoimmune diseases. BDCA-2-induced signaling in PDCs inhibits up-regulation of CD86 and CD40 molecules in CpG-activated PDCs, but not in CD40L-activated PDCs. Furthermore, triggering of BDCA-2 diminished the ability of CpG- and CD40L-stimulated PDCs to process and present antigen to antigen-specific autologous memory T cells. Jähn et al. (2010) suggest that BDCA-2 represents an attractive target for clinical immunotherapy of IFN-I dependent autoimmune diseases influencing both, IFN-I production and antigen-specific T-cell stimulation by PDCs.

TLR7 and TLR9 ligands can induce the secretion of biologically active TNF-related apoptosis-inducing ligand (TRAIL) by PDCs. Accordingly PDC supernatant is endowed with TRAIL-mediated cytotoxic activity when tested on a TRAIL-sensitive Jurkat cell line. Importantly, both TRAIL secretion and cytotoxic activity of PDC supernatants are completely abolished by BDCA2 ligation. These results document a negative regulatory pathway of PDC cytotoxic activity that may be relevant in pathological situations such as tumors and autoimmune diseases (Riboldi et al. 2009).

35.11.4 Functions of BDCA-2

Regulation of IFN-α Production: PDCc are the natural type I IFN-producing cells that produce large amounts of IFN-α in response to viral stimulation. Cross-linking BDCA-2, BDCA-4, and CD4 on PDC regulates IFN-α production at the level of IRF-7, while the decrease in IFN-α production after CD123 cross-linking is due to stimulation of the IL-3R and induction of PDC maturation (Fanning et al. 2006).

Pregnancy: Dendritic cells are involved in the immune regulation during physiological pregnancy. $CD1c^+$ and BDCA-2^+ cells can influence the Th2 phenomenon which is observed during physiological pregnancy. It seems possible that lower BDCA-2^+ cells percentage and higher $CD1c^+$: BDCA-2^+ ratio can be associated with increased Th1-type immunity in patients with pre-eclampsia (Darmochwal-Kolarz et al. 2003). DCs within the decidua have been implicated in pregnancy maintenance. Ban et al. (2008) identified three DC subsets in normal human first-trimester decidua: BDCA-1^+ $CD19^-$ $CD14^-$ myeloid DC type 1 (MDC1), BDCA-3^+ $CD14^-$ myeloid DC type 2 (MDC2) and BDCA-2^+ $CD123^+$ plasmacytoid DC (PDC).

BDCA-2^+ in SLE: Type 1 IFN is thought to be implicated in the autoimmune process of SLE. Plasmacytoid DC, which are natural IFN-α producing cells, play a pivotal epipathogenic role in SLE. The phenotypic characteristics of peripheral blood DC in SLE patients show a reduced number of both BDCA-2^+ plasmacytoid DC and $CD11c^+$ myeloid DC. These alternations of the DC subset may drive the autoimmune response in SLE (Blomberg et al. 2003; Migita et al. 2005). BDCA-2-expressing pDCs are termed natural IFN α-producing cells, IFN α production could be inhibited by anti-BDCA-2/4 mAb (Blomberg et al. 2003; Wu et al. 2008).

Hematopoietic Malignancies: $CD4^+CD56^+$ hematodermic neoplasms are rare, aggressive hematopoietic malignancies usually presenting with cutaneous masses followed by a leukemic phase. Accumulating evidence suggests that these neoplasms represent malignant counterparts to the plasmacytoid DCs. BDCA-2, expressed predominantly on plasmacytoid DCs in $CD7^+$ subset of hematodermic neoplasms, and similar to non-neoplastic plasmacytoid dendritic cells, indicates a relatively more mature differentiation state. Clinical follow-up data confirm the aggressiveness of these tumors and suggests that BDCA-2 immunoreactivity may herald a significant reduction in survival (Jaye et al. 2006).

35.12 CLECSF8

Murine Clecsf8 was first identified through a differential display PCR screen of numerous cell lines for macrophage-specific genes (Balch et al. 1998). The molecule, named mouse macrophage C-type lectin, is a 219-amino acid, type II transmembrane protein with a single extracellular C-type lectin domain and expressed in cell lines and normal mouse tissues in a macrophage-restricted manner. The expression of this receptor was upregulated on these cells by IL-6, IL-10, TNF-α or IFN-γ, but was downreglated with LPS. The cDNA and genomic sequences of mouse macrophage C-type lectin indicate that it is related to the Group II animal C-type lectins. The mcl gene locus has been mapped between the genes for the interleukin-17 receptor and CD4 on mouse chromosome 6, the same chromosome as the mouse NK cell gene complex (Arce et al. 2004).

Arce et al. (2004) characterized the human orthologue of the mouse Mcl/Clecsf8. Human CLECSF8 codes for a type II membrane glycoprotein of 215 amino acids that belongs to human C-type lectin family. The cytoplasmic tail of CLECSF8 lacks consensus signaling motifs and its extracellular region shows a single CRD. The CLECSF8 gene has been localized on the telomeric region of the NK gene complex on chromosome 12p13 close to MINCLE. CLECSF8 mRNA shows a monocyte/ macrophage expression pattern. Biochemical analysis of CLECSF8 on transiently transfected cells showed a glycoprotein of 30 kDa. Cross-linking of the receptor leads to a rapid internalization suggesting that CLECSF8 constitutes an endocytic receptor. Preliminary studies suggest that Clecsf8 could be upregulated in proinflammatory settings, as has been described for Mincle and Dectin-2 (Arce et al. 2004).

35.13 Macrophage Galactose/N-acetylgalactosamine Lectin (MGL)

The hMGL is a marker of immature DCs and it functions as an endocytic receptor for glycosylated antigens (Higashi et al. 2002). Van Die et al. (2003) identified an exclusive specificity for terminal α- and β-linked GalNAc residues that naturally occur as parts of glycoproteins or glycosphingolipids. Specific glycan structures containing terminal GalNAc moieties, expressed by helminth parasite *Schistosoma mansoni* as well as tumor antigens and a subset of gangliosides, were found as ligands for MGL. Studies indicate an endogenous function for DC-expressed MGL in the clearance and tolerance to self-gangliosides, and in the pattern recognition of tumor antigens and foreign glycoproteins derived from helminth parasites. The dendritic cell-specific C-type lectin DC-SIGN is a receptor for Schistosoma 8 MGL recognition of terminal GalNAc residues (Van Die et al. 2003). The MGL has been discussed in greater details in Chap. 33

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DC-SIGN Family of Receptors

36

Rajesh K. Gupta and G.S. Gupta

36.1 DC-SIGN (CD209) Family of Receptors

In the immune system, C-type lectins and CTLDs have been shown to act both as adhesion and as pathogen recognition receptors. The **D**endritic **c**ell-**s**pecific **I**CAM-3 **g**rabbing **n**on-integrin (DC-SIGN) and its homologs in human and mouse represent an important C-type lectin family. DC-SIGN contains a lectin domain that recognizes in a Ca^{2+}-dependent manner carbohydrates such as mannose-containing structures present on glycoproteins such as ICAM-2 and ICAM-3. DC-SIGN is a prototype C-type lectin organized in microdomains, which have their role as pathogen recognition receptors in sensing microbes. Although the integrin LFA-1 is a counter-receptor for both ICAM-2 and ICAM-3 on DC, DC-SIGN is the high affinity adhesion receptor for ICAM-2/-3. While cell–cell contact is a primary function of selectins, collectins are specialized in recognition of pathogens. Interestingly, DC-SIGN is a cell adhesion receptor as well as a pathogen recognition receptor. As adhesion receptor, DC-SIGN mediates the contact between dendritic cells (DCs) and T lymphocytes, by binding to ICAM-3, and mediates rolling of DCs on endothelium, by interacting with ICAM-2. As pathogen receptor, DC-SIGN recognizes a variety of microorganisms, including viruses, bacteria, fungi and several parasites (Cambi et al. 2005). The natural ligands of DC-SIGN consist of mannose oligosaccharides or fucose-containing Lewis-type determinants. In this chapter, we shall focus on the structure and functions of DC-SIGN and related CTLDs in the recognition of pathogens, the molecular and structural determinants that regulate the interaction with pathogen-associated molecular patterns. The heterogeneity of carbohydrate residues exposed on cellular proteins and pathogens regulates specific binding of DC-expressed C-type lectins that contribute to the diversity of immune responses created by DCs (van Kooyk et al. 2003a; Cambi et al. 2005).

The DC-SIGN, originally described in 1992 as a C-type lectin able to bind the HIV surface protein, gp120 (Curtis et al. 1992), is important for efficient infection with HIV (Geijtenbeek et al. 2000c). Recent advances on a broad perspective concerning DC-SIGN structure, signaling and immune function have appeared in excellent reviews (den Dunnen et al. 2009; Gringhuis and Geijtenbeek 2010; Svajger et al. 2010). Interaction of DC-SIGN with the viral envelope glycoproteins may evoke cellular signal transduction implicated in viral pathogenesis.

36.1.1 CD209 Family Genes in Sub-Human Primates

Two *CD209* family genes identified in humans, *CD209* (*DC-SIGN*) and *CD209L* (*DC-SIGNR*/*L-SIGN*/*LSECtin*), encode C-type lectins that serve as adhesion receptors for ICAM-2 and ICAM-3 and participate in the transmission of HIV and SIV respectively to target cells in vitro. The *CD209* gene family that encodes C-type lectins in primates includes CD209 (DC-SIGN), CD209L (L-SIGN) and CD209L2. The *CD209* gene family in sub-human primates showed evolutionary alterations that occurred in this family across primate species. All of the primate species, specifically, Old World monkeys (OWM) and apes, have orthologues of human *CD209*. In contrast, *CD209L* is missing in OWM but present in apes. A third family member, that has been named *CD209L2*, was cloned from rhesus monkey cDNA and subsequently identified in OWM and apes but not in humans. Rhesus *CD209L2* mRNA was prominently expressed in the liver and axillary lymph nodes. Despite a high level of sequence similarity to both human and rhesus CD209, rhesus CD209L2 was substantially less effective at binding ICAM-3 and poorly transmitted HIV type 1 and SIV to target cells relative to CD209. The Toll-like receptor (TLR) gene family shares with CD209 genes a common

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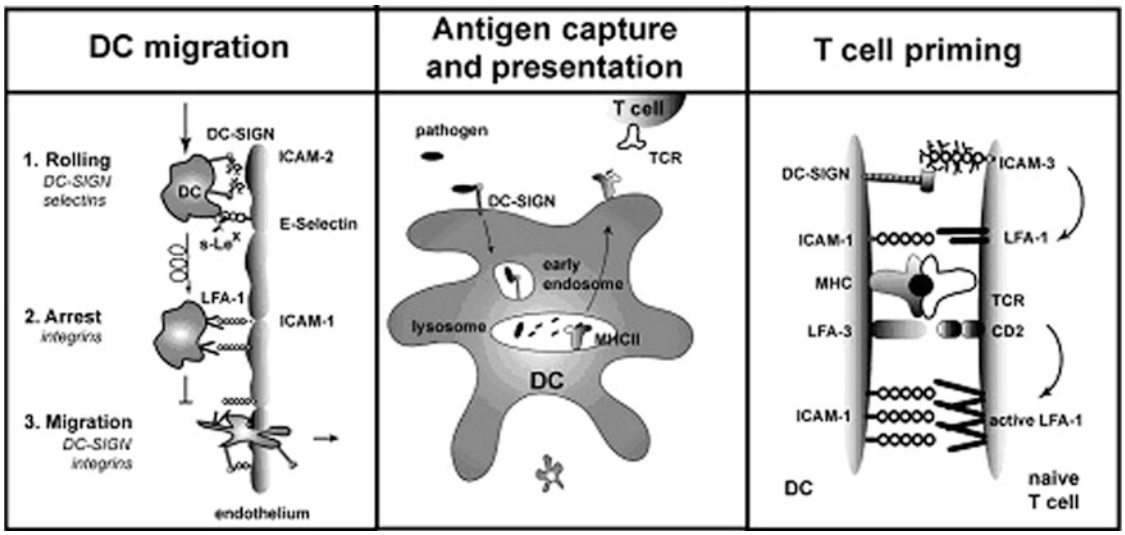

Fig. 36.1 DC-SIGN controls many functions of DC to elicit immune responses. The egress of precursor DC from blood into tissues is mediated partly by DC-SIGN. DC-SIGN facilitates rolling and transendothelial migration of DC-SIGN_ precursor DC, whereas arrest is mediated by integrin-mediated interactions. DC-SIGN also functions as an antigen receptor. DC-SIGN internalizes rapidly upon binding soluble ligand and is targeted to late endosomes/lysosomes, where antigens are processed and presented by MHC class II molecules. Moreover, initial DC-T-cell clustering, necessary for an efficent immune response, is mediated by transient interactions between DC-SIGN and ICAM-3. This interaction facilitates the formation of low-avidity LFA-1/ICAM-1 interaction and scanning of the antigen-MHC repertoire. It is becoming clear that other C-type lectins also participate in these processes. Selectins and the MR may regulate DC migration. Many other C-type lectins on DC, such as MR, DEC205, DC-ASPGR, BCDA-2, and dectin-1, are antigen receptors that recognize various distinct carbohydrate-containing antigens. It has been postulated that dectin-1 regulates T-cell priming, however its interaction with T cells is not carbohydrate-dependent (Reprinted with permission from Geijtenbeek et al. 2002a © Journal of Leukocyte Biology)

profile of evolutionary constraint (Bashirov et al. 2003b; Ortiz et al. 2008).

36.2 DC-SIGN (CD209): An Adhesion Molecule on Dendritic Cells

DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin, where ICAM is intercellular adhesion molecule) or CD209 is a type II C-type lectin expressed by DCs. DC-SIGN is specifically expressed on DCs and has been identified on monocyte-derived DCs in vitro and on DC subsets of skin, mucosal tissues, tonsils, lymph nodes, and spleen in vivo (Geijtenbeek et al. 2000a, c). DC-SIGN on DC binds the intercellular adhesion molecule (ICAM)-3 (CD50) with very high affinity. Although ICAM-3, a member of the immunoglobulin (Ig) superfamily, is known to be a ligand for β2 integrins lymphocyte function-associated antigen-1 (LFA-1; αLβ2) and $\alpha_D\beta_2$ (Bleijs et al. 2001; Geijtenbeek et al. 2002a; van Kooyk and Geijtenbeek 2002) (Fig. 36.1), these receptors did not contribute to the binding activity of ICAM-3 by DC. Many C-type lectins have been identified on DCs: (1) Type I multi-CRD lectins are represented by the mannose receptor (Sallusto et al. 1995) and DEC-205 (Kato et al. 2000); and (2) type II single-CRD lectins by DC-SIGN, dectin-1, dectin-2, Langerin, BCDA-2, DCIR, DLEC, CLEC-1, and DC-ASGPR (Geijtenbeek et al. 2000a) (Chap. 35). Within the CRD, the highly conserved EPN or QPD sequences are essential in recognizing mannose- and galactose-containing structures, respectively.

DC-SIGN contains a short, cytoplasmic N-terminal domain with several intracellular sorting motifs, an extracellular stalk of seven complete and one partial tandem repeat, and a terminal lectin or CRD (Geijtenbeek et al. 2000a; Curtis et al. 1992). The DC-SIGN has three conserved cytoplasmic tail motifs: the tyrosine (Y)-based, dileucine (LL), and triacidic cluster (EEE), which are believed to regulate ligand binding, uptake, and trafficking. The full-length porcine DC-SIGN cDNA encodes a type II transmembrane protein of 240 amino acids. Phylogenetic analysis revealed that porcine DC-SIGN, together with bovine, canis and equine DC-SIGN, is more closely related to mouse SIGNR7 and SIGNR8 than to human DC-SIGN. Porcine DC-SIGN has the same gene structure as bovine, canis DC-SIGN and mouse SIGNR8 with eight exons. Porcine DC-SIGN mRNA expression was detected in spleen, thymus, lymph node, lung, bone marrow and muscles. Porcine DC-SIGN protein was found to express on the surface of monocyte-derived macrophages and dendritic cells, alveolar macrophages, lymph node sinusoidal macrophage-like, dendritic-like and endothelial cells but not of monocytes, peripheral blood lymphocytes or lymph node lymphocytes (Huang et al. 2009). Bovine ortholog of human DC-SIGN,

within the bovine genome, exists as a single copy with a sequence similar to that of SIGNR7 (Yamakawa et al. 2008).

36.3 Ligands of DC-SIGN

36.3.1 Carbohydrates as Ligands of DC-SIGN

DC-SIGN and its close relative DC-SIGNR recognize various oligosaccharide ligands found on human tissues as well as on pathogens including viruses, bacteria, and parasites through the receptor lectin domain-mediated carbohydrate recognition. The DC-SIGN and DC-SIGNR bind to high-mannose carbohydrates on a variety of viruses. Studies have shown that these receptors bind the outer trimannose branch Manα1-3(Manα1-6)Manα present in high mannose structures. Although the trimannoside binds to DC-SIGN or DC-SIGNR more strongly than mannose, additional affinity enhancements are observed in presence of one or more Manα1-2Manα moieties on nonreducing termini of oligomannose structures. The molecular basis of this enhancement was investigated in crytstals of DC-SIGN bound to a synthetic six-mannose fragment of a high mannose N-linked oligosaccharide, Manα1-2Manα1-3 (Manα1-2Manα1-6)Manα1-6Man and to the disaccharide Manα1-2Man. The structures revealed mixtures of two binding modes in each case. Each mode features typical C-type lectin binding at main Ca^{2+}-binding site by one mannose residue. In addition, other sugar residues form contacts unique to each binding mode. Thus the affinity enhancement of DC-SIGN toward oligosaccharides decorated with Manα1-2Manα structure is due to multiple binding modes at the primary Ca^{2+} site, which provides both additional contacts and a statistical (entropic) enhancement of binding (Feinberg et al. 2007).

36.3.1.1 Lewis Antigen as Ligand

In addition to high-mannose moieties, DC-SIGN recognizes nonsialylated $Lewis^X$ (Le^x) and Le^Y glycans and binds to Le^X-expressing pathogens such as *Schistosoma mansoni* and *Helicobacter pylori* (Appelmelk et al. 2003; van Die et al. 2003). Mouse homolog of human DC-SIGN has similar carbohydrate specificity for high mannose-containing ligands present on both cellular and pathogen ligands and called mSIGNR1or SIGNR1. However, mSIGNR1 interacts not only with $Le^{x/y}$ and $Le^{a/b}$ antigens similar to DC-SIGN, but also with sialylated Le^x, a ligand for selectins. The differential recognition of Lewis antigens suggests differences between mSIGNR1 and DC-SIGN in the recognition of cellular ligands and pathogens that express Lewis epitopes (Koppel et al. 2005a). Using the known 3D structure of the Lewis-x trisaccharide, Timpano et al. (2008) identified some monovalent α-fucosylamides that bind to DC-SIGN. α-fucosylamides work as functional mimics of chemically and enzymatically unstable α-fucosides and describes interesting candidates for the preparation of multivalent systems able to block DC-SIGN with high affinity and with potential biomedical applications.

The binding partner of DC-SIGN on endothelial cells is the glycan epitope Le^Y, expressed on ICAM-2. The interaction between DC-SIGN on DCs and ICAM-2 on endothelial cells is strictly glycan-specific. ICAM-2 expressed on CHO cells only served as a ligand for DC-SIGN when properly glycosylated, underscoring its function as a scaffolding protein (García-Vallejo et al. 2008). Oligosaccharide ligands expressed on SW1116, a typical human colorectal carcinoma are recognized by DC-SIGN, and has similar carbohydrate-recognition specificities as MBP. These tumor-specific oligosaccharide ligands comprise clusters of tandem repeats of Le^a/Le^b glycans on carcinoembryonic Ag (CEA) and CEA-related cell adhesion molecule 1 (CEACAM1). DC-SIGN ligands containing Le^a/Le^b glycans are also highly expressed on primary cancer colon epithelia but not on normal colon epithelia (Nonaka et al. 2008). Fucosylated glycans similar to pathogens are also found in a variety of allergens, but their functional significance remains unclear. Results suggest that allergens are able to interact with DC-SIGN and induce TNF-α expression in monocyte-derived DCs (MDDCs) via, in part, Raf-1 signaling pathways (Hsu et al. 2010).

Structural characterization of glycolipids, in combination with solid phase and cellular binding studies revealed that DC-SIGN binds to carbohydrate moieties of both glycosphingolipid species with Galβ1-4(Fucα1-3)GlcNAc (Le^x) and Fucα1-3Galβ1-4(Fucα1-3)GlcNAc (pseudo- Le^y) determinants. These data indicate that surveying DCs in the skin may encounter schistosome-derived glycolipids immediately after infection. Crystal structure of the CRD of DC-SIGN bound to Le^x provided insight into the ability of DC-SIGN to bind fucosylated ligands. The observed binding of schistosome-specific pseudo-Le^y to DC-SIGN is not directly compatible with the model described (Meyer et al. 2005).

36.4 Structure of DC-SIGN

36.4.1 Neck-Domains

Human DC-SIGN is a type II membrane protein which contains 404 amino acids and is of 44 kDa in molecular weight. DC-SIGN consists of extracellular domain, transmembrane region and cytoplasmic region (Fig. 36.2). The extracellular portion of each receptor contains a membrane-distal CRD and forms tetramers stabilized by an extended neck region consisting of 23 amino acid repeats. Cross-linking analysis of full-length receptors expressed in

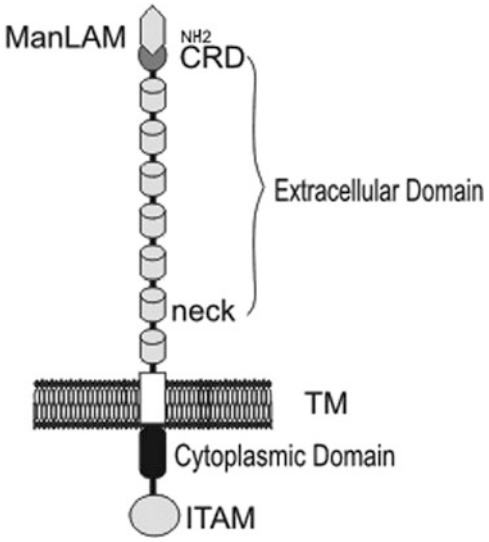

Fig. 36.2 Structure of DC-SIGN. Cytoplasmic domain, transmembrane region (TM) and extracellular domain are the three parts of DC-SIGN. The extracellular domain contains carbohydrate recognition domain (CRD) and neck domain. Cytoplasmic domain contains LL (di-leucine), EEE (tri-acidic clusters) and other internalization motifs and is connected to an incomplete ITAM. CRD recognizes certain carbohydrate-contained antigens like ManLAM and $Lewis^X$ by four amino acids (Glu^{347}, Asn^{349}, Glu^{354} and Asn^{365}) and one Ca^{2+}-binding site in it (Zhou et al. 2006)

fibroblasts confirmed the tetrameric state of the intact receptors. Alternative splicing and genomic polymorphism generate DC-SIGN mRNA variants, which were detected at the sites of pathogen entrance and transmission. Naturally occurring DC-SIGN neck variants differ in multimerization competence in the cell membrane, exhibit altered sugar binding ability, and retain pathogen-interacting capacity, implying that pathogen-induced cluster formation predominates over the basal multimerization capability. Reports highlight the central role of the neck domain in the pH-sensitive control of oligomerization state, in the extended conformation of the protein, and in CRD organization and presentation (Guo et al. 2006; Tabarani et al. 2009). Analysis of DC-SIGN neck polymorphisms indicated that the number of allelic variants is higher than previously thought and that the multimerization of the prototypic molecule is modulated in presence of allelic variants with a different neck structure. Serrano-Gómez et al. (2008) demonstrated that the presence of allelic variants or a high level of expression of neck domain splicing isoforms might influence the presence and stability of DC-SIGN multimers on the cell surface, thus providing a molecular explanation for the correlation between DC-SIGN polymorphisms and altered susceptibility to HIV-1 and other pathogens. Neck domains of DC-SIGN and DC-SIGNR are shown to form tetramers in the absence of the CRDs. Analysis indicates that interactions between the neck domains account full stability of the tetrameric extracellular portions of the receptors. The neck domains are ~40% α-helical based on circular dichroism analysis. However, in contrast to other glycan-binding receptors in which fully helical neck regions are intimately associated with C-terminal C-type CRDs, the neck domains in DC-SIGN and DC-SIGNR act as autonomous tetramerization domains and the neck domains and CRDs are organized independently. Neck domains from polymorphic forms of DC-SIGNR that lack some of the repeat sequences show modestly reduced stability, but differences near the C-terminal end of the neck domains lead to significantly enhanced stability of DC-SIGNR tetramers compared to DC-SIGN (Yu et al. 2009). The length of the neck region shows variable levels of polymorphism, and can critically influence the pathogen binding properties of these two receptors. In Colored South African population of 711 individuals, including 351 tuberculosis patients and 360 healthy controls, Barreiro et al. (2007) revealed that none of the DC-SIGN and L-SIGN neck-region variants or genotypes seems to influence the individual susceptibility to develop tuberculosis.

Surface force measurements between apposed lipid bilayers displaying the extracellular domain of DC-SIGN and a neoglycolipid bearing an oligosaccharide ligand provide evidence that the receptor is in an extended conformation and that glycan docking is associated with a conformational change that repositions the carbohydrate-recognition domains during ligand binding. The results further show that the lateral mobility of membrane-bound ligands enhances the engagement of multiple carbohydrate-recognition domains in the receptor oligomer with appropriately spaced ligands. These studies highlight differences between pathogen targeting by DC-SIGN and receptors in which binding sites at fixed spacing bind to simple molecular patterns (Menon et al. 2009).

36.4.2 Crystal Structure of DC-SIGN (CD209) and DC-SIGNR (CD299)

To understand the tetramer-based ligand binding avidity, the crystal structure of DC-SIGNR was determined with its last repeat region. Compared to the carbohydrate-bound CRD structure, the structure revealed conformational changes in the calcium and carbohydrate coordination loops of CRD, an additional disulfide bond between the N and the C termini of the CRD, and a helical conformation for the last repeat. On the basis of the current crystal structure and other published structures with sequence homology to the repeat domain, Snyder et al. (2005) generated a tetramer model for DC-SIGN/DC-SIGNR using homology modeling and proposed a ligand-recognition index to identify potential receptor ligands.

The CRD of DC-SIGN is a globular structure consisting of 2 α-helices, 12 β-strands, and 3 disulphide bridges. A loop protrudes from the protein surface and forms part of two Ca^{2+}- binding sites. One of such sites is essential for the conformation of CRD, and the other is essential for direct coordination of the carbohydrate structures. Four amino acids (Glu^{347}, Asn^{349}, Glu^{354} and Asn^{365}) interact with Ca^{2+} at this site and dictate the recognition of specific carbohydrate structures. CRD can recognize certain carbohydrate containing antigens like ManLAM and Le^{x}. Crystal structures of CRDs of DC-SIGN and of DC-SIGNR bound to oligosaccharide revealed that these receptors selectively recognize endogenous high-mannose oligosaccharides and represent a new avenue for developing HIV prophylactics (Feinberg et al. 2001). Hydrodynamic studies on truncated receptors demonstrated that the portion of the neck of each protein adjacent to the CRD was sufficient to mediate the formation of dimers, whereas regions near the N terminus were needed to stabilize the tetramers. Some of the intervening repeats are missing from polymorphic forms of DC-SIGNR. Two different crystal forms of truncated DC-SIGNR comprising two neck repeats and the CRD revealed that the CRDs are flexibly linked to the neck, which contains α-helical segments interspersed with non-helical regions. Differential scanning calorimetry measurements indicated that the neck and CRDs were independently folded domains (Feinberg et al. 2005).

The neck domain contains 7 or 8 complete tandem repeats of 23 amino acids each and 1 incomplete repetitive sequence. It is required for oligomerization, which regulates carbohydrate specificity. Transmembrane region is essential in localization of DC-SIGN on cell surface. The cytoplasmic region contains internalization motifs, such as di-leucine (LL) motif, tri-acidic (EEE) clusters and an incomplete immunoreceptor tyrosine (Y) based activation motif (ITAM) which are believed to regulate ligand binding, uptake, and trafficking. The LL motif participates in antigen internalization and EEE clusters participate in signal transduction (Guo et al. 2004; Feinberg et al. 2001; van Kooyk and Geijtenbeek 2003). DC-SIGNR is homologous to DC-SIGN and is also denoted DC-related protein (L-SIGN), which has a similar structure to DC-SIGN. The genes encoding DC-SIGNR are similar to those of DC-SIGN (Pohlmann et al. 2001). Based on crystal structures and hydrodynamic data, models for the full extracellular domains of the receptors have been generated. The observed flexibility of the CRDs in the tetramer, combined with previous data on the specificity of these receptors, suggests an important role for oligomerization in the recognition of endogenous glycans, in particular those present on the surfaces of enveloped viruses recognized by these proteins (Feinberg et al. 2005). Azad et al. (2008) mutated each of the three conserved cytoplasmic tail motifs of DC-SIGN by alanine substitution and tested their roles in phagocytosis and receptor-mediated endocytosis of highly mannosylated ligands, *M. tuberculosis* ManLAM and HIV-1 surface gp120, respectively, in transfected human myeloid K-562 cells. Azad et al. (2008) indicated a dual role for EEE motif as a sorting signal in the secretory pathway and a lysosomal targeting signal in the endocytic pathway. The DC-SIGN and L-SIGN have been shown to interact with a vast range of infectious agents, including *M. tuberculosis*.

36.5 DC-SIGN versus DC-SIGN-RELATED RECEPTOR [DC-SIGNR or L-SIGN (CD 209)/LSEctin] (Refer Section 36.9)

36.5.1 DC-SIGN Similarities with DC-SIGNR/ L-SIGN/LSEctin

DC- SIGNR is homologous to DC-SIGN and is also denoted as DC- related protein (L-SIGN), which has a similar structure to DC-SIGN. The genes encoding DC-SIGNR are similar to those of DC-SIGN (Pohlmann et al. 2001). The mRNA of L-SIGN shows about 90% similarity with DC-SIGN, which has a similar binding specificity to L-SIGN. L-SIGN like DC-SIGN binds to the cellular ligands i.e. ICAM-2 and ICAM-3 (Bashirova et al. 2001). The sequences of CRDs of DC-SIGN and DC-SIGNR show greatest identity to human asialoglycoprotein receptors (41% and 34% at amino acid level, respectively) and rat CD23 (both 33% at amino acid level). Consistent with previous reports (Curtis et al. 1992; Geijtenbeek et al. 2000a, b), DC-SIGN shows features of a mannose binding lectin, as opposed to the features of a protein-binding NK cell lectin (Weis et al. 1998). DC-SIGNR shows 77% identity to DC-SIGN at the amino acid level and also possesses all the residues shown to be required for the binding of mannose (Weis et al. 1998). The closely linked gene, DC-SIGNR, shows 73% identity to DC-SIGN at RNA level and a similar genomic organization.

The DC-SIGN and DC-SIGNR/L-SIGN (or CLEC4M) directly recognize a wide range of micro-organisms of major impact on public health. Both genes have long been considered to share similar overall structure and ligand-binding characteristics. Both DC-SIGN and DC-SIGNR efficiently bind HIV-1 surface glycoproteins of viruses and other viral as well as nonviral pathogens by interacting with high mannose oligosaccharides and assist either cis or trans infection. DC-SIGNR/L-SIGN (Lozach et al. 2004) is specifically expressed by liver sinusoidal endothelial cells (LSEC), a liver-resident APC, by endothelial cells in lymph nodes (Bashirova et al. 2001; Pohlmann et al. 2001) and by placenta (Soilleux 2003b). Similar *trans* activity for other viruses has been reported, and the receptors can also directly mediate infection of cells in *cis* (Soilleux 2003; Alvarez

et al. 2002; Gardner et al. 2003). There is also evidence that these receptors interact with bacterial pathogens and with parasites (Appelmelk et al. 2003). The L-SIGN/DC-SIGNR functions as a HIV-1 trans-receptor similar to DC-SIGN (Bashirova et al. 2001). Moreover, L-SIGN interacts with other pathogens such as *Ebola virus* (Alvarez et al. 2002), to the envelope glycoproteins from *HIV-1, Hepatitis C virus* and *cytomegalovirus* (Alvarez et al. 2002; Bashirova et al. 2001; Bovin et al. 2003; Gardner et al. 2003; Halary et al. 2002), similar to DC-SIGN. L-SIGN is a liver-specific capture receptor for hepatitis C virus (Bovin et al. 2003).

Both DC-SIGN and DC-SIGNR bind ligands bearing mannose and related sugars through CRDs. The CRDs of DC-SIGN and DC-SIGNR bind $Man_9GlcNAc_2$ oligosaccharide 130- and 17-fold more tightly than mannose (Mitchell et al. 2001). Both DC-SIGN and DC-SIGNR possess a neck region, made up of multiple repeats, which supports the ligand-binding domain. Cross-linking analysis of full-length receptors expressed in fibroblasts confirms the tetrameric state of the intact receptors. The extra-cellular domain of DC-SIGN and DC-SIGNR, each comprises seven 23-residue tandem repeats, encoded by a single exon to form a coiled coil neck region. There is very high sequence identity between the repeat units, within each protein, and between DC-SIGN and DC-SIGNR. By analogy to other lectin receptors, such as the asialoglycoprotein receptors and CD23 (Bates et al. 1999; Beavil et al. 1995), it was suggested that this domain could mediate oligomerization, forming an α-helical coiled coil.

A subset of B cells in the blood and tonsils of normal donors also express DC-SIGN, which increased after stimulation in vitro with IL-4 and CD40 ligand, with enhanced expression of activation and co-stimulatory molecules CD23, CD58, CD80, and CD86, and CD22. The activated B cells captured and internalized X4 and R5 tropic strains of HIV-1, and mediated trans- infection of T cells. DC-SIGN serves as a portal on B cells for HIV-1 infection of T cells in trans. Transmission of HIV-1 from B cells to T cells through DC-SIGN pathway could be important in the pathogenesis of HIV-1 infection (Gupta and Rinaldo 2006).

36.5.2 Domain Organization of DC-SIGN and DC-SIGNR

DC-SIGN and DC-SIGNR consist of an N-terminal cytoplasmic domain, a repeat region consisting of seven 23-amino-acid tandem repeats, and a C-terminal C-type CRD that binds mannose-enriched carbohydrate modifications of host and pathogen proteins. They bind with highest affinity to larger glycans that contain 8 or 9 mannose residues (Mitchell et al. 2001; Guo et al. 2004). In addition, DC-SIGN, but not DC-SIGNR, binds to fucose-containing glycans, such as those present on the surfaces of nematode parasites (Appelmelk et al. 2003; Guo et al. 2004). The sugar-binding activity of each protein is conferred by a Ca^{2+}-dependent CRD that is located at the C terminus of the receptor polypeptide. This domain is separated from the membrane anchor by a neck region consisting of multiple 23-amino acid repeats. The neck forms an extended structure that associates to create a tetramer at the cell surface (Feinberg et al. 2001) (Fig. 36.3). Signals in the N-terminal cytoplasmic domain of DC-SIGN direct internalization of the receptor, which can thus mediate endocytosis and degradation of glycoproteins (Guo et al. 2004). DC-SIGNR lacks such signals and seems not to be a recycling receptor. Although these lectins on primary sinusoidal cells support HCV E2 binding, they are unable to support HCV entry. Lai et al. (2006) provided evidence for binding of circulating HCV with DC-SIGN and DC-SIGNR on sinusoidal endothelium within the liver allowing subsequent transfer of the virus to underlying hepatocytes in a manner analogous to DC-SIGN presentation of HIV on DCs (Lai et al. 2006).

36.5.3 Differences Between DC-SIGN and DC-SIGNR/L-SIGN

DC-SIGN and L-SIGN/DC-SIGNR genes have long been considered to share similar overall structure and ligand-binding characteristics. However, biochemical and structural studies show that they have distinct ligand-binding properties and different physiological functions. Of importance in both these genes is the presence of an extra-cellular domain consisting of an extended neck region encoded by tandem repeats that support the CRD, which plays a crucial role in influencing the pathogen-binding properties of these receptors. The notable difference between these two genes is in the extra-cellular domain. Whilst the tandem-neck-repeat region remains relatively constant in size in DC-SIGN, there is considerable polymorphism in L-SIGN. Homo-oligomerization of the neck region of L-SIGN has been shown to be important for high-affinity ligand binding, and heterozygous expression of the polymorphic variants of L-SIGN in which neck lengths differ could thus affect ligand-binding affinity. Despite DC-SIGN and DC-SIGNR bind HIV and enhance infection, comparison of these receptors reveals that they have very different physiological functions.

Screening an extensive glycan array demonstrated that DC-SIGN and DC-SIGNR have distinct ligand-binding properties. Structural and mutagenesis studies explain how both receptors bind high-mannose oligosaccharides on enveloped viruses and why only DC-SIGN binds blood group antigens, including those present on microorganisms. DC-SIGN mediates endocytosis, trafficking as a recycling receptor and releasing ligand at endosomal pH, whereas

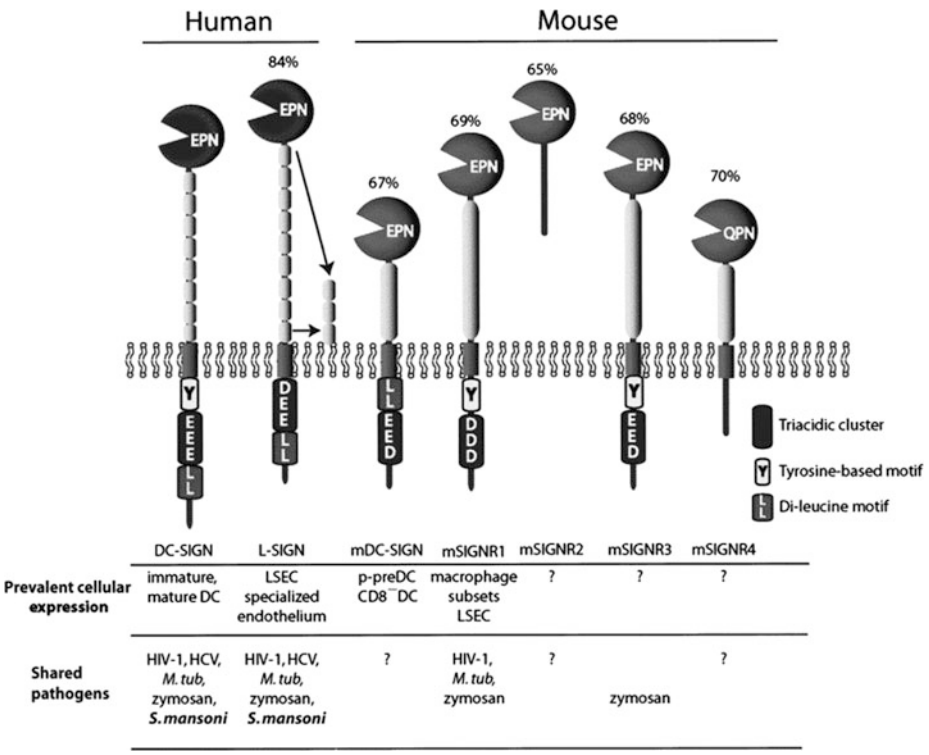

Fig. 36.3 Schematic representation of the structure, expression and binding specificities of DC-SIGN and its human and murine homologs. All SIGN homologs are transmembrane receptors, except for mSIGNR2, which is a soluble receptor. The percentage amino-acid-homology of CRDs of different homologs compared to DC-SIGN is depicted above the CRDs. Within the CRDs the highly conserved EPN sequence is essential for recognizing mannose-containing structures. All SIGN homologs contain the EPN motif, except for mSIGNR4, which has a QPN motif, indicating that mSIGNR4 may have another ligand specificity compared to the other SIGN molecules. In contrast to DC-SIGN that contains seven complete and one incomplete repeats, the number of repeats of L-SIGN is variable and varies between three and nine which is indicated with arrows. Within the cytoplasmic tail several internalization motifs are found. The di-leucin (LL) motif is thought to be important for internalization of DC-SIGN. The tyrosine-based motif and the tri-acidic cluster are also involved in internalization. However, the internalization capacities of the homologs of DC-SIGN have not been extensively explored. Based on its expression pattern, mSIGNR1 seems more similar to the homolog of L-SIGN rather than DC-SIGN whereas the expression of mDC-SIGN seems more homologous to DC-SIGN, although they are expressed by different DC subtypes. Strikingly, DC-SIGN, L-SIGN and mSIGNR1 share the ability to bind a number of pathogens (Reprinted with permission from Koppel et al. 2005 © John Wiley and Sons)

DC-SIGNR does not release ligand at low pH or mediate endocytosis. Thus, whereas DC-SIGN has dual ligand-binding properties and functions both in adhesion and in endocytosis of pathogens, DC-SIGNR binds a restricted set of ligands and has only the properties of an adhesion receptor (Guo et al. 2004). Functional studies on the effect of tandem-neck-repeat region on pathogen-binding, as well as genetic association studies for various infectious diseases and among different populations, have been reported. Worldwide demographic data of the tandem-neck-repeat region showing distinct differences in the neck-region allele and genotype distribution among different ethnic groups have been presented. These findings support the neck region as an excellent candidate acting as a functional target for selective pressures exerted by pathogens (Khoo et al. 2008). Chung et al (2010) identified Trp-258 in the DC-SIGN CRD to be essential for HIV-1 transmission. Although introduction of a K270W mutation at the same position in L-SIGN was insufficient for HIV-1 binding, an L-SIGN mutant molecule with K270W and a C-terminal DC-SIGN CRD subdomain transmitted HIV-1 (Chung et al. 2010).

36.5.4 Recognition of Oligosaccharides by DC-SIGN and DC-SIGNR

Both DC-SIGN and DC-SIGNR bind mannose bearing ligands and related sugars through CRDs. The CRDs of DC-SIGN and DC-SIGNR bind $Man_9GlcNAc_2$ oligosaccharide 130- and 17-fold more tightly than mannose. Results indicate that CRDs contain extended or secondary oligosaccharide binding sites that accommodate mammalian-type glycan structures. When the CRDs are clustered in the tetrameric extracellular domain, their arrangement provides a means of amplifying specificity for multiple glycans on host molecules targeted by DC-SIGN and DC-SIGNR. Binding to clustered oligosaccharides may also explain the interaction of these receptors with the gp120 envelope

protein of HIV-1, which contributes to virus infection (Mitchell et al. 2001). Crystal structures of carbohydrate-recognition domains of DC-SIGN and of DC-SIGNR bound to oligosaccharide, in combination with binding studies, revealed that these receptors selectively recognize endogenous high-mannose oligosaccharides and may represent a new avenue for developing HIV prophylactics (Feinberg et al. 2001) (Fig. 36.4).

Similar to DC-SIGN, DC-SIGNR/L-SIGN can recognize high-mannose type *N*-glycans and the fucosylated glycan epitopes Lewis^{A} (Le^{a}, Galß1-3(Fuc1-4)GlcNAc-), Lewis ^{B} (Le^{b}, Fuc1-2Galß1-3(Fuc1-4)GlcNAc-) and Lewis Y (Le^{Y}, Fuc1-2Galß1-4(Fuc1-3)GlcNAc-) (Geijtenbeek et al. 2003; Guo et al. 2004; van Liempt et al. 2004). L-SIGN, however, does not bind to the Le^{X} epitope, which is one of the major ligands of DC-SIGN, although the formation of crystals between L-SIGN and Le^{X} indicates that a weak interaction is possible (Guo et al. 2004). The inability of L-SIGN to bind to Le^{X} epitopes is mainly due to the presence of a single amino acid in the CRD of L-SIGN, Ser^{363} that prevents interaction with the Fuc(1-3)GlcNAc unit in Le^{X}, but supports binding of the Fuc1-4GlcNAc moiety present in Le^{A} and Le^{B} antigens. The equivalent amino acid residue Val^{351} in DC-SIGN creates a hydrophobic pocket that strongly interacts with the Fuc(1-3/4)GlcNAc moiety of Le^{X}, other Lewis antigens, and probably LDN-F (Guo et al. 2004; van Liempt et al. 2004; van Liempt et al. 2006). The interaction of L-SIGN with *S. mansoni* egg glycoproteins and its location on liver endothelial cells suggest that L-SIGN may function in the recognition of glycan antigens of eggs that are trapped in the liver, thus contributing to glycan-specific immune responses and/or the immunopathology of schistosomiasis.

36.5.5 Extended Neck Regions of DC-SIGN and DC-SIGNR

Two different crystal forms of truncated DC-SIGNR comprising two neck repeats and the CRD reveal that the CRDs are flexibly linked to the neck, which contains α-helical segments interspersed with non-helical regions. Differential scanning calorimetry measurements indicated that the neck and CRDs are independently folded domains. Based on the crystal structures and hydrodynamic data, models for the full extracellular domains of the receptors have been generated. The observed flexibility of the CRDs in the tetramer, combined with reported data on the specificity of these receptors, suggests an important role for oligomerization in the recognition of endogenous glycans, in particular those present on the surfaces of enveloped viruses recognized by these proteins (Feinberg et al. 2005, 2009). To understand the tetramer-based ligand binding avidity, Snyder et al. (2005)

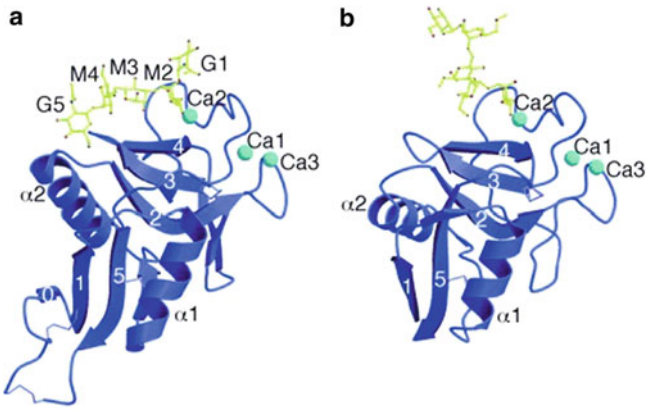

Fig. 36.4 Structure of CRD of DC-SIGN bound to $GlcNAc_2Man_3$. (**a**) Ribbon diagram of the DC-SIGN CRD (*blue*), with the bound oligosaccharide shown in a *ball-and-stick* representation (*yellow-green*, bonds and carbon atoms; *red*, oxygen; *blue*, nitrogen). Oligosaccharide residues are shown with the single letter code G for GlcNAc and M for mannose. Large cyan spheres are three Ca^{2+} ions. Disulfide bonds are shown in *pink*. The DC-SIGNR complex is very similar, except that the fourth disulfide connecting the NH_2 and COOH termini is not visible in either copy. (**b**) Rat serum mannose-binding protein bound to a high-mannose oligosaccharide. The color scheme is same as in (**a**) (Adapted from Feinberg et al. 2001 © American Association for the Advancement of Science)

determined the crystal structure of DC-SIGNR with its last repeat region and showed that compared to the carbohydrate-bound CRD structure, there are conformational differences in the calcium and carbohydrate coordination loops of CRD, an additional disulfide bond between the N and the C termini of the CRD, and a helical conformation for the last repeat. Snyder et al. (2005) generated a tetramer model for DC-SIGN/R using homology modeling and proposed a ligand-recognition index to identify potential receptor ligands. Polymorphisms associated with the length of the extracellular neck region of DC-SIGNR have been linked to differences in susceptibility to infection by enveloped viruses. The heterotetramers provide a molecular basis for interpreting the way polymorphisms affect interactions with viruses (Guo et al. 2006).

CRDs in DC-SIGN and DC-SIGNR are projected from the membrane surface by extended neck domains containing multiple repeats of a largely conserved 23-amino-acid sequence motif. The repeats are largely α-helical. Based on the structure and arrangement of the repeats in the crystal, the neck region can be described as a series of four-helix bundles connected by short, non-helical linkers. Combining the structure of the isolated neck domain with a overlapping structure of the distal end of the neck region with the CRDs attached provides a model of the almost-complete extracellular portion of the receptor. The organization of the neck suggests how CRDs may be disposed differently in DC-SIGN compared with DC-SIGNR and in variant forms of DC-SIGNR assembled from polypeptides with different

numbers of repeats in the neck domain (Feinberg et al, 2009).

CRD of Langerin with Structural Similarity with DC-SIGN: Though DCs are thought to mediate HIV-1 transmission, yet it is becoming evident that different DC subsets at the sites of infection have distinct roles. In the genital tissues, two different DC subsets are present: the LCs and the DC-SIGN$^+$-DCs. Although DC-SIGN$^+$-DCs mediate HIV-1 transmission, recent data demonstrate that LCs prevent HIV-1 transmission by clearing invading HIV-1 particles. However, this protective function of LCs is dependent on the function of Langerin: blocking Langerin function by high virus concentrations enables HIV-1 transmission by LCs. A better understanding of the mechanism of these processes is crucial to understand and develop strategies to combat transmission (de Witte et al. 2008b). The CRD of human Langerin was examined by X-ray analyses for apo-Langerin and for complexes with mannose and maltose. The fold of the Langerin CRD resembles that of DC-SIGN. However, especially in the long loop region (LLR), which is responsible for carbohydrate-binding, two additional secondary structure elements are present: a 3(10) helix and a small β-sheet arising from the extended β-strand 2, which enters into a hairpin and a new strand β2'. However, the crystal structures in presence of maltose and mannose revealed two sugar-binding sites. One is calcium-dependent and structurally conserved in the C-type lectin family whereas the second one represents a calcium-independent type. Based on these data, the differences in binding behavior between Langerin and DC-SIGN with respect to the Lewis X carbohydrate antigen and its derivatives has been explained (Chatwell et al. 2008).

36.5.6 Signaling by DC-SIGN through Raf-1

Adaptive immune responses by DCs are critically controlled by Toll-like receptor (TLR) function. Little is known about modulation of TLR-specific signaling by other pathogen receptors. DC-SIGN has gained an exponential increase in attention because of its involvement in multiple aspects of immune function. Besides being an adhesion molecule, particularly in binding ICAM-2 and ICAM-3, it is also crucial in recognizing several endogenous and exogenous antigens. Additionally, the intracellular domain of DC-SIGN includes molecular motifs, which enable the activation of signal transduction pathways involving serine and threonine kinase Raf-1 and subsequent modulation of DC-maturation status, through direct modification of nuclear factor Nf-kB in DCs. DC-SIGN modulates TLR signaling at the level of the transcription factor NF-kB.

The DC-SIGN has emerged as a key player in the induction of immune responses against numerous pathogens by modulating TLR-induced activation. Upon DC-SIGN engagement by mannose- or fucose-containing oligosaccharides, the latter leads to a tailored Toll-like receptor signaling, resulting in an altered DC-cytokine profile and skewing of Th1/Th2 responses. Gringhuis et al. (2007) demonstrated that pathogens trigger DC-SIGN on human DCs to activate Raf-1, which subsequently leads to acetylation of the NF-kB subunit p65, but only after TLR-induced activation of NF-kB. Acetylation of p65 both prolonged and increased IL10 transcription to enhance anti-inflammatory cytokine responses. Different pathogens such as *M. tuberculosis, M. leprae, Candida albicans, measles virus,* and *HIV-1* interacted with DC-SIGN to activate the Raf-1-acetylation-dependent signaling pathway to modulate signaling by different TLRs. Thus, this pathway is involved in regulation of adaptive immunity by DCs to bacterial, fungal, and viral pathogens (Gringhuis et al. 2007). In addition, other DC-SIGN-ligands induce different signaling pathways downstream of Raf-1, indicating that DC-SIGN-signaling is tailored to the pathogen.

36.6 Functions of DC-SIGN

36.6.1 DC-SIGN Supports Immune Response

Immature DCs are recruited from blood into tissues to patrol for foreign antigens. Cells expressing DC-SIGN stable transfectants were able to mediate phagocytosis of *E. coli*. Ca^{2+} binding sites in the CRD of DC-SIGN were involved in phagocytosis of bacteria as well as multimerization of DC-SIGN, and neck region played a role in efficiency of binding to microbes as well as multimerization of the protein (Iyori et al. 2008; Valera et al. 2008). While analyzing early stages of DC-SIGN-mediated endocytosis, Cambi et al. (2009) demonstrated that both membrane cholesterol and dynamin are required and that DC-SIGN-mediated internalization occurs via clathrin-coated pits. Electron microscopy studies confirmed the involvement of DC-SIGN in clathrin-dependent HIV-1 internalization by DCs. Recent studies showed that some functions of decidual dendritic cells appear to be essential for pregnancy. In humans, decidual dendritic cells are identifiable by their expression of DC-SIGN. In normal decidua, DC-SIGN$^+$ cells expressed antigens associated with immature myeloid dendritic cells. In samples from spontaneous abortions, the decidual DC-SIGN$^+$ cells at a significantly lower proportion compared to normal pregnancies seem to play a role in pathological pregnancy outcomes (Tirado-González et al. 2010).

The dendritic cell-specific DC-SIGN internalizes antigen for presentation to T cells. After antigen uptake and

processing, DCs mature and migrate to the secondary lymphoid organs where they initiate immune responses. As an adhesion molecule, DC-SIGN is able to mediate rolling and adhesion over endothelial cells under shear flow. The normal functions of DC-SIGN and DC-SIGNR include binding to ICAM-2 and ICAM-3. Binding of DC-SIGN to ICAM-2 on endothelial cells facilitates chemokine-induced DC extravasation; binding to ICAM-3 on T lymphocytes provides the initial step for establishing cell-mediated immunity (Liu and Zhu 2005). DCs could activate arrest T cells in the lymph node, but the mechanism is poorly understood. Studies showed that binding of DC-SIGN to both carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1) and Mac1 was required to establish cellular interaction between DCs and neutrophils, and such interaction promoted T cell proliferation and transformation to Th1 cells (van Gisbergen et al. 2005a, c). Subpopulations of human macrophages express DC-SIGN, to which Le^x-carrying CEACAMs may modulate the immune response in normal tissues such as the human placenta or in malignant tumors, for example in colorectal, pancreatic or lung carcinomas (Samsen et al. 2010). Reports suggest that DCs participate in the contact between itself and resting T cells, and also in T cell activation, and such effect is related to its cytoplasmic ITAM signal transduction (Engering et al. 2002). Martinez et al. discovered that DC-SIGN could promote CD3-activated T cells to produce IL-2 and receive a strong TCR signal, thus strengthening TCR-APC interaction and enhancing immune response (Martinez et al. 2005). Inhibiting DC-SIGN on DCs could reduce T cell proliferation and inhibit co-stimulator CD11c, CD83, CD80 and CD86 expression. Such effects are achieved by NF-κB signaling pathway (Zhou et al. 2006a). Recent evidences suggest that there is a cross-talk between DC-SIGN together with its CLRs and TLRs, and such crosstalk could lead to immune activation or T cells depression (Geijtenbeek et al. 2004; Gantner et al. 2003; Zhou et al. 2006b).

36.6.2 DC-SIGN Recognizes Pathogens

Viral and Bacterial Antigens DC-SIGN plays an important role in recognizing and capturing pathogens, DC migration and initiation of T cell responses. The role of DC-SIGN as a broad pathogen receptor has been well established (Geijtenbeek et al. 2000a; Alvarez et al. 2002; Colmenares et al. 2002; Cambi et al. 2003; Geijtenbeek et al. 2003; Lozach et al. 2003). In addition, DC-SIGN functions as a cell adhesion receptor mediating the interaction between DCs and resting T cells by binding to ICAM-3, and the transendothelial migration of DCs by binding to ICAM-2 (Geijtenbeek et al. 2000b). DC-SIGN has been identified as a receptor for HIV-1, HCV, Ebola virus, CMV, dengue virus, and the SARS coronavirus. Evidences suggest that DC-SIGN can function both as an adhesion receptor and as a phagocytic pathogen-recognition receptor, similar to the Toll-like receptors. Although major differences in the cytoplasmic domains of these receptors might predict their function, findings show that differences in glycosylation of ligands can dramatically alter C-type lectin-like receptor usage (Cambi and Figdor 2003; Engering et al. 2002). As a pathogen receptor, DC-SIGN displays affinity for high mannose moieties and functions as an internalization receptor for *HIV-1*, *hepatitis C virus, Mycobacterium tuberculosis, Helicobacter pylori,* and *Schistosoma manson, Leishmania*, and *Candida albicans* (Appelmelk et al. 2003; Bashirova et al. 2003a; Feinberg et al. 2001; Jack et al. 2001; Colmenares et al. 2004; Geijtenbeek et al. 2000a; Lozach et al. 2003; van Kooyk and Geijtenbeek 2003) and other pathogens that express mannose-containing carbohydrates (Alvarez et al. 2002; Halary et al. 2002; Tassaneetrithep et al. 2003; Zhou et al. 2008). The DC-SIGN molecule is used by HIV to attach to DCs in the genitourinary tract and rectum. Geitjenbeek et al. (2000a, c) suggest that DCs then carry HIV particles to lymph nodes, where the infection of T lymphocytes via receptors such as CD4 and CCR5 may occur. The virus may remain bound to DC-SIGN for protracted periods. DC-SIGN may deliver bound HIV to permissive cell types, mediating infection with high efficiency.

In vitro, DC-SIGN specifically interacts with *S. pneumoniae* serotype 3 and 14 in contrast to other serotypes such as 19F. While DC-SIGN interacts with *S. pneumoniae* serotype 14 through a ligand expressed by the capsular polysaccharide, the binding to *S. pneumoniae* serotype 3 appeared to depend on an as yet unidentified ligand (Koppel et al. 2005b). Leptospirosis is a global zoonotic disease, caused by pathogenic Leptospira species including *Leptospira interrogans* that causes public health and livestock problems. *L. interrogans* binds DC-SIGN and induces DCs maturation and cytokine production, which should provide new insights into cellular immune processes during leptospirosis (Gaudart et al. 2008).

Schistosoma Mansoni Antigens: Schistosomiasis is a human parasitic disease caused by helminths of the genus *Schistosoma*. *Schistosoma mansoni* synthesizes a multitude of carbohydrate complexes, which include both parasite-specific glycan antigens, as well as glycan antigens that are shared with the host. One example for a host-like glycan is the Le^X epitope Galß1-4(Fuc1-3)GlcNAc, which is expressed in all schistosomal life stages, but also on human leukocytes. Glycan antigens expressed by schistosomes induce strong humoral and cellular immune responses in their host (Meyer et al. 2007). The recognition of

carbohydrates is mediated by DC-SIGN, and DC-SIGNR bind to glycans of *S. mansoni* soluble egg antigens (SEA) (van Die et al. 2003; van Liempt et al. 2004; Meyer et al. 2005; Saunders et al. 2009; van Die and Cummings 2006, 2010). L-SIGN binds both SEA and egg glycosphingolipids, and can mediate internalization of SEA by L-SIGN expressing cells. L-SIGN predominantly interacts with oligomannosidic *N*-glycans of SEA. L-SIGN binds to a glycosphingolipid fraction containing fucosylated species with compositions of $Hex_1HexNAc_{5-7}dHex_{3-6}Cer$. Results indicate that L-SIGN recognizes both oligomannosidic *N*-glycans and multiply fucosylated carbohydrate motifs within *Schistosoma* egg antigens, which demonstrate that L-SIGN has a broad but specific glycan recognition profile (Meyer et al. 2007).

DC-SIGN Captures Also Fungi: The DC-SIGN is located in the submucosa of tissues, where they mediate HIV-1 entry. Interestingly, the pathogen *C. albicans*, the major cause of hospital-acquired fungal infections, penetrates at similar submucosal sites. The DC-SIGN is able to bind *C. albicans* both in DC-SIGN-transfected cell lines and in human monocyte-derived DC. Moreover, in immature DC, DC-SIGN was able to internalize *C. albicans* in specific DC-SIGN-enriched vesicles, distinct from those containing the ManR. These results demonstrated that DC-SIGN is an exquisite pathogen-uptake receptor that captures not only viruses but also fungi (Cambi et al. 2003). However, DC-SIGN regulation in monocyte-derived macrophages does not singly predict the transmission potential of this cell type (Bashirova et al. 2003; Chehimi et al. 2003).

Microbial Uptake Capacities of DC-SIGN, SIGNR1, SIGNR3 and Langerin: Using transfected non-macrophage cell lines, Takahara et al. (2004) compared the polysaccharide and microbial uptake capacities of three lectins—DC-SIGN, SIGNR1 and SIGNR3—to another homolog mLangerin. Each molecule shares a potential mannose-recognition EPN-motif in its carbohydrate recognition domain. Using an anti-Tag antibody, it was found that each molecule could be internalized, although the rates differed. However, mDC-SIGN was unable to take up FITC-dextran, FITC-ovalbumin, zymosan or heat-killed *C. albicans*. The other three lectins showed distinct carbohydrate recognition properties. Furthermore, only SIGNR1 was efficient in mediating the capture by transfected cells of Gram-negative bacteria, such as *E. coli and S. typhimurium,* while none of the lectins tested were competent to capture Gram-positive bacteria, *S. aureus*. Therefore, these homologous C-type lectins have distinct recognition patters for microbes despite similarities in the carbohydrate recognition domains (Takahara et al. 2004).

36.6.3 DC-SIGN as Receptor for Viruses

DC-SIGN and L-SIGN can function as attachment receptors for *Sindbis (SB) virus*, an arbovirus of the Alphavirus genus. DC-SIGN is a universal pathogen receptor and can be used by *hepatitis C virus* (HCV) and other viral pathogens including *Ebola* virus, *cytomegalovirus* (CMV), *and Dengue virus* to facilitate infection by a mechanism that is distinct from that of HIV-1, leading to inhibition of the immunostimulatory function of DC and, hence, promotion of pathogen survival. Reports show that DC-SIGN not only plays a role in entry into DC, but HCV E2 interaction with DC-SIGN might also be detrimental to the interaction of DC with T cells during antigen presentation (Zhou et al. 2008). The surface membrane glycoprotein of *Borna disease virus* (BDV) is a polypeptide of 57 kDa and N-glycosylated to a precursor glycoprotein (GP) of about 94 kDa. Analysis showed that the precursor GP contains only mannose-rich N-glycans (Kiermayer et al. 2002) and has the potential to bind with DC-SIGN, L-SIGN in addition to other mannose spectic lectins discussed in different Chapters.

Reduced expression of DC-SIGN in spleen specifically characterizes pathogenic forms of simian immunodeficiency virus (SIV) infection, correlates with disease progression, and may contribute to SIV pathogenesis (Yearley et al. 2008).

N-glycan Status Modifies Virus Interaction: The virus-producing cell type is an important factor in dictating both N-glycan status and virus interaction with DC-SIGN/DC-SIGNR (Lin et al. 2003). In contrast, viruses bearing Ebola (Zaire strain) and Marburg (Musoke strain) envelope glycoproteins bind at significantly higher levels to immobilized MBL compared with virus particles pseudo typed with vesicular stomatitis virus glycoprotein or with no virus glycoprotein. Importantly, the tetrameric complexes, in contrast to DC-SIGN monomers, bind with high affinity to high mannose glycoproteins such as mannan or HIV gp120 suggesting that such an assembly is required for high affinity binding of glycoproteins to DC-SIGN (Bernhard et al. 2004).

36.6.4 HIV-1 gp120 and Other Viral Envelope Glycoproteins

DC-SIGN has been described as an attachment molecule for human HIV-1 with the potential to mediate its transmission. About half of the carbohydrates on gp120 are terminally mannosylated, a pattern common to many pathogens. The DC-SIGN binds to HIV and SIV gp120 and mediates the binding and transfer of HIV from monocyte-derived dendritic cells (MDDCs) to permissive

T cells (Geijtenbeek et al. 2002c). However, DC-SIGN binding to HIV gp120 may also be carbohydrate independent. Hong et al. (2002) formally demonstrated that gp120 binding to DC-SIGN and MDDCs is largely if not wholly carbohydrate dependent.

DC-SIGN was mainly expressed in tubular epithelial cells and DC-SIGN$^+$ DCs were primarily distributed in renal tubulo-interstitial areas during the early stage of nephritis. In a rat model of chronic renal interstitial fibrosis, there was a significant correlation of DC-SIGN expression with DC-SIGN$^+$ DC distribution and the degree of tubulo-interstitial lesion. DC-SIGN plays an important role in DC-mediated renal tubular interstitial lesions induced by immuno-inflammatory responses (Zhou et al. 2009). HIV-1 infection of renal cells has been proposed to play a role in HIV-1-associated nephropathy. The HIV-1 internalization was DC-SIGN receptor mediated. It appeared that HIV-1 routing occurred through nonacid vesicular compartments and the clathrin-coated vesicles and caveosomes may not be contributing to HIV-1-associated membrane traffic (Mikulak et al. 2010). However, Hatsukari et al. (2007) showed no expression of DC-SIGN, or mannose receptors in tubular cells and suggested that DEC-205 (Chap.15 and Chap. 35) acts as an HIV-1 receptor that mediates internalization of the virus into renal tubular cells (HK2), from which the virus can be rescued and disseminated by encountering immune cells.

Herpes Simplex Virus and Human Herpes Virus 8: Of the two Herpes simplex virus (HSV) subtypes described, HSV-1 causes mainly oral-facial lesions, whilst HSV-2 is associated with genital herpes. HSV-1 and -2 both interact with DC-SIGN. Analyses demonstrated that DC-SIGN interacts with the HSV glycoproteins gB and gC. In another setting, human herpesvirus 8 (HHV-8) is the etiological agent of Kaposi's sarcoma, primary effusion lymphoma, and some forms of multicentric Castleman's disease. DC-SIGN is an entry receptor for HHV-8 on DC and macrophages. The infection of B cells with HHV-8 resulted in increased expression of DC-SIGN and a decrease in the expression of CD20 and MHC-I. It was indicated that the expression of DC-SIGN is essential for productive HHV-8 infection of and replication in B cells (de Jong et al. 2008; Rappocciolo et al. 2006, 2008).

West Nile Virus: West Nile virus (WNV), a mosquito-borne flavivirus, has recently emerged in North America. The elderly are particularly susceptible to severe neurological disease and death from infection with this virus. DC-SIGN enhances infection of cells by West Nile virus (WNV) glycosylated strains, which may at least in part explain the higher pathogenicity of glycosylated L1 strains versus most non-glycosylated L2 strains (Martina et al. 2008). Kong et al. (2008) found that the binding of the glycosylated WNV envelope protein to DC-SIGN leads to a reduction in the expression of TLR3 in macrophages from young donors via signal transducer and activator of transcription 1 (STAT1)-mediated pathway. This signaling is impaired in the elderly, and the elevated levels of TLR3 result in an elevation of cytokine levels. This alteration of the innate immune response with aging may contribute to the permeability of blood–brain barrier and a possible mechanism for the increased severity of WNV infection in aged individuals.

Corona Virus Infection and SARS: The MBL deficiency is a susceptibility factor for acquisition of severe acute respiratory syndrome (SARS) corona virus (CoV) (Ip et al. 2005). On similar pattern, entry of the serotype II feline corona virus strains feline infectious peritonitis virus (FIPV) and DF2 into nonpermissive mouse 3T3 cells could be rescued by the expression of human DC-SIGN and the infection of a permissive feline cell line (Crandall-Reese feline kidney) was markedly enhanced by the overexpression of DC-SIGN. Treatment with mannan considerably reduced infection of feline monocyte-derived cells expressing DC-SIGN, indicating a role for FIPV infection in vivo (Regan and Whittaker 2008).

Dengue Virus Envelope Glycoprotein and DC-SIGN: Dengue virus (DV) primarily targets immature DCs after a bite by an infected mosquito vector. Navarro-Sanchez et al. (2003) showed that DC-SIGN is a binding receptor for DV that recognizes N-glycosylation sites on the viral E-glycoprotein and allows viral replication. Mosquito-cell-derived DVs may have differential infectivity for DC-SIGN-expressing cells. There is evidence that infection of immature myeloid DCs plays a crucial role in dengue pathogenesis and that the interaction of the viral envelope E glycoprotein with DC-SIGN is a key element for their productive infection (Kwan et al. 2008).

Measles Virus Targets DC-SIGN: DCs are involved in the pathogenesis of measles virus (MV) infection by inducing immune suppression and possibly spreading the virus from the respiratory tract to lymphatic tissues. The DC-SIGN is the receptor for laboratory-adapted and wild-type MV strains. The ligands for DC-SIGN are both MV glycoproteins F and H. DC-SIGN was found important for the infection of immature DCs with MV, since both attachment and infection of immature DCs with MV were blocked in the presence of DC-SIGN inhibitors. Moreover, MV might not only target DC-SIGN to infect DCs but may also use DC-SIGN for viral transmission and immune suppression (de Witte et al. 2006). Thus, DCs play a prominent role during the initiation, dissemination, and clearance of MV infection (de Witte et al. 2008a).

36.7 Subversion and Immune Escape Activities of DC-SIGN

36.7.1 The entry and dissemination of viruses can be mediated by DC-SIGN

Dendritic cells are likely the first cells to encounter invading pathogens. The entry and dissemination of viruses in several families can be mediated by DC-SIGN. Terminal mannoses at positions 2 or 3 in the trisaccharides are the most important moiety and present the strongest contact with the binding site of DC-SIGN (Reina et al. 2008). Although, the mechanism of DC-SIGN and HIV-1 interaction remains unclear, Smith et al. (2007) identified a cellular protein that binds specifically to the cytoplasmic region of DC-SIGN and directs internalized virus to the proteasome degradation. This cellular protein, leukocyte-specific protein 1 (LSP1) is an F-actin binding protein involved in leukocyte motility and found on the cytoplasmic surface of the plasma membrane. LSP1 interacted specifically with DC-SIGN and other C-type lectins, but not the inactive mutant DC-SIGNΔ35, which lacks a cytoplasmic domain and shows altered virus transport in DCs. Thus, LSP1 protein facilitates virus transport into the proteasome after its interaction with DC-SIGN through its interaction with cytoskeletal proteins. Thus, it has been proposed that attachment of HIV to DC-SIGN enables the virus to hijack cellular transport processes to ensure its transmission to adjacent T cells.

Interestingly, not all interactions between DC-SIGN receptors and pathogenic ligands have beneficial results. During the interaction between body and pathogens or tumors, the latter could escape immune surveillance and survive. Such mechanism is related to suppressions of DCs by DC-SIGN. Thus, though DCs are vital in the defense against pathogens, it is becoming clear that some pathogens subvert DC functions to escape immune surveillance. It appears that some pathogens have evolved immunoevasive or immunosuppressive activities through receptors such as DC-SIGN. For example, HIV-1 targets the DC-SIGN to hijack DCs for viral dissemination. Binding to DC-SIGN protects HIV-1 from antigen processing and facilitates its transport to lymphoid tissues, where DC-SIGN promotes HIV-1 infection of T cells. Studies have also demonstrated that different ligand binding and/or sensing receptors collaborate for full and effective immune responses (McGreal et al. 2005; van Kooyk et al. 2003; Wang et al. 2004). It was also demonstrated that DC-SIGN efficiently reduces the amount of gp120 present on the cell plasma membrane, and completely strips off gp120 from the virions produced by the host cells, suggesting that blockage of HIV budding is due to internalization of gp120 by DC-SIGN (Wang and Pang 2008; Solomon Tsegaye and Pohlmann 2010).

Perhaps, binding of DC-SIGN to gp120 may facilitate or stabilize these transitions. Further studies demonstrated that HIV-1 would lose its activity if it is kept in vitro for 24 h, but DC-SIGN-bound HIV-1 could be kept within DCs for more than 4 days, allowing incoming virus to persist for 25 days before infecting target (Zhou et al. 2004). The mechanism of DC-SIGN prolongating viral infectivity is still poorly understood (Lozach et al. 2005). Thus, HIV sequestration by and stimulation of DC-SIGN helps HIV evade immune responses and spread to cells (Hodges et al. 2007; Marzi et al. 2007).

Many Pathogens Target DC-SIGN to Escape Host Immunity: For Hepatitis C Virus, both DC-SIGN and L-SIGN are known to bind envelope glycoproteins E1 and E2. Soluble DC-SIGN and L-SIGN specifically bound HCV virus-like particles. It is also speculated that HBV exploits mannose trimming as a way to escape recognition by DC-SIGN and thereby subvert a possible immune activation response (Op den Brouw et al. 2008). Reduced expression of DC-SIGN in spleen specifically characterizes pathogenic forms of SIV infection, correlates with disease progression, and may contribute to SIV pathogenesis (Yearley et al. 2008). DC-SIGN acts as a capture or attachment molecule for avian H5N1 virus, and mediates infections in cis and in trans (Wang et al. 2008b). Mittal et al. (2009) demonstrated that *Enterobacter sakazakii* (ES) targets DC-specific DC-SIGN to survive in myeloid DCs for which outer membrane protein A expression in ES is critical, although it is not required for uptake. ES interaction with DC-SIGN seems to subvert the host immune responses by disarming MAPK pathway in DCs (Mittal et al. 2009). *Yersinia pestis* is the etiologic agent of bubonic and pneumonic plagues. It is speculated that *Y. pestis* hijacks DCs and alveolar macrophages, in order to be delivered to lymph nodes. DC-SIGN is a receptor for *Y. pestis* that promotes phagocytosis by DCs in vitro (Zhang et al. 2008b). Accumulating evidence supports that certain pathogens target DC-SIGN to escape host immunity. Unlike certain other host pathogen interactions, activation of DCs by *B. pseudomallei* is not dependent on DC-SIGN. Evidence also indicates that the LPS mutant that binds DC-SIGN has a suppressive effect on DC cytokine production (Charoensap et al. 2008).

36.7.2 DC-SIGN and Escape of Tumors

Recognition of Tumor Glycans: Dendritic cells play an important role in the induction of antitumor immune responses. Glycosylation changes during malignant transformation create tumor-specific carbohydrate structures that interact with C-type lectins on DCs. Studies suggest that tumor glycoproteins, such as carcinoembryonic antigen (CEA) and MUC-1, indeed interact with DC-SIGN and

macrophage galactose-type lectin on APCs. DC-SIGN has been detected on immature DCs that were associated with melanoma and myxofibrosarcoma (Soilleux et al. 2003; Vermi et al. 2003). The consequences for anti-cancer immunity or tolerance induction can be extrapolated from the function of C-type lectins in pathogen recognition and antigen presentation. In addition, in vivo studies in mice demonstrated the potency of targeting antigens to C-type lectins on APCs for anti-tumor vaccination strategies (Aarnoudse et al. 2006).

DC-SIGN and Escape of Tumors: DC-SIGN has been related to immune escape of tumors (van Gisbergen et al. 2005b; Gijzen et al. 2008). Immature DCs are located intratumorally within colorectal cancer and intimately interact with tumor cells, whereas mature DCs are present peripheral to the tumor. The majority of colorectal cancers over-express CEA and malignant transformation changes the glycosylation of CEA on colon epithelial cells, resulting in higher levels of Le^X and de novo expression of Le^Y on tumor-associated CEA. Since DC-SIGN has high affinity for nonsialylated Lewis antigens, it is possible that DC-SIGN is involved in recognition of colorectal cancer cells by DCs. It was shown that immature DCs within colorectal cancer express DC-SIGN, which mediates these interactions through binding of Le^X/ Le^Y carbohydrates on CEA of colorectal cancer cells. In contrast, DC-SIGN does not bind CEA expressed on normal colon epithelium due to low levels of Lewis antigens. This indicates that DCs may recognize colorectal cancer cells through binding of DC-SIGN to tumor-specific glycosylation on CEA. Similar to pathogens that target DC-SIGN to escape immunosurveillance, tumor cells may interact with DC-SIGN to suppress DC functions (van Gisbergen et al. 2005b). At the same time, tumor cells can suppress DC maturation by DC-SIGN and escape immune surveillance. Bogoevska et al. (2006) showed that CEA-related cell adhesion molecule 1 (CEACAM1) selectively attaches and specifically interacts with DC-SIGN, and participates in cancer development. DC-SIGN is involved in the interaction of DCs with colorectal tumor SW1116 cells through the recognition of aberrantly glycosylated forms of Le^a/Le^b glycans on CEA and CEACAM. DC-SIGN ligands containing Le^a/Le^b glycans are also highly expressed on primary cancer colon epithelia but not on normal colon epithelia, and DC-SIGN is suggested to be involved in the association between DCs and colorectal cancer cells in situ by DC-SIGN recognizing these cancer-related Le glycan ligands. Observations imply that colorectal carcinomas affecting DC function and differentiation through interactions between DC-SIGN and colorectal tumor-associated Le glycans may induce generalized failure of a host to mount an effective antitumor response (Nonaka et al. 2008). In acute lymphoblastic leukemia (ALL), aberrant glycosylation of blast cells can alter their interaction with DC-SIGN and L-SIGN, thereby affecting their immunological elimination. High binding of B-ALL peripheral blood cells to DC-SIGN and L-SIGN correlates with poor prognosis. Apparently, when B-ALL cells enter the blood circulation and are able to interact with DC-SIGN and L-SIGN the immune response is shifted toward tolerance (Gijzen et al. 2008).

The role of DCs in progression of primary cutaneous T-cell lymphoma (CTCL) is not established. Schlapbach et al. (2010) found a significant infiltration of CTCL lesions by immature DC-SIGN$^+$ DCs with close contact to tumor cells. Matured and activated DCs were only rarely detected in lesions of CTCL. The preponderance of immature DC-DIGN$^+$ DCs in contact with regulatory T cells in lesions of CTCL points to an important role of this subset in the host's immune reaction to the malignant T cells. Since these immature DCs are known to induce immunotolerance, they might play a role in the mediation of immune escape of the proliferating clone (Schlapbach et al. 2010). In contrast, studies showed that there are CEA specific T cells in colorectal cancer patients which have anti-tumor effects (Nagorsen et al. 2000). Such results suggest that DCs could recognize and bind to colorectal tumor cells by DC-SIGN and participate in anti-tumor immune response.

36.7.3 *Mycobacterial* Carbohydrates as Ligands of DC-SIGN, L-SIGN and SIGNR1

Mycobacteria, including *M. tuberculosis* (Mtb), are surrounded by a loosely attached capsule that is mainly composed of proteins and polysaccharides. Although the chemical composition of the capsule is relatively well studied, its biological function is only poorly understood. *M. tuberculosis*, the causative agent of tuberculosis (TB), is recognized by pattern recognition receptors on macrophages and DCs, thereby triggering phagocytosis, antigen presentation to T cells and cytokine secretion. Mtb spreads through aerosol carrying them deep into the lungs, where they are internalized by phagocytic cells, such as neutrophils (PMNs), DCs, and macrophages. The DC- Mtb manipulates cells of the innate immune system to provide the bacteria with a sustainable intracellular niche (Schaefer et al. 2008). Mannosylated moieties of the mycobacterial cell wall, such as mannose-capped lipoarabinomannan (ManLAM) or higher-order phosphatidylinositol-mannosides (PIMs) of Mtb, were shown to bind to DC-SIGN on immature DCs and macrophage subpopulations. This interaction reportedly impaired dendritic cell maturation, modulated cytokine secretion by phagocytes and dendritic cells and was postulated to cause suppression of protective immunity to TB. However, experimental Mtb infections in mice transgenic for human DC-SIGN revealed

that, instead of favoring immune evasion of mycobacteria, DC-SIGN may promote host protection by limiting tissue pathology. Furthermore, infection studies with mycobacterial strains genetically engineered to lack ManLAM or PIMs demonstrated that manLAM/PIM-DC-SIGN interaction was not critical for cytokine secretion in vitro and protective immunity in vivo (Ehlers 2010).

Reports suggest that *M. tuberculosis* targets DC-SIGN to inhibit the immuno-stimulatory function of DC through the interaction of the mycobacterial ManLAM to DC-SIGN, which prevents DC maturation and induces the formation of immuno-suppressive cytokine IL-10 that helps in the survival and persistence of *M. tuberculosis* (Fig. 36.2). The pathogen-derived carbohydrate structure on ManLAM that is recognized by DC-SIGN has been identified. The synthetic mannose-cap oligosaccharides manara, (Man)2-ara and (Man)3-ara specifically bound by DC-SIGN. The human and murine DC-SIGN homolog L-SIGN and SIGNR1, respectively, also interact with mycobacteria through ManLAM. Both homologs have the highest affinity for the (Man)3-ara structure, similar to DC-SIGN. The identification of SIGNR1 as a receptor for ManLAM enabled in vivo studies to investigate the role of DC-SIGN in *M. tuberculosis* pathogenesis (Geijtenbeek et al. 2003; Tailleux et al. 2003; Koppel et al. 2004). In addition to manLAM, Mtb α-glucan is another ligand for DC-SIGN. The recognition of α-glucans by DC-SIGN is a general feature and the interaction is mediated by internal glucosyl residues. As for manLAM, an abundant mycobacterial cell wall-associated glycolipid, binding of α-glucan to DC-SIGN stimulated the production of immunosuppressive IL-10 by LPS-activated monocyte-derived DCs. This IL-10 induction was DC-SIGN-dependent and also required acetylation of NF-kB (Geurtsen et al. 2009).

The mannose cap of LAM is a crucial factor in mycobacterial virulence. Appelmelk et al. (2008) evaluated the biological properties of capless mutants of *M. marinum* and *M. bovis* BCG, made by inactivating homologs of Rv1635c and showed that its gene product is an undecaprenyl phospho-mannose-dependent mannosyltransferase. Compared with parent strain, capless *M. marinum* induced slightly less uptake by and slightly more phagolysosome fusion in infected macrophages but this did not lead to decreased survival of the bacteria in vitro, nor in vivo in zebra fish. Appelmelk et al. (2008) contradicted the current paradigm and demonstrated that mannose-capped LAM does not dominate the M.-host interaction. Although the mannose caps of the mycobacterial surface (ManLAM) are essential for the binding to DC-SIGN, genetic removal of these caps did not diminish the interaction of whole mycobacteria with DC-SIGN and DCs. Like ManLAM, Hexamannosylated PIM (6), which contains terminal α(1—>2)-linked mannosyl residues identical to the mannose cap on ManLAM showed high affinity and represents a bonafide DC-SIGN ligand but that other, as-yet-unknown, ligands dominate in the interaction between *mycobacteria* and DCs (Driessen et al. 2009) (Fig. 36.1, 36.2).

36.7.4 Decreased Pathology of Human DC-SIGN Transgenic Mice During Mycobacterial Infection

Although, the *M. tuberculosis*, the causative agent of pulmonary tuberculosis, interacts with DC-SIGN to evade the immune system, transgenic mice after high dose aerosol infection with the strain *Mtb*-H37Rv, showed massive accumulation of DC-SIGN$^+$ cells in infected lungs, reduced tissue damage and prolonged survival. Based on these results, it was proposed that instead of favoring the immune evasion of mycobacteria, human DC-SIGN may have evolved as a pathogen receptor promoting protection by limiting tuberculosis-induced pathology (Schaefer et al. 2008). Hedlund et al. (2010) demonstrated that DCs can distinguish between normal and infected apoptotic PMNs via cellular crosstalk, where the DCs can sense the presence of danger on the Mtb-infected PMNs and modulate their response accordingly. Balboa et al. (2010) showed that early interaction of Î-irradiated *M. tuberculosis* with Mo subverts DC differentiation in vitro and suggested that *M. tuberculosis* escapes from acquired immune response in tuberculosis may be caused by an altered differentiation into DC leading to a poor Mtb-specific T-cell response. Since, *M. tuberculosis* interacts with DC-SIGN to evade the immune system, the dominant Mtb-derived ligands for DC-SIGN are presently unknown, and a major role of DC-SIGN in the immune response to Mtb infection may lie in its capacity to maintain a balanced inflammatory state during chronic TB (Ehlers 2010).

36.7.5 Genomic Polymorphism of DC-SIGN (CD209) and Consequences

36.7.5.1 CD209 Genetic Polymorphism and HIV Infectivity

DC-SIGN and DC-SIGNR have been thought to play an important role in establishing HIV infection by enhancing trans-infection of CD4$^+$ T cells in the regional lymph nodes. The variation of DC-SIGNR genotypes affects the efficacy of trans-infection by affecting the amounts of the protein expressed on cell surface and augmenting the infection. A potential association of DC-SIGN and DC-SIGNR neck domain repeat polymorphism and risk of HIV-1 infection is currently under debate. Rathore et al. (2008a) showed that polymorphism in DC-SIGN neck repeats region was rare and not associated with HIV-1 susceptibility among North Indians. But sequencing analysis of DC-SIGN gene

confirmed four novel genetic variants in intronic region flanking exon 4 coding region. A total of 13 genotypes were found in DC-SIGNR neck repeat region polymorphism. Among all the genotypes, only 5/5 homozygous showed significant reduced risk of HIV-1 infection in HIV-1-exposed seronegative individuals. A unique genotype 8/5 heterozygous was also found in HIV-1 seropositive individual, which is not reported elsewhere (Rathore et al. 2008a, b).

The association of polymorphism of homolog of DC-SIGNR gene with susceptibility to virus infection suggests that the tandem-repeat polymorphisms of the DC-SIGNR gene in the Chinese Han population exhibit unique genetic characteristics not recognized earlier in the Caucasian population. Genotype 9/5 seems to be a risk factor for HIV-1 infection in the Chinese population (Wang et al. 2008a). To understand the role of DC-SIGN neck-region length variation in HIV-1 transmission, Zhang et al. (2008a) studied 530 HIV-1-positive and 341 HIV-1-negative individuals in China. The carrier frequency of a DC-SIGN allele with <5 repeat units in the neck-region was 0.9% in HIV-1-positive and 3.8% in HIV-1-negative individuals. This observation suggests that DC-SIGN variation plays a role in HIV-1 transmission. These naturally occurring DC-SIGN neck-region variants were significantly more frequent in the Chinese population than in the US population and in a worldwide population. Several transcripts of DC-SIGN have been identified, some of which code for putative soluble proteins. However, little is known about the regulation and the functional properties of such putative sDC-SIGN variants. Based on the analysis of the cytokine/chemokine content of sDC-SIGN culture supernatants, results confirmed that sDC-SIGN, like membrane DC-SIGN counterpart, may play a pivotal role in CMV-mediated pathogenesis (Plazolles et al. 2011). Variations in genes encoding virus recognition and reactivation and patients following allogeneic stem-cell transplantation suggested that two SNP (rs735240, G > A; rs2287886, C > T) in the promoter region of DC-SIGN are significantly associated with an increased risk of development of hCMV reactivation and disease. These genetic markers influence the expression levels of DC-SIGN on immature DCs, as well as infection efficiency of immature DCs by hCMV and might help to predict the individual risk of hCMV reactivation and the disease (Mezger et al. 2008).

Variations in the number of repeats in the neck region of DC-SIGN and DC-SIGNR possibly influence host susceptibility to HIV-1 infection. Chaudhary et al. (2008a) examined the SNP of DC-SIGN and DC SIGNR in healthy HIV seronegative individuals, high risk STD patients seronegative for HIV, and HIV-1 seropositive patients from northern India. DC-SIGN polymorphism was rare and genotype 7/7 was predominant in all groups studied. DC-SIGNR was highly polymorphic and 11 genotypes were observed among the different study groups. The precise role of the polymorphic variants of DC-SIGNR needs to be elucidated in the population (Chaudhary et al. 2008a, b). GG genotype of SDF-1α 3′UTR polymorphism may be associated with susceptibility to PTB in HIV-1 infected patients in south India. Genotype frequencies of DC-SIGN polymorphisms did not differ significantly between HIV patients with or without TB. A better understanding of genetic factors that are associated with TB could help target preventive strategies to those HIV patients likely to develop tuberculosis (Alagarasu et al. 2009). Whether variants in the DC-SIGN encoding CD209 gene are associated with susceptibility to or protection against HIV-1 infection or development of TB among HIV-1 infected south Indian patients, study suggests that -336G/G genotype while associated with protection against HIV-1 infection the same genotype is also associated with susceptibility to HIV-TB among south Indians (Selvaraj et al. 2009). Olesen et al. (2007) support the report that vitamin D receptor (VDR) gene SNPs modulate the risk for TB in West Africans and suggest that variation within DC-SIGN and PTX3 also affect the disease outcome.

Reports suggest that CD209 promoter SNP-336A/G exerts an effect on CD209 expression and is associated with human susceptibility to dengue, HIV-1 and tuberculosis in humans. The CD209 -336G variant allele is also associated with significant protection against tuberculosis in individuals from sub-Saharan Africa and, cases with -336GG were significantly less prone to develop tuberculosis-induced lung cavitation. Previous in vitro work demonstrated that the promoter variant -336G allele causes down-regulation of CD209 mRNA expression. This report suggests that decreased levels of the DC-SIGN receptor may be protective against both clinical tuberculosis in general and cavitory tuberculosis disease in particular. This is consistent with evidence that *Mycobacteria* can utilize DC-SIGN binding to suppress the protective pro-inflammatory immune response (Vannberg et al. 2008).

Wichukchinda et al. (2007) genotyped two SNPs in DC-SIGN promoter (-139A/G and 336A/G), a repeat number of 69 bp in Exon 4 of DC-SIGN and DC-SIGNR, and one SNP in Exon 5 of DC-SIGNR and showed that the proportion of individuals possessing a heterozygous 7/5 and 9/5 repeat and A allele at rs2277998 of DC-SIGNR in HIV-seronegative individuals of HIV-seropositive spouses was significantly higher than HIV-seropositive individuals. These associations were observed only in females but not in males. The proportion of individuals possessing the 5A haplotype in HIV-seronegative females was significantly higher than HIV-seropositive females. These associations suggested that DC-SIGNR might affect susceptibility to HIV infection by a mechanism that is different in females and males (Wichukchinda et al. 2007; Zhu et al. 2010).

36.7.5.2 Human T-Cell Lymphotropic Virus Type 1 (HTLV) Infection

DC-SIGN plays a critical role in HTLV-1 binding, transmission, and infection, thereby providing an attractive target for the development of antiretroviral therapeutics and microbicides (Jain et al. 2009). Kashima et al. (2009) evaluated four polymorphisms located in the DC-SIGN gene promoter region (positions -336, -332 -201 and -139) in DNA samples from Brazilian ethnic groups (Caucasians, Afro-Brazilian, Asians and Amerindians) to establish the population distribution of these SNPs and correlated DC-SIGN polymorphisms and infection in samples from human T-cell lymphotropic virus type 1 (HTLV-1)-infected individuals. The -336A and -139A SNPs were quite common in Asians and that the -201T allele was not observed in Caucasians, Asians or Amerindians. No significant differences were observed between individuals with HTLV-1 disease and asymptomatic patients. However, the -336A variant was more frequent in HTLV-1-infected patients. In addition, the -139A allele was found to be associated with protection against HTLV-1 infection when the HTLV-1-infected patients as a whole were compared with the healthy-control group. Kashima et al. (2009) suggested that the -139A allele might be associated with HTLV-1 infection, although no significant association was observed among asymptomatic and HAM/TSP patients. Koizumi et al. (2007) showed that RANTES -28G was associated with delayed AIDS progression, while DC-SIGN -139C was associated with accelerated AIDS progression in HIV-1-infected Japanese hemophiliacs. While analyzing DC-SIGN and DC-SIGNR polymorphisms in Caucasian Canadian and indigenous African populations, Boily-Larouche et al. (2007) found several novel nucleotide variants within regulatory 5′- and 3′-untranslated regions of the genes that could affect their transcription and translation. Study demonstrated that Africans show greater genetic diversity at these two closely-related immune loci than observed in other major population groups.

36.7.5.3 CD209 Genetic Polymorphism and Tuberculosis Disease

DC-SIGN is a receptor capable of binding and internalizing *M. tuberculosis*. The CD209 promoter single SNP-336A/G exerts an effect on CD209 expression and is associated with human susceptibility to dengue, HIV-1 and tuberculosis in humans. In vitro studies confirmed that this SNP modulates gene promoter activity. An association study was performed in Tunisian patients comprising tuberculosis and healthy controls. Sequencing of the DC-SIGN promoter region detected four polymorphisms (-939, -871, -601, and -336), but no differences in their allelic distribution were observed between the two groups. In addition, the analysis of length variation in the DC-SIGN neck region indicated extremely low levels of polymorphisms and, again, no differences between patients and controls. Results suggested neither promoter variants nor length variation in the neck region of DC-SIGN is associated with susceptibility to tuberculosis in Tunisian patients (Ben-Ali et al. 2007).

Among Caucasians patients suffering from pulmonary tuberculosis, DC-SIGN revealed no significant differences in loci -336A/G and -871A/G with controls. Analysis of MIRU-VNTR patterns identified 50 unique profiles, among which there were genotypes of the families Beijing, T. LAM, Haarlem, "Ural" (Haarlem 4) and X. Among 90 MIRU-VNTR genotypes, 42 profiles belonged to the Beijing family. Moreover, the minimum spanning tree (MST) test revealed a number of Beijing-like strains. The genotypes of the subjects affected with Beijing and Beijing-like strains and those affected with the strains of other families (non-Beijing) were compared. A significance reduction was found in the incidence of the -336G genotype among the subjects affected with Beijing strains versus those infected with non-Beijing strains at a frequency of 0.09 and 0.24, respectively (Ogarkov et al. 2007). CD209 facilitates severe acute respiratory syndrome (SARS)-coronavirus spike protein-bearing pseudotype driven infection of permissive cells in vitro. Genetic association analysis of SNP with clinico-pathologic outcomes in 824 serologic confirmed that the -336AG/GG genotype SARS patients were associated with lower LDH levels compared with the -336AA patients. High LDH levels are known to be an independent predictor for poor clinical outcome, probably related to tissue destruction from immune hyperactivity. Hence, SARS patients with the CD209 -336 AA genotype carry a 60% chance of having a poorer prognosis. This association is in keeping with the role of CD209 in modulating immune response to viral infection. The relevance of these findings for other infectious diseases and inflammatory conditions would be worth investigating (Chan et al. 2010).

36.8 SIGNR1 (CD209b): The Murine Homologues of DC-SIGN

36.8.1 Characterization

The mouse (m) DC-SIGN family consists of several homologous type II transmembrane proteins located in close proximity on chromosome 8 and having a single carboxyl terminal carbohydrate recognition domain. Initial screening of mouse cDNA libraries led to the identification of multiple mouse homologs of DC-SIGN and DC-SIGNR, designated DC-SIGN and SIGNR1 through SIGNR4 by Park et al. (2001). More murine homologs of human SIGNs have been identified, and the biochemical and cell biological properties of all murine SIGNs have been compared (Fig. 36.3). The SIGNR1 is a C-type lectin domain of murine homolog

of DC-SIGN and functions in vivo as a pathogen recognition receptor on macrophages that captures blood-born antigens, which are rapidly internalized and targeted to lysosomes for processing (Geijtenbeek et al. 2002b). In addition to five SIGNR proteins, a pseudogene, encoding a hypothetical SIGNR6, and a further two expressed proteins, SIGNR7 and SIGNR8, have been identified. Screening of a glycan array demonstrated that only mouse SIGNR3 shares with human DC-SIGN the ability to bind both high mannose and fucose-terminated glycans in this format and to mediate endocytosis. SIGNR3 is a differentiation marker for myeloid mononuclear cells and that some DCs, especially in sLNs, are possibly replenished by Ly6C(high) monocytes (Nagaoka et al. 2010). The mouse homologs of DC-SIGN have a diverse set of ligand-binding and intracellular trafficking properties, some of which are distinct from the properties of any of the human receptors (Powlesland et al. 2006).

SIGN Related 1 (SIGNR1) or CD209b is expressed at high levels on macrophages in lymphoid tissues, especially within the marginal zone of the spleen. SIGNR1 can bind and mediate the uptake of various microbial polysaccharides, including dextrans, lipopolysaccharides and pneumococcal capsular polysaccharides. SIGNR1 mediates the clearance of encapsulated pneumococcus, complement fixation via binding C1q independent of antibody and innate resistance to pneumococcal infection. Recently, SIGNR1 has also been demonstrated to bind sialylated antibody and mediate its activity to suppress autoimmunity (Silva-Martin et al. 2009). The CRD of SIGNR1 has been cloned and overexpressed in a soluble secretory form in CHO cells. The single crystal of CRD protein of SIGNR1 belonged to the monoclinic space group C2 with unit-cell parameters $a = 146.72, b = 92.77, c = 77.06$ A, $\beta = 121.66°$, allowed the collection of a full X-ray data set to a maximum resolution of 1.87 A (Silva-Martin et al. 2009).

36.8.2 Functions

SIGNR1 receptor, involved in the uptake of capsular polysaccharides (caps-PS) by APCs, is necessary for the antibody response to pneumococcal caps-PS and phosphorylcholine (PC). Moens et al. (2007) found that SIGNR1 is not involved in the IgM antibody production to PC and caps-PS serotype 3 or 14 and the IgG immune response to PC and caps-PS serotype 14. There is no direct relation between capture and uptake of caps-PS serotype 14 by SIGNR1 and the initiation of the anti-caps-PS antibody production in mice. Resident peritoneal macrophages (PEMs) express SIGNR1 on the cell surface as a major mannose receptor. These cells also ingest oligomannose-coated liposomes (OMLs) in an oligomannose-dependent manner following intraperitoneal administration. SIGNR1 on macrophages acts as a receptor for recognition of OMLs under physiological conditions (Takagi et al. 2009). Sialylated Fc from IgG require SIGNR1 which preferentially binds to 2,6-sialylated Fc compared with similarly sialylated, biantennary glycoproteins, suggesting that a specific binding site is created by sialylation of IgG Fc. A human DC-SIGN displays a binding specificity similar to SIGNR1 but differs in its cellular distribution. These studies thus identify an antibody receptor specific for sialylated Fc, and present the initial step that is triggered by intravenous Ig to suppress inflammation (Anthony et al. 2008).

SIGNR1 also interacts with *M. tuberculosis* similar to DC-SIGN. Peritoneal macrophages from SIGNR1 deficient (KO) mice produce less IL-10 upon stimulation with ManLAM than those from wild-type mice, suggesting that the interaction of ManLAM with SIGNR1 can result in immuno-suppression similar to its human homolog. Studies suggest that although SIGNR1 has a similar binding specificity as DC-SIGN, its role is limited during murine M. tuberculosis infection (Wieland et al. 2007). Resistance to *M. tuberculosis* was impaired only in SIGNR3-deficient animals. SIGNR3 was expressed in lung phagocytes during infection, and interacted with *M. tuberculosis* bacilli and mycobacterial surface glycoconjugates to induce secretion of critical host defense inflammatory cytokines, including TNF. SIGNR3 signaling was dependent on an intracellular tyrosine-based motif and the tyrosine kinase Syk. Thus, the mouse DC-SIGN homolog SIGNR3 makes a unique contribution to protection of the host against a pulmonary bacterial pathogen (Tanne et al. 2009). The rat CD209b mediates the uptake of dextran or CPS14 within the rat splenic marginal zone, similar to SIGNR1. On microglia, rat CD209b also mediates the uptake of CPS14 of *S. pneumoniae*. Findings suggest that both rat CD209b and SIGNR1 on microglia mediate the SIGNR1 complement activation pathway against *S. pneumoniae*, and thereby plays an important role in the pathogenesis of pneumococcal meningitis (Park et al. 2009).

Marginal zone macrophages in the murine spleen play an important role in the capture of blood-borne pathogens and are viewed as an essential component of host defense against the development of pneumococcal sepsis. However, reports described the loss of marginal zone macrophages associated with the splenomegaly that follows a variety of viral and protozoal infections; this finding raises the question of whether these infected mice would become more susceptible to secondary pneumococcal infection. Contrary to expectations, Kirby et al. (2009) demonstrated that the normal requirement for SIGNR1$^+$ marginal zone macrophages to protect against a primary pneumococcal infection can be readily compensated for by activated red pulp macrophages under conditions of splenomegaly. SIGNR1 is crucial for the capture of *S.*

pneumoniae from blood. SIGNR1 is able to interact in vitro with the juxtaposing marginal zone B cell population, which is responsible for the production of early IgM response against the *S. pneumoniae*-epitope phosphorylcholine. Strikingly, SIGNR1-deficient mice display a reduction in the marginal zone B cell population. In addition, ex vivo B cell stimulation assays demonstrate a decrease in phosphorylcholine specificity in the splenic B cell population derived from SIGNR1-deficient mice, whereas the total IgM response was unaffected. Therefore, interaction of SIGNR1 expressed by marginal zone macrophages with marginal zone B cells is essential to early IgM responses against *S. pneumoniae* (Koppel et al. 2008; Saunders et al. 2009).

Inflammatory Bowel Disease: In context of the etiology of inflammatory bowel disease, there is a less defined function for C-type lectins. The CD209 gene is located in a region linked to inflammatory bowel disease (IBD). Though the CD209 functional polymorphism (rs4804803) has been associated to other inflammatory conditions, it does not seem to be influencing Crohn's disease susceptibility. However, it could be involved in the etiology or pathology of Ulcerative Colitis in HLA-DR3-positive individuals (Núñez et al. 2007). Saunders et al. (2010) demonstrated that mice deficient in SIGNR1 have reduced susceptibility to experimental colitis, with a reduction in the disease severity, colon damage, and levels of the proinflammatory cytokines IL-1β, TNF-α, and IL-6. SIGNR1$^{-/-}$ peritoneal macrophages, but not bone marrow-derived macrophages, had a specific defect in IL-1β and IL-18 production, but not other cytokines, in response to TLR4 ligand LPS. SIGNR1 was associated in the regulation of inflammation in a model of experimental colitis and is a critical innate factor in response to LPS.

36.9 Liver and Lymph Node Sinusoidal Endothelial Cell C-Type Lectin (LSECtin) (or CLEC4G or L-SIGN or CD209L)

36.9.1 Characterization and Localization

The liver is an organ with paradoxic immunologic properties and is known for its tolerant microenvironment, which holds important implications for hepatic diseases. Liver and lymph node sinusoidal endothelial cell C-Type lectin (LSECtin) (or CLEC4G or L-SIGN or CD209L) displays 77% amino acid identity with DC-SIGN, and is expressed on endothelial cells in lymph node sinuses, capillary endothelial cells in the placenta and on liver sinusoidal cells (LSECs) (Soilleux et al. 2000; Bashirova et al. 2001; Pohlmann et al. 2001; Engering et al. 2004). The LSECtin (CD209L), gene encodes a protein of 293 amino acids and maps to chromosome 19p13.3 adjacent to C-type lectin genes, CD23, DC-SIGN, and DC-SIGNR. The four genes form a tight cluster in an insert size of 105 kb and have analogous genomic structures. The LSECtin is a type II integral membrane protein of approximately 40 kDa in size with a single C-type lectin-like domain at the C-terminus, close in homology to DC-SIGNR, DC-SIGN, and CD23. LSECtin mRNA was expressed in liver and lymph node among 15 human tissues tested, intriguingly neither expressed on hematopoietic cell lines nor on monocyte-derived DCs. Colmenares et al. (2007) detected LSECtin expression in human peripheral blood and thymic dendritic cells. LSECtin is also detected in monocyte-derived macrophages and dendritic cells at RNA and protein level (Liu et al. 2004). LSECtin could also be detected in the MUTZ-3DC cell line at mRNA and protein level. Human liver revealed its presence in Kupffer cells coexpressing the myeloid marker CD68 (Domínguez-Soto et al. 2009). In vitro, IL-4 induces the expression of 3 LSECtin alternatively spliced isoforms, including a potentially soluble form (Δ2 isoform) and a shorter version of the prototypic molecule (Δ3/4 isoform).

Full-length porcine (p) CLEC4G (L-SIGN) cDNA encodes a type II transmembrane protein of 290 amino acids. The pCLEC4G gene has same gene structure as human and the predicted bovine, canis, mouse and rat CLEC4G genes with nine exons. The pCLEC4G mRNA expresses in liver, lymph node and spleen tissues. A series of sequential intermediate products of pCLEC4G pre-mRNA were also identified during splicing from pig liver. The chromosomal regions syntenic to the human cluster of genes CD23/CLEC4G/DC-SIGN/L-SIGN have been compared in mammalian species including primates, domesticated animal, rodents and opossum. The L-SIGN homologs do not exist in non-primates mammals (Huang and Peng 2009).

In the liver LSECs functions as liver-resident antigen presenting cells (Knolle and Gerken 2000) and is important in tolerance induction (Knolle and Limmer 2001). LSECs may mediate the clearance of antigens from the circulation in same manner as DCs (Bashirova et al. 2001; Karrar et al. 2007). DC-SIGN and L-SIGN (LSECtin) share a di-leucine motif and a cluster of three acidic amino acids in their cytoplasmic tails, which are known to be essential for antigen uptake (Bashirova et al. 2001; Engering et al. 2002). Recent studies with Ebola virus, Severe Acute Respiratory Syndrome (SARS) virus or antibodies against L-SIGN, clearly demonstrated that L-SIGN indeed is able to internalize antigens (Klimstra et al. 2003; Liu et al. 2004; Jeffers et al. 2004; Ludwig et al. 2004; Dakappagari et al. 2006). DC-SIGN transient expression in HEK293T is a useful model for investigating p38 MAPK pathway

triggered by hepatitis C virus glycoprotein E2, which may provide information for understanding cellular receptors-mediated signaling events and the viral pathogenesis (Chen et al. 2010).

36.9.2 Ligands of LSECtin

LSECtin binds to mannose, GlcNAc, and fucose in a Ca^{2+}-dependent manner but not to galactose (Liu et al. 2004). The DC-SIGN and DC-SIGNR (DC-SIGN/R) bind to high-mannose carbohydrates on a variety of viruses. In contrast, the related lectin LSECtin does not recognize mannose-rich glycans and interacts with a more restricted spectrum of viruses. LSECtin and DC-SIGNR, which are co-expressed by liver, lymph node and bone marrow sinusoidal endothelial cells, bind to soluble Ebola virus glycoprotein (EBOV-GP) with comparable affinities. Similarly, LSECtin, DC-SIGN and Langerin readily bound to soluble HIV-1 GP. However, only DC-SIGN captured HIV-1 particles, indicating that binding to soluble GP is not necessarily predictive of binding to virion-associated GP. Results reveal important differences between pathogen capture by DC-SIGN/R and LSECtin and hint towards different biological functions of these lectins (Gramberg et al. 2008). To compare the sugar and pathogen binding properties of LSECtin with those of related but more extensively characterized receptors, such as DC-SIGN, a soluble fragment of LSECtin consisting of the C-terminal CRD was expressed in bacteria and used to probe a glycan array and to characterize binding to oligosaccharide and glycoprotein ligands. LSECtin binds with high selectivity to glycoproteins terminating in GlcNAcβ1-2Man. Glycan analysis of the surface glycoprotein of Ebola virus reveals the presence of such truncated glycans, explaining the ability of LSECtin to facilitate infection by Ebola virus (Powlesland et al. 2008). A systematic study of DC-SIGN, DC-SIGNR and LSECtin suggested that 'agalactosylated N-glycans' are candidate ligands common to these lectins (Dominguez-Soto et al. 2010).

Polymorphisms of *CLEC4M* have been associated with predisposition for infection by severe acute respiratory syndrome coronavirus (SARS-CoV). LSECtin not only acts as an attachment factor for pathogens, but also recognizes "endogenous" activated T cells. The CD44 on Jurkat T cells is a candidate ligand of LSECtin. Moreover, LSECtin selectively bound CD44s, CD44v4 and CD44v8-10 by screening a series of typical CD44 isoforms. The interaction between CD44 and LSECtin is dependent on protein-glycan recognition. Findings indicate that CD44 is the first endogenous ligand of LSECtin, and that LSECtin is a ligand of CD44 (Tang et al. 2010).

36.9.3 Functions

LSECtin functions as a pathogen receptor, because its expression confers Ebola virus-binding capacity to leukemic cells. Sugar-binding studies indicate that LSECtin specifically recognizes N-acetyl-glucosamine, whereas no LSECtin binding to Mannan- or N-acetyl-galactosamine-containing matrices are observed. Antibody or ligand-mediated engagement triggers a rapid internalization of LSECtin, which is dependent on tyrosine and diglutamic-containing motifs within the cytoplasmic tail. Therefore, LSECtin is a pathogen-associated molecular pattern receptor in human myeloid cells. In addition, LSECtin participates in antigen uptake and internalization, and might be a suitable target in vaccination strategies (Colmenares et al. 2007). In liver, LSECtin specifically recognized activated T cells and negatively regulated their immune responses. In mice with T-cell-mediated acute liver injury, the lack of LSECtin accelerated the disease owing to an increased T-cell immune response, whereas the exogenous administration of recombinant LSECtin protein or plasmid ameliorated the disease via down-regulation of T-cell immunity. Results reveal that LSECtin is a novel regulator of T cells and expose a crucial mechanism for hepatic T-cell immune suppression, perhaps opening up a new approach for treatment of inflammatory diseases in the liver (Tang et al. 2009).

The L-SIGN (or LSECtin) is also expressed in human lung in type II alveolar cells and endothelial cells, both potential targets for SARS-CoV. Since, several other enveloped viruses including *Ebola* and *Sindbis* use CD209L as a portal of entry, and HIV and *hepatitis C virus* can bind to L-SIGN on cell membranes but do not use it to mediate virus entry, it appears that the large S glycoprotein of SARS-CoV may use L-SIGN, in addition to ACE2 in infection and pathogenesis (Jeffers et al. 2004). Capture of *Hepatitis C virus* (HCV) by L-SIGN results in *trans*-infection of hepatoma cells. L-SIGN polymorphism could influence the establishment and progression of HCV infection (Falkowska et al. 2006). There is no significant correlation between the genetic polymorphism of DC-SIGN's exon 4 and HCV infection susceptibility. 9/5 genotype distribution frequency of DC-SIGNR's exon 4 in patients with hepatitis C is significantly higher and may be associated with HCV infection susceptibility (Wang et al. 2007). The L-SIGN binds mycobacterial ManLAM but not AraLAM, suggesting that L-SIGN may bind *M. tuberculosis*. Binding assays suggest that L-SIGN interacts strongly with the (Man)2-ara and (Man)3-ara, but not with the man-ara, similar to DC-SIGN. It indicates that L-SIGN may be involved in the pathogenesis of *M. tuberculosis* infection and that the L-SIGN captures the infection through ManLAM and rapidly internalizes it to lysosomes. This shows that L-SIGN may be involved in the clearance of mycobacteria since L-SIGN is expressed on those sites in lymph nodes and

liver which are ideally suited for antigen capture and clearance. However, mycobacteria may target L-SIGN to invade those tissues. More research is necessary to investigate the specific role of L-SIGN in these infections.

36.9.4 Role in Pathology

LSECtin enhances infection driven by filovirus glycoproteins (GP) and the S protein of SARS coronavirus, but does not interact with HIV-1 and hepatitis C virus envelope proteins. Ligand binding to LSECtin was inhibited by EGTA but not by mannan, suggesting that LSECtin unlike DC-SIGN/R does not recognize high-mannose glycans on viral glycoproteins. LSECtin is N-linked glycosylated and glycosylation is required for cell surface expression. In nut-shell, LSECtin is an attachment factor that in conjunction with DC-SIGNR might concentrate viral pathogens in liver and lymph nodes (Gramberg et al. 2005). Li et al. (2008) genotyped 23 tagSNPs in 181 SARS patients and reported no significant association with disease predisposition. Genetic variations in this cluster also did not predict disease prognosis. However, Li et al. (2008) detected a population stratification of the VNTR alleles in a sample of 1145 Han Chinese collected from different parts of China. Li et al. (2008) indicated that the genetic predisposition allele was not found in this lectin gene cluster and population stratification might have caused the previous positive association.

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Part XII

C-Type Lectins: Proteoglycans

Lectican Protein Family

37

G.S. Gupta

37.1 Proteoglycans

Proteoglycans (PGs) are a diverse group of glycoproteins, which are formed through covalent and noncovalent aggregation of proteins and glycosaminoglycans (GAGs) in the extracellular matrix. GAGs are linear polysaccharides, whose building blocks (disaccharides) consist of an amino sugar (either GlcNAc or GalNAc) and an uronic acid (GlcA and IdoA). Virtually all mammalian cells produce proteoglycans and either secrete them into the ECM, insert them into the plasma membrane, or store them in secretory granules. The matrix proteoglycans include small interstitial proteoglycans (decorin, biglycan, fibromodulin), a proteoglycan form of type IX collagen, and one or more members of the aggrecan family of proteoglycans (aggrecan, brevican, neurocan, or versican). Some of these proteoglycans contain only one GAG chain (e.g., decorin), whereas others have more than 100 chains (e.g., aggrecan). The matrix proteoglycans typically contain the GAGs known as chondroitin sulfate (CS) or dermatan sulfate (DS). Exceptions to this generalization exist, since the HS proteoglycans perlecan and agrin are major species found in basement membranes. The size and shape of PGs vary widely that suggests numerous functions for them. The protein components of PGs are synthesized by ribosomes and translocated into the lumen of the rough endoplasmic reticulum. Glycosylation of the proteoglycan occurs in the Golgi apparatus in multiple enzymatic steps. The point of attachment is a Serine (Ser) residue to which the glycosaminoglycan is joined through a tetrasaccharide bridge (For example: chondroitin sulfate-GlcA-Gal-Gal-Xyl-PROTEIN). The Ser residue is generally in the sequence -Ser-Gly-X-Gly- (where X can be any amino acid residue), although not every protein with this sequence has an attached glycosaminoglycan. Thus, a PG contains a core protein and one to many glycosaminoglycans or carbohydrate chains. Along with glycoproteins and glycolipids proteoglycans on the noncytosolic side of the cell membrane form the carbohydrate layer. This layer on top of the bilipid layer serves to protect the cell from both chemical and mechanical damage. These components absorb water, giving the cell a slimy surface. Another main function of PGs is to regulate the movement of molecules through the matrix. Proteoglycans also contribute to the activity and stability of proteins within the matrix.

Proteoglycans being the major component of the animal extracellular matrix, are called the "filler" substances between cells in an organism. Here they form large complexes, both to other proteoglycans, to hyaluronan and to fibrous matrix proteins (such as collagen). They are also involved in binding cations (such as sodium, potassium and calcium) and water, and also regulating the movement of molecules through the matrix. Evidence also shows that they can affect the activity and stability of proteins and signalling molecules within the matrix. Individual functions of proteoglycans can be attributed to either the protein core or the attached GAG chain and serve as lubricants.

37.1.1 Nomenclature of PGs

Nomenclature for PGs is based on the source of glycosylation or the functions of PG. Many PGs can be classified on the basis of the size of core protein. Size is also used for classification of PGs (Table 37.1). Large PGs are considered aggrecans, which are a major part of cartilage (Fig. 37.1). Certain members are considered members of the "small leucine-rich proteoglycan family". These include decorin, biglycan, fibromodulin and lumican. Small interstitial PGs have a high degree of homology in their protein core sequence. Each has between 10 and 12 highly conserved leucine rich tandem repeats which make up the central portion of the core protein. The fibromodulin and lumican are keratan sulfate substituted where as decorin and biglycan are both chondroitin sulfate substituted.

Decorins are small extracellular PGs usually within 90–140 kDa in size. Decorins have a core protein of ~42 kDa in size and one GAG chain consisting of either CS or DS. They were named so because they appeared decorating collagen fibers under EM. Biglycans are

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DOI 10.1007/978-3-7091-1065-2_37,

Table 37.1 Classification of proteoglycans on the basis of their localization and type of core protein

Localization	GAG-chain	M_r of core protein (kDa)	Principal members
ECM	hyaluronic acid, chondroitin sulfate, keratan sulfate	225–250	Aggrecan (the major proteoglycan in cartilage), versican (present in many adult tissues including blood vessels and skin)
Collagen-associated	chondroitin sulfate, keratan sulfate, dermatan sulfate	40	decorin, biglycan fibromodulin[c]
Basement membrane	hyaluronic acid	120	perlecan
Cell-surface	hyaluronic acid, chondroitin sulfate	33[a]; 60[b]; 92[c]	syndecans[a]; glypican[b]; betaglycan[c], CD44E, cerebroglycan
Intracellular granules	heparin, chondroitin sulfate	17–19	serglycin

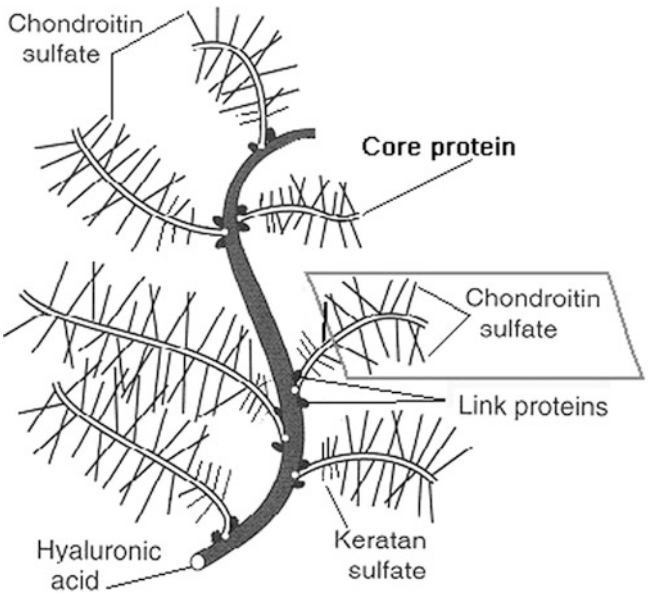

Fig. 37.1 **Proteoglycan Complex**. In the cartilage matrix, individual proteoglycans (in *box*) are linked to a nonsulfated GAG, hyaluronic acid, to form a giant complex with a molecular mass of about 3,000,000. The *box* indicates one of the proteoglycans of the type shown in figure

also small extracellular PGs that are similar to decorins. They are about 150–240 kDa in size and are generally found on the surface of cells, bones, and cartilage. Among small PGs, fibromodulin is the most abundant. Fibromodulin is about 60 kDa in size with a core protein of ~42 kDa. Fibromodulin is very similiar to decorins and biglycans and contains 4 keratan sulfate (KS) chains.

Large proteoglycans are versicans, neurocans, brevicans, and collagens. Chondroitin sulfate proteoglycan or versican is a sulfated glycosaminoglycan attributed for possessing good hydration potential. This property allows for interaction with surrounding cells in cell adhesion, proliferation, and migration. Another type of chondroitin sulfate proteoglycan called neurocan is located in the extracellular matrix within the brain. Proteoglycans can be a main contributor for all types of amyloidosis. High density proteoglycans are also linked to joint autoimmune responses and arthritis in mice. Joint problems such as these may improve with glucosamine and chondroitin added to the diet (Baeurle et al. 2009). Heparan sulfate proteoglycans (HSPGs) interact with many proteins and protein receptors that influence growth factors and structural proteins. Mutations in genes relating to HSPGs are associated with increased cancer risk. HSPGs are involved in several human diseases and affect the cartilage and musculoskeletal systems like hereditary multiple exostoses (HME).

37.1.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) or mucopolysaccharides are long unbranched polysaccharides consisting of a repeating disaccharide unit as a building block. The disaccharides consist of an amino sugar (either GlcNAc or GalNAc) and an uronic acid (GlcA and IdoA) (Figs. 37.2 and 37.3). Protein cores made in the rough endoplasmic reticulum are posttranslationally modified by glycosyltransferases in the Golgi apparatus, where GAG disaccharides are added to protein cores to yield proteoglycans; the exception is hyaluronan, which is uniquely synthesized without a protein core and is "spun out" by enzymes at cell surfaces directly into the extracellular space. This family of carbohydrates is essential or important for life. GAGs form an important component of connective tissues and may be covalently linked to a protein to form proteoglycans. Water sticks to GAGs; this is where the resistance to pressure comes from. The density of sugar molecules and the net negative charge attract salts. Water does not compress, unlike gas. Some examples of glycosaminoglycan uses in nature include heparin as an anticoagulant, hyaluronate as a component in the synovial fluid lubricant in body joints, and chondroitins which can be found in connective tissues, cartilage and tendons. Classification and examples of GAGs are given in Table 37.1.

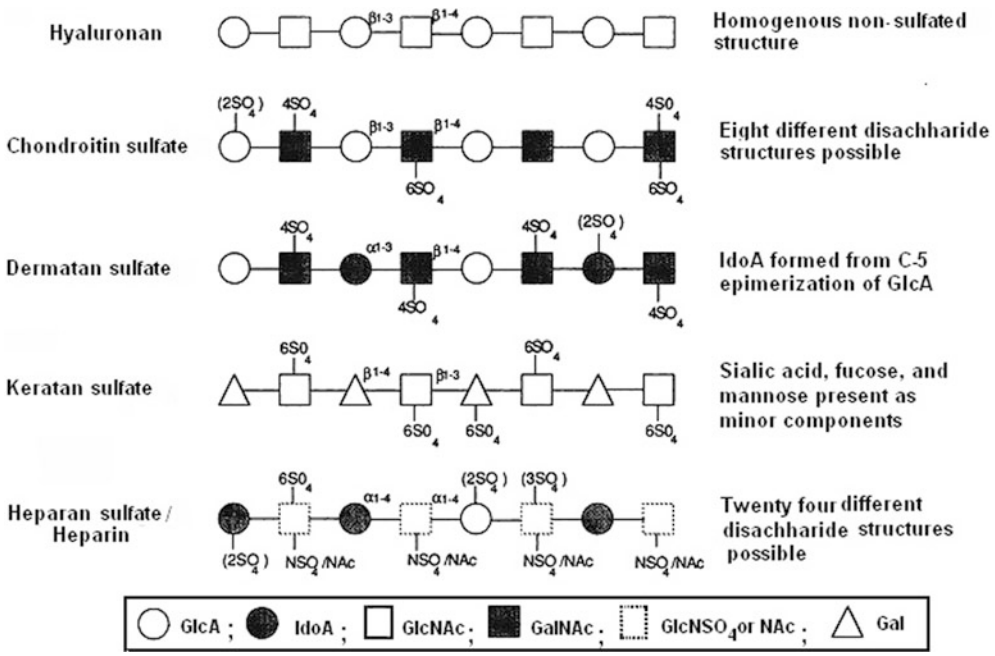

Fig. 37.2 Repeating disaccharide structures of glycosaminoglycans. Glycosaminoglycans, consisting repeating disaccharide units, are composed of an N-acetylated or N-sulfated hexosamine and either a uronic acid (glucuronic acid or iduronic acid) or galactose. Hyaluronan is synthesized without a covalent link to any protein. Hyaluronan lacks sulfate groups, but the rest of the glycosaminoglycans contain sulfates at various positions. Dermatan sulfate is formed from chondroitin sulfate by intracellular eprimenzation of glucuronate (GlcA) to iduronate (IdoA) and distinguished from chondroitn sulfate by the presence of iduronic acid. 2-sulfated hexuronate residues are more common in dermatan sulfate than in chondroitin-sulfate. Keratan sulfates lack uronic acids and instead consist of sulfated galactose and *N*-acetylglucosamine residues. Heparin is a more extensively epimerized and sulfated form of heparan sulfate. Heparan sulfate frequently contains some chain segments with little or no epimerization or sulfation. Heparin is synthesized only on a mast cell granule proteoglycan, whereas heparan sulfate is found on many cell surfaces and on some matrix proteoglycans. Abbreviations: GlcNAc, N-acetyl glucosamine; GaINAc, N-acetyl galactosamine; GlcA, glucuronic acid; Xyl, xylose; Gal, galactose; (Figure adapted with permission from Hardingham and Fosang 1992 © Federation of American Societies for Experimental Biology)

37.1.3 Chondroitin

Chondroitin is a chondrin derivative without "sulfate" and has been used to describe a fraction with no sulfation. Types of chondroitin include: (1) Chondroitin sulfate and (2) Dermatan sulfate. Chondroitin's functions depend largely on the properties of overall proteoglycan of which it is a part. These functions can be broadly divided into structural and regulatory roles. However, this division is not absolute, and some proteoglycans have both structural and regulatory roles (see versican). Chondroitin is an ingredient found commonly in dietary supplements and used as an alternative medicine to treat osteoarthritis and also approved and regulated as a symptomatic slow-acting drug for this disease. It is commonly sold together with glucosamine.

Chondroitin Sulfate: Chondroitin sulfate is a major component of extracellular matrix, and is important in maintaining the structural integrity of the tissue. Chondroitin sulfate is a sulfated GAG composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid). It is usually found attached to proteins as part of a proteoglycan. A chondroitin chain can have over 100 individual sugars, each of which can be sulfated in variable positions and quantities. Chondroitin sulfate is an important structural component of cartilage and provides much of its resistance to compression. Members of the glycosaminoglycan family vary in the type of hexosamine, hexose or hexuronic acid unit they contain (e.g. glucuronic acid, iduronic acid, galactose, galactosamine, glucosamine). They also vary in the geometry of the glycosidic linkage (Figs. 37.2 and 37.3).

Chondroitin sulfate is important in maintaining the structural integrity of the tissue. This function is typical of the large aggregating proteoglycans: aggrecan, versican, brevican, and neurocan, collectively termed the lecticans. As part of aggrecan, chondroitin sulfate is a major component of cartilage. The tightly packed and highly charged sulfate groups of chondroitin sulfate generate electrostatic repulsion that provides much of the resistance of cartilage to compression. Loss of chondroitin sulfate from the cartilage is a major cause of osteoarthritis. Chondroitin sulfate readily interacts with proteins in the extracellular matrix due to its negative charges.

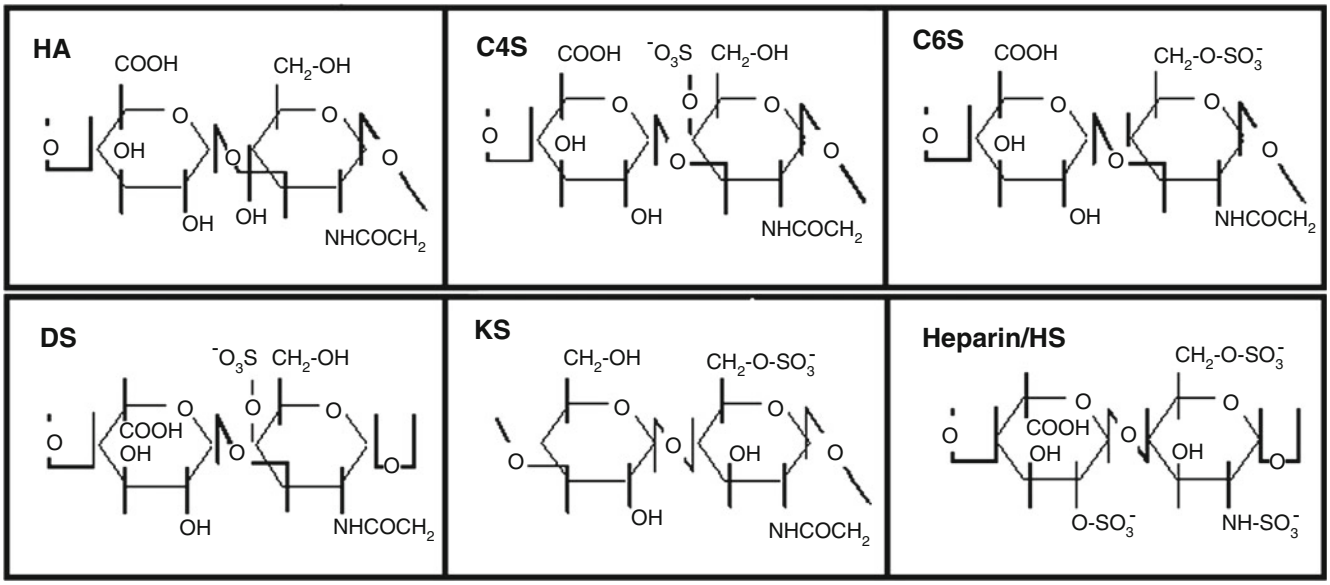

Fig. 37.3 Glycosidic bonds in disachharides present in GAGs. Heparin/HS and hyaluronic acid (HA) are glycosaminoglycans. Heparin has highest negative charge density among any known biological molecule and the only GAG that is exclusively non-sulfated; HS: Highly similar in structure to heparin, however heparan sulfates disaccharide units are organised into distinct sulfated and non-sulfated domains. Since heparin/HS structures are highly heterogeneous, IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3), the most abundant disaccharide unit of HS, is shown here; Chondroitin-4-sulfate (C4S) and chondroitin-6-sulfate (C6S) are most prevalent GAG; C6S and dermatan sulfate (DS) are galactosaminoglycans. DS can be distinguished from chondroitin sulfate by the presence of iduronic acid, although some hexuronic acid monosaccharides may be glucuronic acid; Keratan sulfate (KS) is a sulfated polylactosamine

These interactions are important for regulating a diverse array of cellular activities. The lecticans are a major part of the brain extracellular matix, where the chondroitin sugar chains function to stabilize normal brain synapses as part of perineuronal nets. The levels of chondroitin sulfate proteoglycans are vastly increased after injury to the CNS where they act to prevent regeneration of damaged nerve endings. Although these functions are not as well characterized as those of heparan sulfate, new roles continue to be discovered for the chondroitin sulfate proteoglycans. The dosage of oral chondroitin used in human clinical trials is 800–1,200 mg per day. Most chondroitin appears to be made from extracts of cartilaginous cow and pig tissues (cow trachea and pig ear and nose), but other sources such as shark, fish, and bird cartilage are also used. CSPG is secreted by human B cell lines that closely resembles in its structure the serum-derived C1q inhibitor (C1qI). Strong binding of B cell CSPG to C1q, its inhibition of C1q activity, and its structural similarities to a human serum C1qI indicate that B cells produce a soluble CSPG, which may act as C1qI under physiologic conditions (Kirschfink et al. 1997).

The benefit of chondroitin sulfate in patients with osteoarthritis is likely the result of a number of effects including its anti-inflammatory activity, the stimulation of the synthesis of proteoglycans and hyaluronic acid, and the decrease in catabolic activity of chondrocytes inhibiting the synthesis of proteolytic enzymes, nitric oxide, and other substances that contribute to damage cartilage matrix and cause of death of articular chondrocytes (Monfort et al. 2008). The rationale behind the use of chondroitin sulfate is based on the belief that osteoarthritis is associated with a local deficiency in some natural substances, including chondroitin sulfate. In several prospective controlled trials, chondroitin sulfate had been thought to decrease pain, improve functional disability, reduce NSAID or acetaminophen consumption, and provide good tolerability with an additional carry-over effect. Bruyere and Reginster (2007) suggest that glucosamine and chondroitin sulfate act as valuable symptomatic therapies for osteoarthritis disease with some potential structure-modifying effects.

Structure of Chondroitin Sulfate: Chondroitin sulfate chains are unbranched polysaccharides of variable length containing two alternating monosaccharides: D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc). Some GlcA residues are epimerized into L-iduronic acid (IdoA); the resulting disaccharide is then referred to as dermatan sulfate (Fig. 37.3). Chondroitin sulfate chains are linked to hydroxyl groups on serine residues of certain proteins. Glycosylated serines are often followed by a glycine and have neighboring acidic residues, but this motif does not always predict glycosylation. Attachment of GAG chain begins with four monosaccharides in a fixed pattern: Xyl – Gal – Gal – GlcA. Each sugar is attached by a specific enzyme, allowing for multiple levels of control over GAG

synthesis. Xylose begins to be attached to proteins in endoplasmic reticulum, while rest of the sugars is attached in Golgi apparatus. Each monosaccharide may be left unsulfated, sulfated once, or sulfated twice. In the most common scenario, the hydroxyls of the four and six positions of the N-acetyl-galactosamine are sulfated, with some chains having the two position of glucuronic acid.

Dermatan Sulfate: Dermatan sulfate is a glycosaminoglycan (formerly called a mucopolysaccharide) found mostly in skin, but also in blood vessels, heart valves, tendons, and lungs. Chondroitin sulfate B is an old name for dermatan sulfate, and is no longer classified as a form of chondroitin sulfate. Dermatan sulfate may have roles in coagulation, cardiovascular disease, carcinogenesis, infection, wound repair, and fibrosis. Dermatan sulfate accumulates abnormally in several of the mucopolysaccharidosis disorders. An excess of dermatan sulfate in the Mitral Valve is characteristic of myxomatous degeneration of the leaflets leading to redundancy of valve tissue and ultimately, Mitral Valve Prolapse (into the Left atrium) and insufficiency. This chronic prolapse occurs mainly in women over the age of 60, and can predispose the patient to mitral annular calcification. Mitral valve insufficiency can lead to eccentric (volume dependent or dilated) hypertrophy and eventually Left Heart failure if untreated (Table 37.1).

37.1.4 Heparan Sulfate Proteoglycans (HSPG)

PGs can be classified on the basis of their localization, GAG-chain composition, and on the type of the core protein. As shown in Table 37.1, HSPGs are localized mainly in the basement membrane and on the cell surface (Figs. 37.2 and 37.3). The final structure of heparin/HS depends upon the incompleteness of the reactions that occur during the biosynthetic process. The modification process is more complete in heparin where the final disaccharide IdoA (2-OSO_3)-GlcNSO_3(6-OSO_3) represents up to 70% of the chain, leading to a heavily *O*-sulfated polysaccharide with a high IdoA/GlcA ratio.

Typical concentrations of HSPGs on the cell surface are in the range of 10^5–10^6 molecules/cell. HSPGs can link to plasma membrane through a hydrophobic transmembrane domain of their core protein or through a glycosylphosphatidylinositol (GPI) anchor covalently bound to core protein (transmembrane HSPGs). Also, HSPGs can interact with the cell by non-covalent linkage to different cell-surface macromolecules (peripheral membrane HSPGs). Transmembrane HSPGs are glypican, cerebroglycan, betaglycan, CD44, and the members of the syndecan family: syndecan 1, fibroglycan (syndecan 2), N-syndecan (syndecan 3) and ryudocan (syndecan 4). It is important to know that cell-associated HSPGs can be internalized via endocytosis and metabolized in the lysosomal compartment. In some cell types oligosaccharides originated during intracellular degradation appear to be delivered specifically to the nucleus (Gallagher 2001).

Glypican and cerebroglycan are typical GPI-anchored HSPGs. Syndecans and betaglycan are typical transmembrane HSPGs characterized by a core protein composed of an extracellular domain, a single membrane-spanning domain and a short cytoplasmic domain. In the extracellular domain are present the consensus sequences for glycosylation and a conserved putative proteolytic cleavage site. The cytoplasmic domain of syndecans can interact with the cytoskeleton and contains four conserved tyrosine residues, one of them being a substrate for enzymatic phosphorylation. Perlecan is a typical peripheral membrane HSPG that interacts with the cell surface through its core protein. The cell-adhesion motif Arg-Gly-Asp within the core protein of perlecan binds integrins β_1 or β_3 present on endothelial cell surface. However, HSPGs may associate to the cell surface and/or ECM also through their GAG-chain. In contrast, the modifications that occur during the biosynthesis of HS are less extensive, leading to HS molecules characterized by lower IdoA content and a lower overall degree of *O*-sulfation and resulting in high heterogeneity of distribution of the sulfate groups along the chain. Eventually, disaccharides containing GlcNAc or $GlcNSO_3$ may form clusters ranging from 2 to 20 adjacent GlcNAc-containing disaccharides and from 2 to 10 adjacent $GlcNSO_3$-containing disaccharides. However, about 20–30% of the chain contains alternate GlcNAc- and $GlcNSO_3$-disaccharides units. HSPGs are necessary for structural and functional integrity of endothelium.

37.2 Proteoglycans in Tissues

Skin Proteoglycans: Expression of versican is observed in various adult tissues such as blood vessels, skin, and developing heart. Smooth muscle cells of blood vessels, epithelial cells of skin, and the cells of central and peripheral nervous system are a few examples of cell types that express versican physiologically. Dramatic changes occur in skin as a function of age, including changes in morphology, physiology, and mechanical properties. The major proteoglycans detected in extracts of human skin are dermaton sulfate (DS) proteoglycons, decorin and versican. Studies indicate that human fetal skin is structurally different from adult skin in terms of both the distribution and the composition of the large, aggregating chondroitin sulfate proteoglycan versican (Sorrell et al. 1999). Versican is a significant component of the interstitial extracellular matrix of skin development. An apparent codistribution of versican with the various fiber forms of the elastic network of the dermis suggested an

association of versican with microfibrils. Both dermal fibroblasts and keratinocytes express versican in culture during active cell proliferation. General function of versican is in cell proliferation processes that may not solely be confined to the skin (Zimmermann et al. 1994; Carrino et al. 2003). Human skin fibroblasts, in addition to versican, express a second large chondroitin sulfate/dermatan sulfate proteoglycan, which is produced in cultures of fetal fibroblasts. Proteoglycans have been described in adult human hair follicles. Versican may play an essential role both in mesenchymal condensation and in hair induction (Kishimoto et al. 1999). Glycosaminoglycan (GAG) and PG expression along the human anagen hair follicle has been characterized by Malgouries et al. (2008).

Brain: Many CSPGs have been shown to influence CNS axon growth in vitro and in vivo. These interactions can be mediated through the core protein or through CS GAG side chains. Degrading CS GAG side chains using chondroitinase ABC enhances dopaminergic nigrostriatal axon regeneration in vivo and interfering with complete CSPGs limit axon growth in vivo. Neurocan, versican, aggrecan, and brevican CSPGs may be anchored within extracellular matrix through hyaluronan glycosaminoglycan. Partial degradation of hyaluronan and chondroitin sulfate and depletion of hyaluronan-binding CSPGs enhances local sprouting of cut CNS axons, but long-distance regeneration fails in regions containing residual hyaluronan-binding CSPGs. Hyaluronan, chondroitin sulfate and hyaluronan-binding CSPGs may, therefore, contribute toward the failure of spontaneous axon regeneration in the injured adult mammalian brain and spinal cord (Moon et al. 2003) (see Chap. 38).

Human Eye: Apart from significant amounts of collagen, hyaluronan and sialylated glycoproteins, the human vitreous gel also contains low amounts of versican-like proteoglycan with a molecular mass of 380 kDa. Its core protein is substituted by CS side chains (37 kDa), in which 6-sulfated disaccharides predominated. Versican, which is able to bind lectins via its C-terminal region, may bridge or interconnect various constituents of the extracellular matrix via its terminal domains in order to stabilize large supramolecular complexes at the vitreous, contributing towards the integrity and specific properties of the tissue (Theocharis et al. 2002).

Glomerular Mesangial Cells Synthesize Versican: Mesangial cells derived from human adult glomeruli synthesize a number of proteoglycans including a large chondroitin sulfate proteoglycan (CSPG), two DS proteoglycans (biglycan and decorin) and two HS proteoglycans. Experiments with ^{3H}-labelled mesangial-cell proteoglycans showed that only the large CSPG, with core protein molecular masses of 400 kDa and 500 kDa, interacted with HA. Thus human mesangial large CSPG is a member of the versican family of proteoglycans. The interaction of CSPG and HA within the glomerulus may be important in glomerular cell migration and proliferation (Thomas et al. 1994). Both basement membrane and interstitial PGs are secreted by Madin-Darby canine kidney (MDCK) cells. HSPGs expressed by MDCK cells are perlecan, agrin, and collagen XVIII. Various CSPG core proteins are made by MDCK cells and have been identified as biglycan, bamacan, and versican/PG-M. These PGs are also associated with mammalian kidney tubules in vivo (Erickson and Couchman 2001).

Human Follicular Fluid and Umbilical Cord Vein: Two proteoglycans differing in size and composition are present in human follicular fluid. The larger one of high density had a molecular mass of 3.0×10^6 Da, and was substituted with 15–20 CS chains (Mr 60–65 kDa). EM of CS proteoglycan revealed a versican-like structure, with one globular domain at each end of a long extended segment substituted with CS side chains. The smaller proteoglycan had a molecular mass of 1.1×10^6 Da that showed a globular-protein core structure. The protein core was found to be heterogeneous, with bands occurring at 215, 330 and 400-kDa after enzymic degradation of the glycosaminoglycan chains (Eriksen et al. 1999). PGs from human umbilical cord arteries (UCAs) are especially enriched in CS/DS PGs. The predominant PG fraction included small PGs with molecular mass of 160–200 kDa and 90–150 kDa, i.e. typical for biglycan and decorin, respectively (Gogiel et al. 2007). Wharton's jelly contains mainly small CS/DS proteoglycans, with decorin strongly predominating over biglycan (Gogiel et al. 2003).

Tendons/Ligaments: The major proteoglycans of tendons are decorin and versican. Other species that were detected were biglycan and the large proteoglycans versican and aggrecan. Majority of the large proteoglycans present in the matrix of tendon are degraded and did not contain the G1 globular domain (Samiric et al. 2004a, b). Sulfated glycosaminoglycan content increases in pathologic tendons compared to normal (Tom et al. 2009). Bovine collateral ligament synthesizes a large proteoglycan which contained only chondroitin sulfate chains with a M_r 32 kDa and core proteins with a range of molecular masses above 200 kDa. Findings indicate that the large CS proteoglycan synthesized by bovine collateral ligament may be a versican-like proteoglycan which exhibited the potential to form like protein-stabilized complexes. Approximately 90% of the total proteoglycans in fresh ligament was decorin. Other species that were detected were biglycan and the large proteoglycans versican and aggrecan (Ilic et al. 2005).

Human Articular Cartilage, Intervertebral Disc, and Bone: Splicing variation of versican and size heterogeneity of versican core protein has been observed in human articular cartilage and intervertebral disc. All articular

cartilage extracts from the fetus to the mature adult contained multiple core protein sizes of greater than 200 kDa. The adult cartilage extracts tended to have an increased proportion of the smaller sized core proteins and osteoarthritic cartilage possessed similar core protein sizes to the normal adult. The increased presence of versican in the disc relative to articular cartilage may suggest a more pronounced functional role for this PG, particularly in the nucleus pulposus (Sztrolovics et al. 2002). Large hyaluronate-binding proteoglycans were characrerized in developing mandible of fetal rats at embryonic day 15 (E15) to E18. The large proteoglycan having smaller molecular weight is preferentially localized to bone nodules and may correlate with bone matrix mineralization (Lee et al. 1998). The Versican has been found in osteoarthritic cartilage. Though the control cartilage showed no staining, but in osteoarthritic cartilage there was strong staining of the cytoplasm of chondrocytes with abnormal morphology (Nishida et al. 1994). Cleavage of aggrecan by aggrecanase in articular cartilage characterizes cartilage degeneration in rheumatoid arthritis (Milz et al. 2002).

Tooth: Biglycan, decorin, versican, and link protein mRNAs are expressed in human dental pulp cells. Assuming expression of link protein and versican in vivo, the larger proteoglycans in the dental pulp are capable of forming large proteoglycan aggregates (Yamada et al. 1997; Abiko et al. 2001). Cementum is believed to play a regulatory role in periodontal regeneration through a variety of macromolecules including PGs present in its ECM. Immunoreactivity to versican, decorin, biglycan and lumican was evident at the borders and lumina of a proportion of lacunae and canaliculi surrounding cementocytes in cellular cementum. Versican, decorin, biglycan and lumican are components of the ECM of cellular, but not of acellular cementum. The distribution of PG epitopes around a proportion of cementocytes suggests the existence of different cementocyte subpopulations, or a differential response of these cells to yet undefined stimuli (Ababneh et al. 1999).

37.3 Hyaluronan: Proteoglycan Binding Link Proteins

Hyaluronan (HA) is a ubiquitous component of extracellular matrices, and in several systems it plays a central role in regulating cellular proliferation and differentiation. In the mammalian CNS, HA is present throughout development and into adulthood. The HA plays an important role in tissue reorganization in response to injury. Cell or tissue-specific functions of HA are likely to be mediated by cell or tissue-specific HA-binding proteins. Link proteins are glycoproteins in cartilage that are involved in the stabilization of aggregates of proteoglycans and hyaluronic acid. Link protein (LP), an extracellular matrix protein in cartilage, stabilizes aggregates of aggrecan and hyaluronan, giving cartilage its tensile strength and elasticity. Targeted mutations in mice in the gene encoding LP (Crtl1) showed defects in cartilage development and delayed bone formation with short limbs and craniofacial anomalies. Thue LP is important for the formation of proteoglycan aggregates and normal organization of hypertrophic chondrocytes (Watanabe et al. 1999) (Fig. 37.1).

37.4 Lecticans (Hyalectans)

The term *hyalectans* (or *lecticans*) defines a family of large hyaluronan-binding proteoglycans whose members include versican, aggrecan, neurocan, and brevican. Lecticans are a family of chondroitin sulfate proteoglycans, encompassing aggrecan (abundant in cartilage), brevican and neurocan (nervous system proteoglycans) and versican (also known as chondroitin sulfate proteoglycan core protein 2 or chondroitin sulfate proteoglycan 2). These proteoglycans are characterized by the presence of a hyaluronan-binding domain and a C-type lectin domain in their core proteins. Through these domains, lecticans interact with carbohydrate and protein ligands in the extracellular matrix and act as linkers of these extracellular matrix molecules. Molecular cloning has allowed identification of genes encoding core proteins of various proteoglycans, leading to a better understanding of the diversity of proteoglycan structure and function, as well as the evolution of a classification of proteoglycans. The postulated functions of proteoglycans in basements include: a structural role in maintaining tissue histoarchitecture, or aid in selective filtration processes; sequestration of growth factors; and regulation of cellular differentiation. Furthermore, expression of PGs has been found to vary in several disease states (Yamaguchi 2000). Lecticans contain N-terminal G1 domains and C-terminal G3 domains. Only aggrecan contains the G2 domain. The G1 domain consists of an Ig-like loop and two link modules, whereas the G2 domain consists only of two link modules. The G3 domain consists of one or two EGF repeats, a C-type lectin domain and CRP-like domain (Fig. 37.4). Lecticans share a number of structural features. The amino-terminal domains mediate the interaction with hyaluronic acid (HA) as well as with the link protein, a relatively small polypeptide involved in the aggregation of proteoglycans. The link protein consists basically of a HA binding domain. BEHAB, a protein deduced from its cDNA sequence, has been suggested to constitute a brain-specific link protein. Members of the aggrecan/versican family are soluble chondroitin sulfate proteoglycans whereas most of the membrane-spanning or glycosylphosphatidylinositol (GPI)-

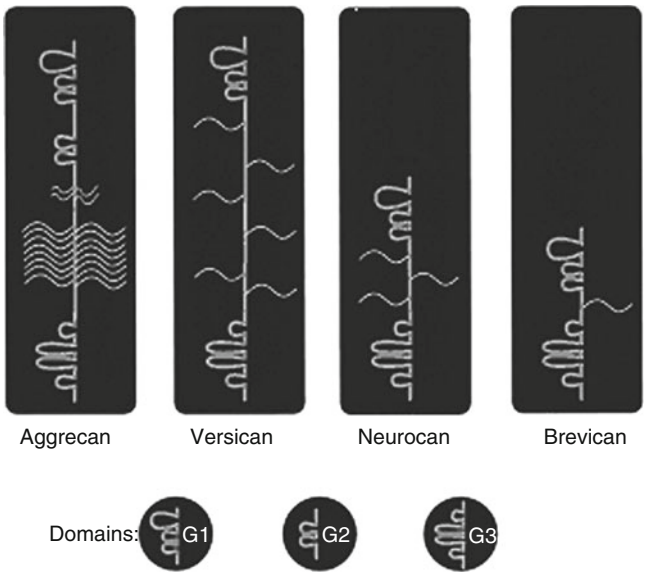

Fig. 37.4 Domain structures of lecticans. Lecticans contain N-terminal G1 domains and C-terminal G3 domains. Only aggrecan contains the G2 domain. The G1 domain consists of an Ig-like loop and two link modules, whereas the G2 domain consists only of two link modules. The G3 domain consists of one or two EGF repeats, a C-type lectin domain and CRP-like domain. All lecticans contain chondroitin sulfate chains (*yellow*) in the central domain. Aggrecan also contains keratan sulfate chains (*pink*) in the N-terminal part of the central domain (Reprinted with permission from Yamaguchi 2000 © Birkhauser Verlag). The number of chondroitin sulfate side chains present in brain aggrecan is not known but appears to be lower than the 100 GAG chains attached to cartilage aggrecan. Because the versicans, neurocan, and brevican form an almost perfect array of functionally related but differently sized molecules, it seems conceivable that the spatiotemporally regulated expression of these hyalectans (lecticans) fine tunes the inhibitory capacity of specialized hyaluronan-rich matrices in the developing and mature CNS (Bandtlow and Zimmermann 2000)

anchored proteoglycans carry heparan sulfate or keratan sulfate side chains.

37.5 Cartilage Proteoglycan: Aggrecan

37.5.1 Skeletogenesis

Skeletogenesis proceeds through a complex process involving patterning signals, cell differentiation, and growth. Most of the skeletal elements including those of limbs, as well as many parts of the craniofacial and axial skeleton, develop through the specialized process of endochondral ossification, in which a cartilaginous rudiment is first formed, then converted to bone. The growth and differentiation of cartilage into bone is controlled in part by interactions between the Indian hedgehog and parathyroid hormone-related peptide signaling pathways in the growth plate, and the induction of the bone- and cartilage-specific transcription factor Cbfa1 (Takeda et al. 2001). Presently, the best candidate for a general chondrogenic transcription factor', Sox9, is implicated in the control of several cartilage-specific genes (Bi et al. 2001). Aggrecan has been shown to interact with versican (Matsumoto et al. 2003). There is association of aggrecan and tenascin in tissues. The expression of aggrecan is mainly restricted to cartilages while tenascin mRNA is present at variable levels in most of the tissues. In the newborn mouse skeleton tenascin and aggrecan mRNAs were expressed essentially in a mutually exclusive manner, tenascin transcripts being present in osteoblasts, periosteal and perichondrial cells, and in cells at articular surfaces. None of these cells expressed the cartilage specific collagen or aggrecan genes. Patterns of gene expression depend on the location of chondrocytes in different cartilages (Glumoff et al. 1994).

37.5.2 Human Aggrecan

37.5.2.1 Domain Organization of Core Protein

The aggregating group of CSPGs is characterized by N-terminal and C-terminal globular (or selectin-like) domains, known as G1, and G3 domains, respectively (Fig. 37.4). The proteoglycans bind hyaluronan through their N-terminal G1 domains, and other ECM proteins through C-type lectin repeat in their C-terminal G3 domains. Members of CSPG family exhibit structural similarity: a G1 domain at the N-terminus and a G3 domain at the C-terminus, with a central sequence for modification by CS chains. However, a unique feature of aggrecan is the insertion of three additional domains, an inter-globular domain (IGD), a G2 domain and a keratan sulfate (KS) domain (sequence modified by KS chains), between the G1 domain and the CS domain (sequence modified by CS chains). The G1 region at the amino terminus of the core protein can be further sub-divided into three functional domains, termed A, B1 and B2, with the B-type domains being responsible for the interaction with HA (Watanabe et al. 1997). The G2 region also possesses two B-type domains, but does not appear to interact with HA (Fosang and Hardingham, 1989) (Fig. 37.4). The domain structure of core proteins of aggrecan/versican family members is reflected at the genomic level. The genes for rat and mouse aggrecan as well as human versican have a very similar exon/intron organization that resembles the arrangement of functional domains of the corresponding proteins (Schwartz et al. 1999). The human form of aggrecan is 2,316 amino acids long and can be expressed in multiple isoforms due to alternative splicing (Doege et al. 1994). Along with Type-II collagen, aggrecan forms a major structural component of cartilage, particularly articular cartilage. Aggrecan is detected as an early event in

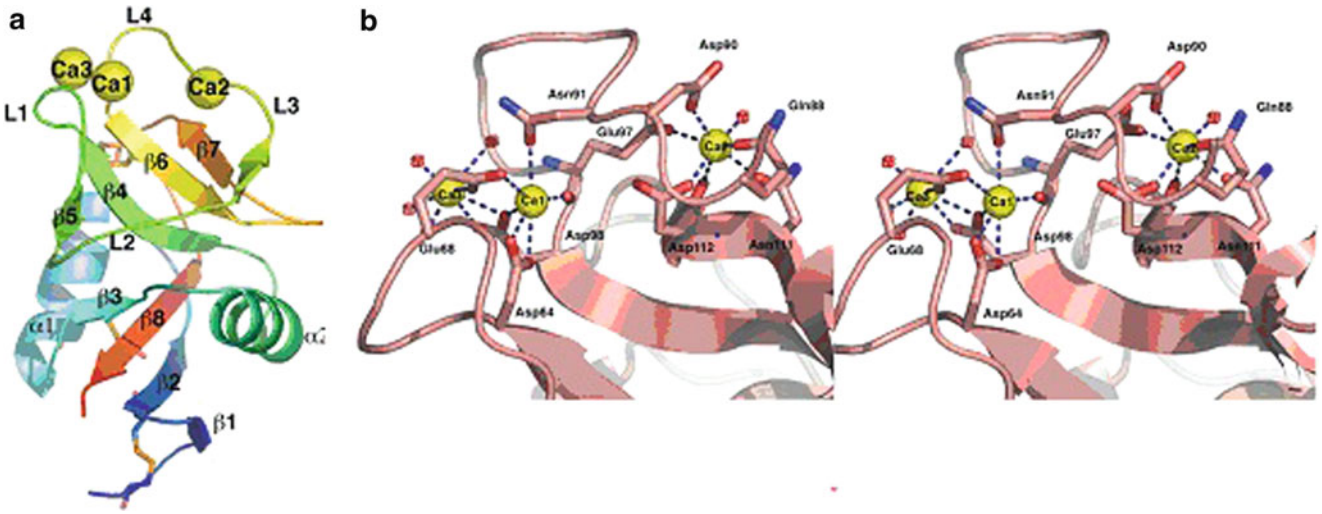

Fig. 37.5 The Aggrecan CLD fold and comparison to other C-type lectin domains (CLD) and Ca^{2+} binding. (**a**) The aggrecan CLD: The elements are colored from *blue* at the N terminus to *red* at the C terminus. The disulfide bridges are highlighted, and the three Ca^{2+} ions are shown as *yellow* spheres. (**b**) A stereoview of Ca^{2+} coordination in the aggrecan CLD. Water molecules are drawn as solid crosses (Reprinted with permission from Lundell et al. 2004 © Elsevier)

chondrocyte differentiation (Sandell 1994). Together with the CLDs of the other lecticans brevican, neurocan, and versican, the aggrecan CLD forms group I of the C-type lectin family. The overall structure is highly canonical for this family (Fig. 37.5). Aggrecan contains a long-form CLD (126 residues) with three disulfide bridges, like, e.g., tetranectin and asialoglycoprotein receptor H1 subunit (ASPGR). A search revealed strong similarity to a variety of other Ca^{2+} binding and non-Ca^{2+} binding CTLDs, including lithostatine, mannose binding protein MBP, and E-selectin (Lundell et al. 2004).

37.5.2.2 The Human Aggrecan Gene

The aggrecan gene has been characterized in several species including rat, human, mouse, and chicken (Schwartz et al. 1999). The basal promoter has been defined and shown to be active in various cell types. The human aggrecan gene, which resides at chromosome 15q26, consists of 19 exons (Doege et al. 2002; Valhmu et al. 1995), with each exon encoding a distinct structural domain of the core protein. Exons 3–6 encode the G1 region and exons 8–10 encode the G2 region. The CS-rich domain and much of the KS-rich domain are encoded by large exon 12. The G3 region is encoded by exons 13–18. It can give rise to different aggrecan transcripts due to alternative splicing (Fülöp et al. 1996), though it was not clear whether this is of any functional consequence. The promoter activity has been shown to be influenced by first exon sequences (Valhmu et al. 1998a), biomechanical stimuli (Valhmu et al. 1998b), sox9 (Sekiya et al. 2000), and a region that inhibits expression in chondrocytes (Pirok et al. 2001).

Glycosaminoglycan (GAG) Chain Attachment Region: The KS-attachment domain residing adjacent to G2 region is composed largely of repeat motifs whose number varies between species (Barry et al. 1994; Funderburgh 2000). The neighbouring CS-attachment domain is divided into two subdomains – the CS1 and CS2 domains. The CS1 domain lies adjacent to the KS-rich domain and is also composed largely of repeat motifs whose number varies between species. In addition, the human CS1 domain exhibits size polymorphism between individuals due to a variable number of 19 amino acid repeats (Doege et al. 1997). This results in the aggrecan molecules of different individuals being able to bear different numbers of CS chains. Irrespective of the number of CS chains present, their structure varies throughout life due to changes in length and sulphation pattern (Roughley and White 1989), though the functional consequence of this change is not clear. The GAG-attachment region also possesses sites for the attachment of O-linked oligosaccharides (Nilsson et al. 1982), which with age may become substituted with KS (Santer et al. 1982). KS may also be present within the G1 region, the IGD and the G2 region, attached to either O-linked or N-linked oligosaccharides (Barry et al. 1995). The KS chains also show age-related changes in structure (Brown et al. 1998).

The GAG-attachment region provides the high anionic charge density needed for unique osmotic properties of aggrecan. Normal cartilage function depends on a high aggrecan content, high GAG substitution and large aggregate size. Loss of cartilage integrity in arthritis is associated with impaired aggrecan function due either to proteolytic cleavage of the aggrecan core protein, which decreases aggrecan charge, or to cleavage of the HA, which decreases

aggregate size. It has also been suggested that aggrecan charge and hence function could be affected by size polymorphism within the CS1 domain, as those individuals with the shortest core protein length would possess aggrecan with the lowest CS substitution. Such individuals might be at risk for premature cartilage degeneration. CS2 domain processing by aggrecanases would result in aggrecan fragments enriched in the CS1 domain and therefore enhance any influence that CS1 domain polymorphism may have on aggrecan function. While size polymorphism in the aggrecan CS1 domain has been associated with both articular cartilage and intervertebral disc degeneration (Horton et al. 1998; Kawaguchi et al. 1999), the reason for the linkage is not clear as it is not always the shorter CS1 domains that have been associated with disease. It is possible that the presence of one short aggrecan allele may be of little functional consequence, and only individuals with two short alleles would be at risk. Such individuals represent less than 1% of the population and have been of little focus.

The G1 and G3 domains have been implicated in product secretion, but G2, although structurally similar to the tandem repeats of G1, performs an unknown function. Each aggrecan domain has been cloned and expressed in various combinations in COS-7 cells (Kiani et al. 2001). Yang et al. (2000) indicated that the presence of G1 domain was sufficient to inhibit product secretion, while the G3 domain enhanced this process. The inhibition of secretion by G1 was mediated by its two tandem repeats, while G3's promotion of glycosaminoglycan chain attachment was apparently dependent on G3's complement-binding protein (CBP)-like motif. The modulatory effects of these two molecular domains contribute to versican's biological activities. Thus, G3 domain enhances product secretion, alone or in combination with the KS or CS domain, and promotes GAG chain attachment (Kiani et al. 2001; Yang et al. 2000; Zheng et al. 1998) (Fig. 37.6).

Effect of Age on Content of C-Terminal Region of Aggrecan: The C-terminal region of aggrecan contains G3 domain which is removed from aggrecan in mature cartilage, by proteolytic cleavage. The content of the C-terminal region decreases with age relative to G1 domain content. The content of this region of the molecule shows a fall of 92% from newborn to 65 years of age. Hence, the content of C-terminal region gives a measure of abundance of newly synthesized aggrecan. The loss of the C-terminal region is not direct part of the process of aggrecan turnover, but it is a slow independent matrix process that occurs more extensively with aging as turnover rates become slower. Young cartilage with the fastest turnover contains least molecules lacking the C-terminal region, whereas in old tissue with slow turnover few molecules retain this region. An increase in the cleavage of this region with age may also contribute to this change (Dudhia et al. 1996; Trowbridge and Gallo 2002).

37.5.3 Rat Aggrecan

Overlapping cDNA clones for coding sequence of rat cartilage proteoglycan core protein from rat chondrosarcoma hybridize to two sizes of RNA transcripts of 8.2 and 8.9 kb pairs, which contain large 3′-untranslated sequences. The entire contiguous cDNA is 6.55 kb pairs in size, and codes for a 2124-residue protein, including a 19-residue signal peptide. The sequence forms a series of eight structural domains including two globules, (Mr = 37,000 and 22,000) at the NH2 terminus of the molecule, one a complete and one a partial copy of the cartilage link protein. The deduced sequence is a 1,104-residue protein, containing 117 Ser-Gly sequences, possible CS attachment sites. These are arranged in three domains of 428, 503, and 173 amino acids. The first domain contains 11 complete or partial repeats of a 40-residue unit, and the second domain is composed of six copies of a 100-residue repeating sequence. The first pattern is the more highly conserved, and may have given rise to the second. The carboxyl-terminal domain is a third globule which has homology with animal lectins (Doege et al. 1987). Two sets of cDNAs encoding isoforms of aggrecan/versican core proteins expressed in rat brain have been characterized. One isoform constitutes most likely the rat homolog of bovine soluble brevican, whereas another group of isoforms represents the first example of GPI-anchored proteins of the aggrecan/versican family. GPI-anchored brevican isoforms are up-regulated late during postnatal development (see Chap. 38).

Nucleotide sequencing of 2 kb of coding sequences for the human protein in comparison with same region in rat and chick indicated that domain 8, the lectin-like domain, is highly conserved among species. In contrast, domain 7 is poorly conserved among species. Some of the cDNA clones also contained an additional structural domain between domains 7 and 8 which was not described in the rat or chick sequences. The additional domain of 38 amino acids was highly homologous to EGF-like sequences seen in other proteins. Because some cDNA clones contained codons for the EGF-like domain and some did not, the results suggested that the EGF-like domain underwent alternative RNA splicing (Baldwin et al. 1989).

37.5.3.1 Rat Aggrecan Gene

Aggrecan DNA sequencing shows 18 exons, most of which encode structural or functional modules; exceptions are domains G1-B and G2-B, which are split into two exons and the G3 lectin domain, which is encoded by three exons. There is one expressed EGF-like exon and in addition a non-

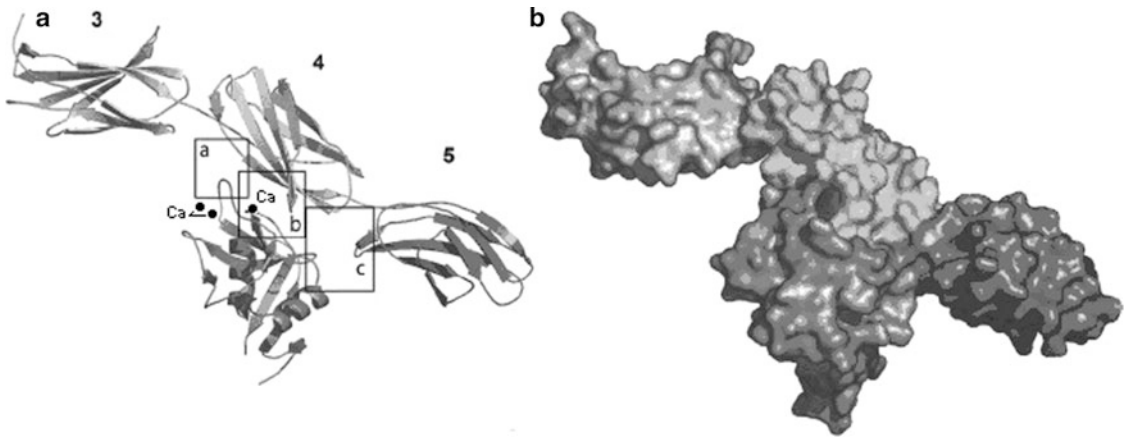

Fig. 37.6 **(a)** Overall structure of the complex between the aggrecan CTLD and FnIII repeats 3–5 of Tenascin-R, TN3–5. A consistent coloring scheme is used throughout for the different domains. Surfaces, cartoon elements, and carbon atoms from domain 3 are colored beige, those in domain 4 are colored cyan, and those in domain 5 are purple. The three Ca^{2+} ions are shown as yellow spheres. The boxes marked *a–c* denote the interaction areas. **(b)** Surface representation colored by domain. The aggrecan CLD amino acid residues mediating interaction with tenascin-R form a surface corresponding to the sulfopeptide binding surface of P-selectin in complex with PSGL-1 (Somers et al. 2000) (Adapted with permission from Lundell et al. 2004 © Elsevier)

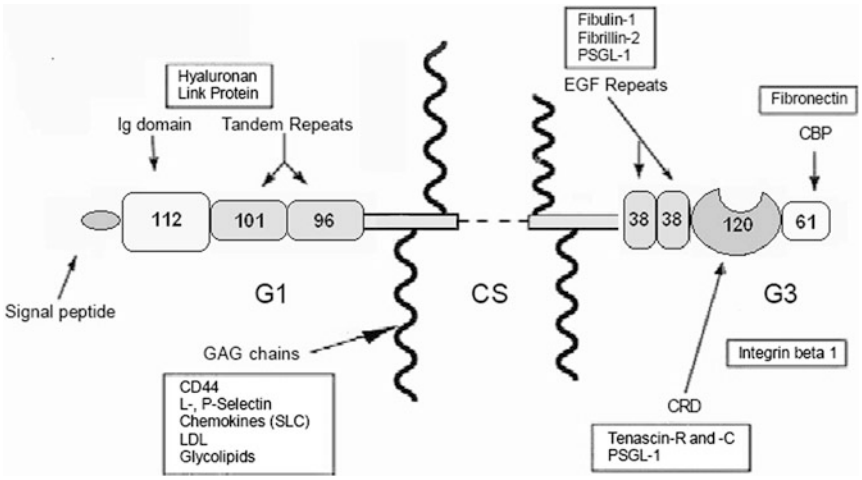

Fig. 37.7 The interaction of versican with various molecules. The locations of versican motifs that interact with other molecules are shown in the figure. Structure shows presence of G1 domain comprising of an Ig domain and two tandom repeats; a proteoglycan with Chondroitin sulfate (CS) region; and G3 domain comprising of an EGF repeats, a carbohydrate recognition domain (CRD) and a complementary binding protein (CBP) region. Molecules interactiong with various domains are boxed (Wu et al. Cell Res 2005)

expressed "pseudo-exon" encoding a heavily mutated EGF-like domain. A 30-kb intron separates exons 1 and 2. Exon 1 encodes 381 bp of 5′-untranslated sequence. There is a minor promoter which initiates transcription of an additional 68 bp 5′ of the major promoter start site. DNA sequence is reported for a 529-bp fragment encompassing exon 1, including 120 bp of 5′-flanking DNA comprising the promoter. This promoter is lacking the TATAA or CCAAT elements but has several putative binding sites for transcription factors. A 922-bp DNA fragment with 640-bp 5′-flanking DNA and 282-bp exon 1 sequence showed higher promoter activity in transfected chondrocytes, is completely inactive in the reverse orientation, and is strongly enhanceable in the forward direction by the SV40 enhancer (Doege et al. 1994).

DNA comprising aggrecan gene (83 kb including the 30-kb first intron) was surveyed for active elements in rat. A 4.7-kb DNA fragment (P3) with cell-specific enhancer activity was discovered ~12 kb upstream of the transcription start site; this active DNA fragment strongly stimulates aggrecan promoter expression in chondrocytes and weakly suppressing transcription in fibroblasts. Most of this activity has been localized to P3-7, a 2.3-kb internal

fragment of P3. Another enhancer element (A23), which is not tissue-specific, was discovered about 70 kb downstream of the transcription start site. Several lines of transgenic mice revealed that neither a short (900 bp) nor a long (3.7 kb) promoter alone showed detectable expression in 14.5-day embryos, whereas placing the P3 tissue-specific enhancer together with P0 gave strong expression restricted to embryonic cartilage of transgenic mice. The A23 downstream enhancer in conjunction with P0 did not confer expression. This gene confers authentic tissue-specific regulation of aggrecan in vitro or in vivo (Doege et al. 2002).

37.5.4 Mouse Aggrecan

Overlapping cDNA clones encoding the entire core protein of aggrecan localized the aggrecan gene in mouse chromosome 7. Walcz et al. (1994) determined 7386 bp of the cDNA sequence, including 132 and 854 nt of 5′ and 3′ untranslated regions, respectively. The core protein precursor is 2,132 amino acids long (M_r 222,008), including a 19-residue secretory signal peptide. The overall amino acid sequence of the mouse aggrecan shows 91.6% identity to rat and 72.5% to human aggrecan. Comparison of the amino acid sequences of various domains and subdomain structures of mouse aggrecan to known sequences of other species and related proteins (versican, neurocan, link protein, and lymphocyte homing receptor CD44) revealed high levels of identity of the G1, G2, and G3 globular domains and relatively less conserved structures in the interglobular and glycosaminoglycan-attachment regions (Walcz et al. 1994).

The mouse aggrecan gene spans at least 61 kb and contains 18 exons. Exon 1 encodes 5′-untranslated sequence and exon 2 contains a translation start codon, methionine. The coding sequence is 6545 bp for a 2,132-amino-acid protein with $M_r = 259{,}131$ including an 18-amino-acid signal peptide. The chondroitin sulfate domain consisting of 1,161 amino acids is encoded by a single exon of 3.6 kb. Although link protein has similar structural domains and subdomains, the sequence identity and the organization of exons encoding the subdomains B and B′ of G1 and G2 domains revealed a strong similarity of mouse aggrecan to both human versican and rat neurocan. Primer extension analysis identified four transcription start sites which are close together. The promoter sequence showed high G/C content and contained several consensus binding motifs for transcription factors including Sp-1 and the glucocorticoid receptor. There are stretches of sequences similar to the promoter region of both the type-II collagen and link protein genes. These sequences may be important for cartilage gene expression (Watanabe et al. 1995, 2006).

37.5.5 Chick Aggrecan

Pirok et al. (1997) cloned and sequenced the 1.8-kb genomic 5′ flanking sequence of the chick aggrecan gene. Sequence analysis revealed potential Sp1, AP2, and NF-I related sites, as well as several putative transcription factor binding sites, including the cartilage-associated silencers CIIS1 and CIIS2. A number of these transcription factor binding motifs are embedded in a sequence flanked by prominent inverted repeats. Although lacking a classic TATA box, there are two instances in the 1.8-kb genomic fragment of TATA-like TCTAA sequences, as have been defined previously in other promoter regions. Primer extension and S1 protection analyses revealed three major transcription start sites, also located between the inverted repeats. Expression of aggrecan is both cell-specific and developmentally regulated. Seven functionally defined *cis* elements in aggrecan promoter region are known to repress aggrecan gene expression. A functional repressor *cis* element, (T/C) TCCCCT(A/C)RRC occurs at multiple locations within the chick aggrecan regulatory region (Pirok et al. 2005).

37.6 Versican or Chondroitin Sulfate Proteoglycan Core Protein 2

Versican, also known as chondroitin sulfate proteoglycan core protein 2 (or chondroitin sulfate proteoglycan 2 (CSPG2), or PG-M), is a large extracellular matrix proteoglycan that is present in a variety of human tissues. It is encoded by the *VCAN* gene. It is a large chondroitin sulfate proteoglycan with M_r of more than 1,000-kDa. Versican is encoded by a single gene, which is located on chromosome 5q12-14 in the human genome. Versican occurs in four isoforms: V0, V1, V2, V3. The central domain of versican V0 contains both the GAG-α and GAG-β domains. V1 isoforms has the GAG-β domain, V2 has the GAG-α domain, and V3 is void of any GAG attachment domains. The GAGs, being composed of repeating disaccharide units, contribute to the negative charge and many other properties of proteoglycans. These proteoglycans share a homologous globular N-terminal, C-terminal, and glycosaminoglycan (GAG) binding regions. The N-terminal (G1) globular domain consists of Ig-like loop and two link modules, and has Hyaluronan binding properties. The C-terminal (G3) globular domain consists of one or two EGF repeats, a C-type lectin domain and complement regulatory protein (CRP)-like domain. The C-terminal domain binds a variety

of ligands in ECM which contribute significantly to the functions of lecticans (Fig. 37.1).

37.6.1 Expression

Expression of versican is observed in various adult tissues such as blood vessels, skin, and developing heart. Smooth muscle cells of blood vessels, epithelial cells of skin, and the cells of central and peripheral nervous system are a few examples of cell types that express versican physiologically. In adult CNS, versican is found in perineuronal nets, where it may stabilize synaptic connections. Also, there is differential temporal and spatial expression of versican by multiple cell types and in different developmental and pathological time frames. To fully appreciate the functional roles of versican as it relates to changing patterns of expression in development and disease, an in depth knowledge of versican's biosynthetic processing is necessary. Rahmani et al. (2006) evaluated the current status of our knowledge regarding the transcriptional control of versican gene regulation with a focuss on the signal transduction pathways, promoter regions, cis-acting elements, and trans-factors that have been characterized.

cDNA Clones: The cDNA clones of versican expressed by human fibroblasts code for 2,389 amino acid long core protein and the 20-residue signal peptide. The sequence predicts a potential hyaluronic acid-binding domain in the amino-terminal portion. This domain contains sequences virtually identical to partial peptide sequences from a glial hyaluronate-binding protein. Putative glycosaminoglycan attachment sites are located in the middle of the protein. The carboxy-terminal portion includes two (EGF) epidermal growth factor-like repeats, a lectin-like sequence and a complement regulatory protein-like domain. Amino- and carboxy-terminal portions of the fibroblast core protein are closely related to the core protein of a large chondroitin sulfate proteoglycan of chondrosarcoma cells. However, the glycosaminoglycan attachment regions in the middle of the core proteins are different and only the fibroblast core protein contains EGF-like repeats. Based on the similarities of its domains with various binding elements of other proteins, it was suggested that the large fibroblast proteoglycan, herein be referred to as versican (Zimmermann and Ruoslahti 1989).

Alternative Splice Variants of Human Versican: Alternative splicing of versican generates at least four isoforms named V0, V1, V2, and V3. An alternatively spliced glycosaminoglycan attachment domain (GAG-α) of human versican was cloned from cDNA libraries. Inserted carboxyl-terminal of the hyaluronan-binding region, this domain adds another 987 amino acids to the original versican (V1) core protein giving rise to the large V0 isoform with 3,396 amino acids and 17–23 putative glycosaminoglycan attachment sites. The GAG-α domain is encoded by exon 7 of the human versican gene. Sequence comparisons revealed a slight similarity to the alternative splice domain of PG-M, further supporting the notion that PG-M is the chicken homologue of versican. Both V0 and V1 isoforms were detected in cerebral cortex, aorta, intervertebral disc, liver, myometrium, and prostate, whereas keratinocytes exclusively expressed versican V1. In brain tissue, a short versican variant (V2) including only the GAG-α domain was identified (Dours-Zimmermann and Zimmermann 1994).

Multiple Forms of Mouse Versican: Versicon from chick limb buds, named as PG-M, was shown to be expressed in the prechondrogenic condensation area of the developing chick limb buds. PG-M shows the presence of a hyaluronic acid binding domain at the amino-terminal side and two EGF-like domains, a lectin-like domain, and a complement regulatory protein-like domain at the carboxyl-terminal side. These domains show high homology to corresponding domains of a human fibroblast versican. The chondroitin sulfate attachment domain of PG-M core protein is about 100 kDa larger than that of versican core protein. The finding of alternatively spliced forms of PG-M core protein suggests that versican might be one of the multiple forms of PG-M (Shinomura et al. 1993). Ito et al. (1995) sequenced cDNA clones that encode the core protein of PG-M-like proteoglycan produced by cultured mouse aortic endothelial cells. A homology search of cDNA revealed that the core protein is a mouse equivalent of chick PG-M-V1, one of the alternatively spliced forms of the PG-M core protein, which may correspond to human versican. Northern blot analysis revealed three mRNA species of 10, 9, and 8 kb in size. The analysis of PG-M mRNA species in embryonic limb buds and adult brain indicated the presence of other mRNA species with different sizes; the one with the largest size (12 kb) was found in embryonic limb buds, and the ones with smaller sizes of 7.5 and 6.5 kb were present in adult brain. Sequencing of cDNA for smaller forms from adult brain showed that they were different from PG-M-V1 in encoding the second chondroitin sulfate attachment domain (CSα) alone. The mRNA of 12 kb in size corresponded to a transcript without the alternative splicing (V0), PG-M-V0. It is likely that multiforms of the PG-M core protein may be generated by alternative usage of either or both of the two different CS attachment domains (α and β) and that molecular forms of PG-M may vary from tissue to tissue by such an alternative splicing. The ectopic expression of versican V1 isoform induced mesenchymal-epithelial transition (MET) in NIH3T3 fibroblasts, and inhibition of

endogenous versican expression abolished the MET in metanephric mesenchyme. Reports indicate the involvement of versican in MET: expression of versican is sufficient to induce MET in NIH3T3 fibroblasts and reduction of versican expression decreased MET in metanephric mesenchyme (Sheng et al. 2006).

Versican Gene: The human VCAN gene is divided into 15 exons over 90–100 kb (Naso et al. 1994). The entire primary structures of versican have been generated from human, murine, bovine and chick cDNA clones; the chick form was originally named PG-M (Shinomura et al. 1993). The exon organization corresponds to the protein subdomains encoded by homologous proteins, with a remarkable conservation of exon size and intron phase. An additional exon is present just proximal to the glycosaminoglycan-binding region that is identical to a splice variant of versican (Dours-Zimmermann and Zimmermann 1994). The versican promoter harbors a typical TATA box located approximately 16 bp upstream of the transcription start site and binding sites for a number of transcription factors involved in regulated gene expression. Stepwise 5′ deletions identified a strong enhancer element between −209 and −445 bp and a strong negative element between −445 and −632 bp (Naso et al. 1994).

37.6.2 Structure

Versican is a large (1–2 × 10^6 Da) chondroitin-sulfate proteoglycan that can form large aggregates by means of interaction with hyaluronan and also binds to a series of other extracellular matrix proteins, chemokines and cell-surface molecules. A recombinant construct corresponding to the C-type lectin domain of versican demonstrates a calcium-dependent self-association of this region. This binding reaction could contribute to the ability of versican to organize formation of the proteoglycan extracellular matrix by inducing binding of individual versican molecules or by modulating binding reactions to other matrix components (Ney et al. 2006) (Fig. 37.4). The large aggregating chondroitin sulfate proteoglycans are characterized by N-terminal and C-terminal globular (or selectin-like) domains known as the G1 and G3 domains, respectively. The modulatory effects of G1 and G3 molecular domains may contribute to versican's biological activities (Yang et al. 2000). Versican, like other members of its family, has unique N- and C-terminal globular regions, each with multiple motifs. A large glycosaminoglycan-binding region lies between them. These structures have been discussed in the context of ECM proteoglycans. All isoforms of versican have homologous N-terminal (HA binding) and C-terminal (lectin-like) domains. The central domain of versican V0 contains both the GAG-α and GAG-β domains. V1 isoforms has the GAG-β domain, V2 has the GAG-α domain, and V3 is void of any GAG attachment domains, and only consists of the N-terminal and C-terminal globular domains.

N-Terminus: The N-terminal (G1) globular domain consists of Ig-like loop and two link modules, and has hyaluronan (HA) binding properties. The N-terminal of versican has an important role in maintaining the integrity of the ECM by interacting with hyaluronan. Its interactions with link protein have also been observed.

C-Terminus: The C-terminal (G3) globular domain consists of one or two EGF repeats, a C-type lectin domain and complement regulatory protein (CRP)-like domain. The C-terminal domain binds a variety of ligands in ECM which contribute significantly to the functions of lecticans. One important family of ligands is the tenascin family. For example, The C-lectin domain of versican interacts with Tenascin R through its fibronectin type III (FnIII) repeat 3–5 domain in a calcium dependent manner, in vivo. Different tenascin domains interact with a wide range of cellular receptors, including integrins, cell adhesion molecules and members of the syndecan and glypican proteoglycan families (Fig. 37.3B). Versican's C-terminal domain interacts with Fibulin-2, a protein whose expression is associated with that of versican in the developing heart. The EGF domain of the C-terminal of Versican also binds the EGF-receptor molecule, in vivo.

GAG Binding Region: The central domain of Versican is decorated with glycosaminoglycans. The structural and functional diversity of Versican is increased by variations in GAG sulfation patterns and the type of GAG chains bound to the core protein. The central domain of versican V0 contains both the GAG-α and GAG-β domains. V1 isoforms has the GAG-β domain, V2 has the GAG-α domain, and V3 is void of any GAG attachment domains. The GAGs, being composed of repeating disaccharide units, contribute to the negative charge and many other properties of proteoglycans.

37.6.3 Interactions of Versican

The diverse binding partners afforded to versican by virtue of its modular design include ECM components, such as hyaluronan, type I collagen, tenascin-R, fibulin-1, and -2, fibrillin-1, fibronectin, P- and L-selectins, and chemokines.

Versican also binds to the cell surface proteins CD44, integrin β1, epidermal growth factor receptor, and PSGL-1. These multiple interactors play important roles in cell behaviour. Roles of versican in modulating such processes have been discussed.. Fibulin-1 is strongly expressed in tissues where versican is expressed, and also expressed in developing cartilage and bone. The interactions with fibulin-1 are Ca^{2+}-dependent with K_D values in the low nM range. The binding site for aggrecan and versican lectin domains was mapped to the EGF-like repeats in domain II of fibulin-1. Solid phase assays with known ligands demonstrated that fibulin-2 and tenascin-R bind the same site on the proteoglycan lectin domains (Wu et al. 2005) (Fig. 37.7).

The interactions of the CS/DS chains of versican with L- and P-selectin and chemokines are sulfation-dependent but the interaction with CD44 is sulfation-independent. The binding of versican to L- and P-selectin was inhibited by CS and HS. On the other hand, the binding to CD44 was inhibited by hyaluronic acid, chondroitin (CH), CS A, CS B, CS C, CS D, and CS E but not by HS or keratan sulfate. A cross-blocking study indicated that L- and P-selectin recognize close or overlapping sites on versican, whereas CD44 recognizes separate sites. Structural analysis showed that versican is modified with at least CS B and CS C (Kawashima et al. 2000). Studies suggest that oversulfated CS/DS chains containing GlcAβ1/IdoAα1-3GalNAc(4,6-O-disulfate) are recognized by L- and P-selectin and chemokines, and imply that these chains are important in selectin- and/or chemokine-mediated cellular responses (Kawashima et al. 2002).

37.6.4 Functions

Versican is a multifunctional molecule with roles in cell adhesion, matrix assembly, cell migration proliferation and angiogenesis, and hence plays a central role in tissue morphogenesis and maintenance. Versican is involved in development, guiding embryonic cell migration important in the formation of the heart and outlining the path for neural crest cell migration. Versican is a key factor in inflammation through interactions with adhesion molecules on the surfaces of inflammatory leukocytes and interactions with chemokines that are involved in recruiting inflammatory cells. Versican is often considered an anti-adhesive molecule. Considering the large size (>1,000 kDa) and hydration capability of versican, it is possible that the interaction of integrins with their cell surface receptors is sterically hindered. In addition, because of negatively charged sulfates or carboxyl groups, chondroitin sulfate chains are attracted to various positively charged molecules such as certain growth factors, cytokines, and chemokines. This interaction in the ECM or on the cell surface is important in the formation of immobilized gradients of these factors, their protection from proteolytic cleavage, and their presentation to specific cell-surface receptors. The binding of versican with leukocyte adhesion molecules L-selectin, P-selectin, and CD44 is also mediated by the interaction of CS chains of versican with the carbohydrate-binding domain of these molecules. Both CD44 and L-selectin have been implicated in leukocyte trafficking. The ability of versican to bind a large panel of chemokines and the biological consequences of such binding has also been examined. Versican can bind specific chemokines through its CS chains and this interaction down-regulates the chemokines function. In light of results that V1 and V2 isoforms of versican have opposite effects on cell proliferation, glycosaminoglycan domain GAG-β has been implicated in versican-enhanced cell proliferation and versican-induced reduction of cell apoptosis.

Versican Guides Migratory Neural Crest Cells: Selective expression of versican V0 and V1 in barrier tissues impede the migration of neural crest cells during embryonic trunk development (Landolt et al. 1995). Pure preparations of either a mixture of versican V0/V1 or V1 alone strongly inhibit the migration of multipotent Sox10/p75NTR-positive early neural crest stem cells. This inhibition is largely core glycoprotein-dependent. Findings support the notion that versican variants V0 and V1 act, possibly in concert with other inhibitory molecules such as aggrecan and ephrins, in directing the migratory streams of neural crest cells to their appropriate target tissues (Dutt et al. 2006).

The versican accumulates in lesions of atherosclerosis and restenosis. The unique structural features make this molecule to bind growth factors, enzymes, lipoproteins, and a variety of other ECM components to influence fundamental events involved in vascular diseases. Versican is upregulated after vascular injury and is a prominent component in stented and nonstented restenotic lesions. The synthesis of versican is highly regulated by specific growth factors and cytokines and the principal source of versican is the smooth muscle cell. Versican interacts with hyaluronan to create expanded viscoelastic pericellular matrices that are required for arterial smooth muscle cell (ASMC) proliferation and migration. Versican is also prominent in advanced lesions of atherosclerosis, at the borders of lipid-filled necrotic cores as well as at the plaque-thrombus interface, suggesting roles in lipid accumulation, inflammation, and thrombosis. Versican influences the assembly of ECM and controls elastic fiber fibrillogenesis, which is of fundamental importance in ECM remodeling during vascular disease. Collectively, studies highlight the importance of this specific ECM component in atherosclerosis and restenosis (Wight and Merrilees 2004).

37.7 Pathologies Associated with PGS

37.7.1 Proteoglycans Facilitate Lipid Accumulation in Arterial Wall

The accumulation of extracellular matrix components such as proteoglycans is a hallmark of an atherosclerotic lesion. Proteoglycans are considered to facilitate lipid accumulation in the arterial wall, as part of the injury and repair process in atherogenesis. Srinivasan et al. (1995) determined (1) characteristics of arterial tissue CS monomers of versican type that vary in binding affinity to low-density lipoproteins (LDL), and (2) the ability of these variants to modulate LDL metabolism by macrophages. A large CS devoid of DS was identified and purified from bovine aorta intima-media under dissociative conditions. Variations in LDL-binding affinity of CS could modulate the lipid accumulation in atherogenesis. LDL enhances mesangial cell (MC) matrix deposition by modulating the production of PG and hyaluronan (HA) in the culture medium and to a lesser extent in the cell layer. The GAG chains did not show difference in either their size or charge. The synthesis of both CS and HS was enhanced as was the production of versican, perlecan and to a lesser extent decorin. An increase in HA synthesis was also demonstrated following LDL stimulation (Chana et al. 2000).

Heparan sulfate in ECM of artery wall possesses antiatherogenic properties and interferes in lipoprotein retention, by suppression of inflammation, and by inhibition of smooth muscle cell (SMC) growth. The amount of HS in atherosclerotic lesions from humans and animals is known to be reduced. The atherosclerotic lesions retrieved from patients undergoing surgery for symptomatic carotid stenosis showed a selective reduction in perlecan gene expression, whereas, expression of other HS proteoglycans in the artery wall, agrin and collagen XVIII, the large CSPG and the versican remained unchanged. This suggests a reduction of perlecan mRNA-expression and protein deposition in human atherosclerosis, which in part explains the low levels of HS in this disease (Tran et al. 2007).

Fatty Acids Modulate the Composition of ECM in Cultured Human Arterial SMCs: In diabetes-associated angiopathies and atherosclerosis, there are alterations in the intima of small and large arteries. High concentrations of nonesterified fatty acids (NEFAs) might alter the basement membrane composition of endothelial cells. In arteries, SMCs are the major producers of proteoglycans and glycoproteins in the intima, which is the site of lipoprotein deposition and modification during atherogenesis. Exposure of human arterial SMCs (ASMCs) to albumin-bound linoleic acid lowered their rate of proliferation and altered cell morphology. ASMCs expressed 2–10 times more mRNA for the core proteins of versican, decorin, and syndecan 4. Studies suggest that some of the NEFA effects are mediated by PPAR-γ. These effects of NEFAs, in vivo, could contribute to changes of the matrix of arterial intima associated with micro- and macroangiopathies (Olsson et al. 1999).

Human hASMC matrix PGs contribute to the retention of apoB lipoproteins in the intima, a possible key step in atherogenesis. The response of ASMC to NEFA could induce ECM alterations favoring apoB lipoprotein deposition and atherogenesis (Rodríguez-Lee et al. 2006). Extracellular matrix changes occur in heart valve pathologies. Decreased expression of the hyaluronan receptor for endocytosis in myxomatous mittral valves suggested that hyaluronan metabolism could be altered in myxomatous mitral valve disease (Gupta et al. 2009).

Perlecan in Atherosclerosis-Prone and Atherosclerosis-Resistant Human Arteries: A large HSPG, perlecan, dramatically increases in the advanced lesion, and vascular SMC are the cell type responsible for the accumulation. In cultured human coronary SMCs indicate that thrombin increases the cell layer-associated HSPG with a core protein size of approximately 400 kDa without any change in the length of the glycosaminoglycan chains when the cell density is high. The HSPG was identified as perlecan. In addition, thrombin elevated the steady-state level of perlecan mRNA but not that of versican, decorin, and syndecan-1 mRNAs, although biglycan mRNA was moderately elevated. It is suggested that thrombin induces the perlecan core protein synthesis without influencing the formation of the heparan sulfate chains in human coronary SMCs at a high cell density. The regulation of proteoglycan synthesis by thrombin may be involved in the accumulation of perlecan in advanced lesions of atherosclerosis (Yamamoto et al. 2005).

A proteomics-based approach indicated that intimal proteoglycan composition in preatherosclerotic lesions was more complex than previously appreciated with up to eight distinct core proteins present, including the large versican and aggrecan, the basement membrane proteoglycan perlecan, the class I small leucine-rich proteoglycans biglycan and decorin, and the class II small leucine-rich proteoglycans lumican, fibromodulin, and prolargin/PRELP (proline arginine-rich end leucine-rich repeat protein). Although most of these proteoglycans seem to be present in similar amounts at the two locations, there was a selective enhanced deposition of lumican in the intima of the atherosclerosis-prone internal carotid artery compared with the intima of the atherosclerosis-resistant internal thoracic artery. The enhanced deposition of lumican in the intima of an atherosclerosis prone artery has

important implications for the pathogenesis of atherosclerosis (Talusan et al. 2005).

Molecular Structure and Organization of Versicon in Human Aneurysmal Abdominal Aortas: Differences have been noted in matrix metalloproteinase expression patterns in cultures of medial smooth muscle cells from tissue affected by abdominal aortic aneurysm (AAA) or atherosclerotic occlusive disease and from normal arterial tissue. Versican, perlecan, and biglycan levels were significantly elevated in AAA smooth muscle cell cultures. Two populations of smooth muscle cell versican were identified. Because heparan sulfate proteoglycans can bind growth factors, their elevated synthesis by AAA smooth muscle cells in combination with an increased expression of matrix metalloproteinases may at least partly explain the differential proliferative capacity of the AAA smooth muscle cells and may govern the pattern of abnormal cellular proliferation and matrix protein synthesis observed in the pathogenesis of vascular disease (Melrose et al. 1998).

Versican in aortic wall participates in various biological functions of the tissue. In human aorta and aneurysmal aortic tissue versican is exclusively substituted with chondroitin sulfate chains, in contrast to other human tissues where both chondroitin and dermatan sulfate chains are attached onto versican core proteins. Except for the significant decrease in the concentration of versican in the aneurysmal tissue, this PG undergoes specific alterations in the aneurysmal tissue. The molecular size of versican isolated from diseased tissue is decreased with a simultaneous increase in the ratio of glycosaminoglycan to protein in this tissue. Although the size of chondroitin sulfate chains was identical in both versican preparations, a significant increase in the percentage of 6-sulfated disaccharides was observed in chondroitin sulfate chains of versican in aneurysmal aortas, which is accompanied by decrease in 4-sulfated and non-sulfated units (Theocharis et al. 2003a). Mature human aorta contains a 70-kDa versican fragment, which appears to represent G1 domain of versican V1 and has been generated in vivo by proteolytic cleavage at the Glu(441)-Ala(442) bond, within the sequence DPEAAE (441)-A(442)RRGQ (Sandy et al. 2001). Increased versican expression is often observed in tumor growth in tissues such as breast, brain, ovary, gastrointestinal tract, prostate, and melanoma, Sarcoma, and mesothelioma. In addition, versican contributes to the development of a number of pathologic processes including atherosclerotic vascular diseases, tendon remodeling, hair follicle cycling, CNS injury, and neurite outgrowth.

37.8 Diseases of Aggrecan Insufficiency

37.8.1 Aggrecanase-Mediated Cartilage Degradation

The linker domain between the N-terminal globular domains, called the interglobular domain, is highly sensitive to proteolysis. Such degradation has been associated with the development of arthritis. Proteases capable of degrading aggrecans are called aggrecanases, and they are members of the ADAM (A Disintegrin And Metalloprotease) protein family. Aggrecanases and matrix metalloproteinases (MMPs) are associated with aggrecan proteolysis (East et al. 2007). The aggrecanases are of particular interest because of their selectivity for aggrecan. Five aggrecanase cleavage sites have been described in aggrecan (Tortorella et al. 2000), with one residing in the IGD domain and four in the CS2 domain. Increasing evidence is accumulating for the importance of the aggrecanases ADAMTS-4 and ADAMTS-5 in cartilage degradation in arthritis. A number of laboratories have provided insight into the regulation of the expression and activity of these proteins and the molecular basis of their role in aggrecan catabolism. Recombinant ADAMTS-4 and ADAMTS-5 cleave aggrecan at five distinct sites along the core protein and aggrecan fragments generated by cleavage at all of these sites have been identified in cartilage explants undergoing matrix degradation. This proteolytic activity of the aggrecanases can be modulated by several means, including altered expression, activation by proteolytic cleavage at a furin-sensitive site, binding to the aggrecan substrate through the C-terminal thrombospondin motif, activation through post-translational processing of a portion of the C-terminus and inhibition of activity by the endogenous inhibitor TIMP-3. ADAMTS-4 and ADAMTS-5 activity is detected in joint capsule and synovium in addition to cartilage, and may be upregulated in arthritic synovium at either the message level or through post-translational processing. Additional substrates include the CSPG brevican and versican. Advances are occurring in the development of selective aggrecanase inhibitors designed to serve as therapeutics for the treatment of arthritis (Arner 2002)

Site of Catabolism of Aggrecan: Characterization of aggrecan core protein peptides present in the matrix of adult human articular cartilage showed that at least 11 aggrecan core proteins were present with M_r between 300,000 and 43,000. All these core proteins were found to

have the same N-terminal sequences as that observed in human aggrecan. Articular cartilage in explant culture in medium containing 10^{-6} M retinoic acid showed a 3.5-fold increase in the loss of aggrecan into the medium compared to tissue maintained in absence of retinoic acid. Analysis showed the presence of N-terminal sequence ARGS-, which starts at residue 393 of the human aggrecan core protein, located within the interglobular region between the G1 and G2 domains and is the site of aggrecan catabolism by the putative protease aggrecanase. The G1 regions may accumulate in the cartilage matrix for many years (Ilic et al. 1995; Maroudas et al. 1998). The structure of aggrecan fragments in human synovial fluid suggests the involvement of a proteinase in osteoarthritis which cleaves the Glu^{373}-Ala^{374} bond of the interglobular domain (Sandy et al. 1992; Fosang et al. 1995).

Induction of Arthritis in BALB/c Mice by Cartilage Link Protein: Both type II collagen and the proteoglycan aggrecan are capable of inducing an erosive inflammatory polyarthritis in mice. The link protein (LP) from bovine cartilage can produce a persistent, erosive, inflammatory polyarthritis when injected repeatedly intraperitoneally into BALB/c mice. Another cartilage protein, LP, like type II collagen and the proteoglycan aggrecan, is capable of inducing an erosive inflammatory arthritis in mice and the immunity to LP involves recognition of both T- and B-cell epitopes. This immunity may be of importance in the pathogenesis of inflammatory joint diseases, such as juvenile rheumatoid arthritis, in which cellular immunity to LP has been demonstrated (Zhang et al. 1998).

37.8.2 A Mutation in Aggrecan Gene Causes Spondyloepiphyseal Dysplasia

Spondyloepiphyseal dysplasia (SED) encompasses a heterogeneous group of disorders characterized by shortening of the trunk and limbs. Mutations in the aggrecan gene leading to chondrodyplasias have been described in the human, mouse and chicken. The autosomal dominant SED type Kimberley (SEDK) is associated with premature degenerative arthropathy and has been mapped in a multigenerational family to a novel locus on 15q26.1. This locus contains the gene AGC1, which encodes aggrecan, the core protein of proteoglycan of cartilage. In the human, A single-base-pair insertion, within the variable repeat region of exon 12 affected individuals from the family with SEDK, that introduces a frameshift of 212 amino acids, including 22 cysteine residues, followed by a premature stop codon and results in a form of spondyloepiphyseal dysplasia (Gleghorn et al. 2005). Roughley et al. (2006) suggested the involvement of aggrecan polymorphism in degeneration of human intervertebral disc and articular cartilage.

In the mouse, a 7 bp deletion in exon 5 results in a premature termination codon arising in exon 6 (Watanabe et al. 1994, 1998). In the chicken, a premature stop codon is present within exon 10 encoding the CS-attachment region (Li et al. 1993), resulting in decreased message accumulation and under-production of a truncated aggrecan. It is likely that the absence of a G3 region impairs secretion of the mutant aggrecan molecules. In the human, chondrodystrophic phenotypes have also been associated with the under-sulphation of aggrecan due to gene defects in a sulfate transporter (Superti-Furga et al. 1996). These disorders illustrate the importance of aggrecan content and charge in embryonic cartilage development and growth.

37.9 Expression of Proteoglycans in Carcinogenesis

Glycosaminoglycans (GAGs) in PG forms or as free GAGs are implicated in the growth and progression of malignant tumors. Human gastric carcinoma (HGC) contained about twofold increased amounts of GAGs in comparison to normal gastric mucosa (HNG). Specifically, increase in chondroitin sulfate (CS) and hyaluronan (HA) contents was associated with HGC. In HGC, the amounts of versican and decorin were significantly increased. Analysis of Δ-disaccharide of versican and decorin from HGC showed an increase of 6-sulfated Δ-disaccharides (Δ di-6 S) and non-sulfated Δ-disaccharides (Δ di-0 S) with a parallel decrease of 4-sulfated Delta-disaccharides (Δdi-4 S) as compared to HNG. In addition, the accumulation of core proteins of versican and decorin in HGC was also associated with many post-translational modifications, referring to the number, size, degree and patterns of sulphation and epimerization of CS/DS chains (Theocharis et al. 2003b). Prostate tumor cells induce host stromal cells to secrete increased versican levels via a paracrine mechanism mediated by transforming growth factor beta1 (Sakko et al. 2001).

Although, versican expression is not an independent prognostic factor in pharyngeal squamous cell carcinoma (Pukkila et al. 2004), Skandalis et al. (2006) provided direct evidence for a significant and stage-related accumulation of versican and decorin in the tumor-associated stroma of laryngeal squamous cell carcinoma (LSCC) in comparison to normal larynx. The accumulated versican and decorin were markedly modified on both protein core and glycosaminoglycan (GAG) levels. Versican was found to undergo stage-related structural modifications. The modified chemical structure of both PGs could be associated with the degree of aggressiveness of laryngeal squamous cell carcinomas (Skandalis et al. 2006).

37.10 Regulation of Proteoglycans

Dex stimulates cell-associated and soluble chondroitin 4-sulfate proteoglycans. During differentiation into adipocytes, there was a 1.68-fold increase in the ^{35} S in medium of 3 T3-L1 preadipocytes, whereas cell-associated proteoglycan showed no increase. Analyses indicated that all ^{35} S label was recovered as two major species of chondroitin 4-sulfate proteoglycans (CSPG-I and CSPG-II) and 7% as heparan sulfate proteoglycan. Cell differentiation was associated with a specific increase in CSPG-I (Calvo et al. 1991). Platelet-derived growth factor (PDGF) and TGF-β1 increase ^{35}Sulfate incorporation into PG by monkey arterial smooth muscle cells but have opposite effects on cell proliferation. Although both of these growth factors increase the net synthesis of a large versican like CSPG, they differ in their effects on the structure of the glycosaminoglycan chains (Schönherr et al. 1991). TGF-β1 markedly enhanced the expression of biglycan and versican mRNAs and the enhancement of biglycan expression was coordinate with elevated type I procollagen gene expression in the same cultures. In contrast, the expression of decorin mRNA was markedly inhibited by TGF-β1. Results indicate differential regulation of PG gene expression in fibroblasts by TGF-β1 (Kähäri et al. 1991; Barry et al. 2001). Changes have been observed in synthesis of proteoglycans by vascular SMCs in response to controlled mechanical strains. Following strain mRNA and protein for versican, biglycan, and perlecan increased. Thus, mechanical deformation increases specific vascular SMC proteoglycan synthesis and aggregation, indicating a highly coordinated extracellular matrix response to biomechanical stimulation (Lee et al. 2001).

37.11 CD44: A Major Hyaluronan Receptor

37.11.1 CD44: A Hyaluronan Receptor

The CD44 is a transmembrane surface glycoprotein widely distributed in both epithelial and nonepithelial normal tissues (Ponta et al. 2003). This protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis. CD44 functions as a major hyaluronan receptor, which mediates cell adhesion and migration in a variety of physiological processes (Sillanpää et al. 2003). It can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). A specialized sialofucosylated glycoform of CD44 called HCELL is found natively on human hematopoietic stem cells, and is a highly potent E-selectin and L-selectin ligand. CD44 is encoded by a single gene located on human chromosome 11. However, alternative splicing of mRNA produces several larger CD44 isoforms in addition to the standard isoform, CD44s. The extracellular region of CD44 gives rise to multiple CD44 isoforms. Alternative splicing is the basis for the structural and functional diversity of this protein, and may be related to tumor metastasis All isoforms contain an amino-terminal domain, which is homologous to cartilage link proteins. The cartilage link protein-like domain of CD44 is important for hyaluronan binding. Splice variants of CD44 on colon cancer cells display the HCELL glycoform, which mediates binding to vascular E-selectin under hemodynamic flow conditions, a critical step in colon cancer metastasis.

CD44 is a lectin cell adhesion molecule that is also expressed in keratinocytes. Amino sugars such as NAG may competitively bind to CD44, modulating keratinocyte cellular adhesion. These amino sugars may modulate keratinocyte cellular adhesion and differentiation, leading to the normalization of stratum corneum exfoliation (Mammone et al. 2009). CD44, along with CD25, is used to track early T cell development in the thymus. Its expression is an indicative marker for effector-memory T-cells. It is tracked with CFSE chemical tagging.

37.11.2 Hyaluronan Binding Sites in CD44

CD44 is a receptor for hyaluronic acid and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). CD44 function is controlled by its posttranslational modifications. CD44 binds hyaluronan ligand via a lectin-like fold termed the Link module, but only after appropriate functional activation. Among twenty-four point mutants, eight residues were identified as critical for binding or to support the interaction. These residues form a coherent surface, the location of which approximately corresponds to the carbohydrate binding sites in MBP and E-selectin (CD62E) (Bajorath et al. 1998). The interaction with hyaluronan is dominated by shape and hydrogen-bonding complementarity. Banerji et al. (2007) identified two conformational forms of the murine Cd44 that differ in orientation of a crucial hyaluronan-binding residue (Arg^{45}, equivalent to Arg^{41} in human CD44). NMR studies indicated that the conformational transition can be induced by hyaluronan binding, the possible mechanisms for regulation of Cd44.

Although full activity of CD44 requires binding to ERM (ezrin/radixin/moesin) proteins, the CD44 cytoplasmic region, consisting of 72 amino acid residues, lacks the Motif-1 consensus sequence for ERM binding found in ICAM-2 and other adhesion molecules of the Ig superfamily. CD44 exists as an extended monomeric form of the cytoplasmic peptide in solution. The crystal structure of the radixin FERM domain complexed with a CD44 cytoplasmic peptide reveals that the KKKLVIN sequence of the peptide

forms a β strand followed by a short loop structure that binds subdomain C of the FERM domain. Like Motif-1 binding, the CD44 β strand binds the shallow groove between strand β5C and helix α1C and augments the β sheet β5C- β7C from subdomain C. Two hydrophobic CD44 residues, Leu and Ile, are docked into a hydrophobic pocket with the formation of hydrogen bonds between Asn of the CD44 short loop and loop β4C- β5C from subdomain C. This binding mode resembles that of neutral endopeptidase 24.11 (NEP) rather than ICAM-2. These results reveal a characteristic versatility of peptide recognition by the FERM domains from ERM proteins, a possible mechanism by which the CD44 tail is released from the cytoskeleton for nuclear translocation by regulated intra-membrane proteolysis, and provide a structural basis for Smad1 interactions with activated CD44 bound to ERM protein (Mori et al. 2008).

37.11.3 CD44 in Cancer

CD44 is involved in cell proliferation, cell differentiation, cell migration, angiogenesis, and as growth factors to the corresponding receptors, and docking of proteases at the cell membrane, as well as in signaling for cell survival. All these biological properties are essential for physiological activities of normal cells, but they are also associated with the pathologic activities of cancer cells. CD44 is remarkable for its ability to generate alternatively spliced forms, many of which differ in their activities. This remarkable flexibility has led to speculation that CD44, via its changing nature, plays a role in some of the methods that tumor cells use to progress successfully through growth and metastasis. Experiments in animals have shown that targeting of CD44 by antibodies, antisense, and CD44-soluble proteins markedly reduces the malignant activities of various neoplasms, stressing the therapeutic potential of anti-CD44 agents (Naor et al. 2002). Variations in CD44 are reported as cell surface markers for some breast and prostate cancer stem cells (Li et al. 2007) and has been seen as an indicator of increased survival time in epithelial ovarian cancer patients (Martin et al. 2004; Sillanpää et al. 2003; Yasuda et al. 2003). The role of CD44 in prostate cancer development and progression is controversial with studies showing both tumor-promoting and tumor-inhibiting effects. However, Patrawala et al. (2006) suggest that the $CD44^+$ prostate cancer cell population is enriched in tumorigenic and metastatic progenitor cells. $CD44^+$ cells displayed clustered growth within colorectal cancer. Knockdown of CD44, strongly prevented clonal formation and inhibited tumorigenicity in xenograft model. Results indicated that CD44 is a robust marker and is of functional importance for colorectal CSC for cancer initiation (Du et al. 2008).

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Proteoglycans of the Central Nervous System 38

G.S. Gupta

38.1 Proteoglycans in Central Nervous System

The extracellular matrix (ECM) is a complex molecular framework that provides physical support to cells and tissues, while also providing signals for cell growth, migration, differentiation and survival. Proteoglycans, as part of the extracellular or cell-surface milieu of most tissues and organ systems, play important roles in morphogenesis by modulating cell-matrix or cell-cell interactions, cell adhesiveness, or by binding and presenting growth and differentiation factors. The basic concept, that specialized extracellular matrices rich in hyaluronan (HA), chondroitin sulfate proteoglycans (CS-PG: aggrecan, versican, neurocan, brevican, phosphacan), link proteins and tenascins (TN-R and TN-C) can regulate cellular migration and axonal growth and thus, actively participate in the development and maturation of nervous system, has gained rapidly expanding experimental support (Zimmermann and Dours-Zimmermann 2008). The distribution of ECM molecules displays area-specific differences along the dorso-ventral axis, delimiting functionally and developmentally distinct areas. In gray matter, laminae I and II lack perineuronal nets (PNN) of extracellular matrix and contain low levels of chondroitin sulfate glycosaminoglycans (CS-GAGs), brevican, and tenascin-R, possibly favoring the maintenance of local neuroplastic properties. Conversely, CS-GAGs, brevican, and phosphacan were abundant, with numerous thick PNNs, in laminae III-VIII and X. Motor neurons (lamina IX), surrounded by PNNs, containd various amounts of CS-GAGs (Vitellaro-Zuccarello et al. 2007).

38.1.1 Large Proteoglycans in Brain

Proteoglycans (PGs) of aggrecan family include extracellular PGs: aggrecan, versican (and avian homologue PG-M), neurocan, brevacan and cell surface specific receptor CD44. They are modular PGs containing combinations of structural motifs such as EGF-like domains, lectin like domains, complement regulatory like domains, immunoglobulin folds, and proteoglycan tandem repeats that are found in other proteins (Fig. 38.1). The protein-hyaluronate aggregates in brain ECM contain versican. The bovine versican splice variant, versican V2 is together with brevican, a major component of the mature brain ECM. Versicans V0 and V1 are only present in relatively small amounts (Schmalfeldt et al. 1998). Several other proteins are related to this family of molecules including link proteins, BEHAV and TSG-6 and contain some of the same highly conserved motifs but lack GAG side chains. Aggrecan and CD44 have been characterized and discussed in general in Chap. 37. In this chapter brain specific PGs, related to aggrecan have been discussed. In adult brain, lecticans are thought to interact with hyaluronan and tenascin-R to form a ternary complex. It is proposed that hyaluronan-lectican-tenascin-R complex constitutes the core assembly of adult brain ECM, which is found mainly in pericellular spaces of neurons as 'perineuronal nets'. Brevican is a member of the aggrecan/versican family of proteoglycans, containing a hyaluronic acid-binding domain in its N-terminus and a lectin-like domain in its C-terminus.

PGs in Developing Brain: A diverse set of proteoglycans (phosphocan, neurocan, versican, aggrecan, and neuron-glia antigen 2 (NG2) proteoglycan) is expressed in developing and adult brain. In developing rat brain from embryonic day 14 (E14), the concentration of aggrecan increased steadily up to 5 months of age, when it reached a level that was 18-fold higher than at E14, where as spliced versican isoforms were present at a relatively low level during the late embryonic and early postnatal period, decreased by approximately 50% between 1 and 2 weeks postnatal, and then increased steadily to a maximum at 100 days (Milev et al. 1998a). In contrast, versican isoforms containing β domain doubled between E14 and birth, after which

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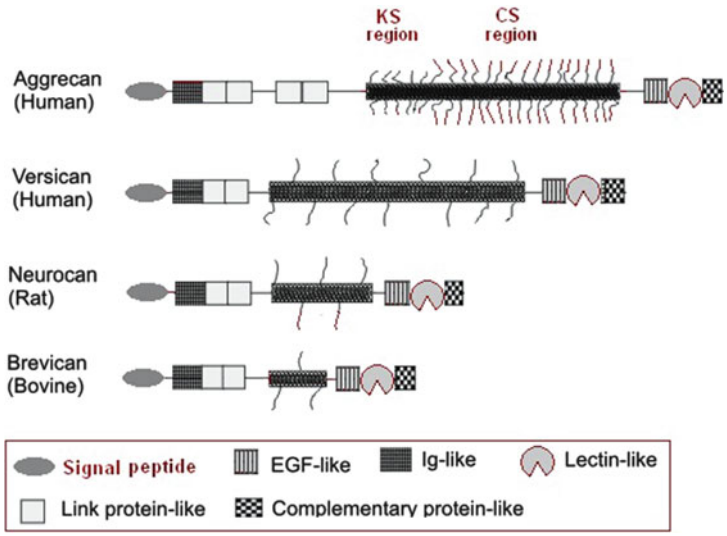

Fig. 38.1 The modular structures of the proteoglycans aggrecan, versican 2, neurocan, and brevican. Aggrecan shows both keratin sulfate (KS) and chondritin sulfate (CS) regions where as versican, neurocan and brevican have CS. Boxes represent putative functional domains shared by all the members of the aggrecan/versican family (Yamada et al. 1994; Fosang and Hardingham 1996)

versican isoforms decreased to reach a low "mature" level that remained unchanged between 2 and 8 months. As in aggrecan, brevican was detected in traces in embryonic brain and increased steadily after birth to reach an adult level that was ~14-fold higher than that in neonatal brain.

Astrocytes as primary site for synthesis of PGs: The CS-PG is primarily synthesized by astrocytes and is believed to influence astroglial motility during development and under certain pathological conditions. The CS-PGs expressed by reactive astrocytes may contribute to the axon growth-inhibitory environment of the injured central nervous system (CNS). In the glial scar, after cortical injury, neurocan and phosphacan can be localized to reactive astrocytes 30 days after CNS injury, whereas brevican and versican are not expressed in the chronic glial scar. Because these CS-PGs are capable of inhibiting neurite outgrowth in vitro, reports suggest that phosphacan and neurocan in areas of reactive gliosis may contribute to axonal regenerative failure after CNS injury. Reports also indicate that brevican is upregulated in areas of brain damage as well as in areas denervated by a lesion and involved in the synaptic reorganization of denervated brain areas (McKeon et al. 1999; Thon et al. 2000). Oligodendrocyte (OL) and their progenitors secrete brevican prior to and during active developmental myelination whereas NG2 is a transmembrane CS-PG produced by early OL progenitors (Table 38.1).

Perineuronal Nets (PNN): Perineuronal nets (PNN) are specialized ECM structures enwrapping CNS neurons, which are important regulators for neuronal and synaptic functions. The decrease in plasticity in CNS during postnatal development is accompanied by appearance of PNNs around the cell body and dendrites of many classes of neuron. These structures are composed of ECM molecules, such as CS-PGs, HA, tenascin-R, and link proteins. In rat deep cerebellar nuclei, only large excitatory neurons were surrounded by nets, which contained the CS-PGs aggrecan, neurocan, brevican, versican, and phosphacan, along with tenascin-R and HA. In the cerebellar cortex, Golgi neurons possessed PNNs and also synthesized HA synthases (HASs), cartilage link protein, and BRAL2 mRNAs. Carulli et al. (2007) proposed that HASs, which can retain HA on the cell surface, may act as a link between PNNs and neurons. Thus, HAS and link proteins might be key molecules for PNN formation and stability. During postnatal development the expression of link protein and aggrecan mRNA is up-regulated at the time of PNN formation, and these molecules may therefore trigger their formation (Galtrey et al. 2008).

38.1.2 Ligands of Lecticans

Interaction Between Tenascin-R and Phosphacan and Neurocan: CS PGs in perineuronal nets include neurocan and phosphacan. Morphological alterations were noticed in Wisteria floribunda labelled nets around cortical interneurones both in tenascin-R knockout and tenascin-R/parvalbumin double knockout mice. This alteration reflects the loss of phosphacan and neurocan from cortical nets in mice deficient in tenascin-R. No effect on the membrane related cytoskeleton, as revealed by ankyrin(R), was observed in any of the mice. These results on mice lacking tenascin-R substantiate in vitro interactions between tenascin-R and phosphacan and neurocan (Haunsø et al. 2000) (Table 38.2).

Table 38.1 Structure and expression of lecticans (Hyalectans) in central nervous system (CNS)[a]

Name	Type	Core protein size (kD) calculated/SDS-PAGE		GAG type	number	Cell origin in CNS	CNS specificity	Sources out side CNS
Brevican	ECM-PG	97	145	CS	0–5	Glial cell/neurons	yes	
GPI-Brevican	GPI-PG	64	140/125	CS	0–5	Glial cells		
Neurocans	ECM-PG	133	245	CS	3	Neurons/Astrocytes	yes	
Versican V0	ECM-PG	370	~550	CS	17–23	Astrocytes	No	Blood vessels; mesenchymes, etc.
Versican V1	ECM-PG	262	500	CS	12–15	Astrocytes	No	Blood vessels; mesenchymes, etc.
Versican V2	ECM-PG	180	400	CS	5–8	Oligodendrocytes	Yes	
Aggrecan	ECM-PG	234	370	CS	?	Neurons	No	Cartilage, notochord

[a]Bbandtlow and Zzimmermann (2000)

Table 38.2 Ligands of proteoglycans in central nervous system[a]

Name	Growth factors	ECM molecules	Cell surface molecules
Brevican		Hyaluronan; tenacin-R	Sulfatides (glycolipids)
Neurocan	FGF2	Hyaluronan; tenacin-C; Tenacin-R	N-CAM; Ng-CAM/L1
			Tag-1/axonin, sulfatides
Versicans		Hyaluronan; tenacin-R; Fibronectin	Sulfatides
Aggrecan		Hyaluronan	Sulfatides

FGF fibroblast growth factor, *NCAM* neural cell adhesion molecule, *L1* cell adhesion molecule highly enriched on axon, *Ng-CAM* neuron-glia cell adhesion molecule (a chick homolog of L1)
[a]Bbandtlow and Zzimmermann (2000)

Sulfoglucuronylglycolipids: Two classes of sulfated glycolipids: sulfatides and HNK-1-reactive sulfoglucuronyl-glycolipids (SGGLs) act as cell surface receptors for brevican. The lectin domain of brevican binds sulfatides and SGGLs in a calcium-dependent manner as expected of a CTLD. The interaction between the lectin domains of lecticans and sulfated glycolipids suggests that lecticans in ECMs serve as substrate for adhesion and migration of cells expressing these glycolipids in vivo (Miura et al. 1999). The Ig chimera of the brevican lectin domain binds to the surface of SGGL-expressing rat hippocampal neurons. The substrate of the brevican chimera promotes adhesion and neurite outgrowth of hippocampal neurons. The full-length brevican also promotes neuronal cell adhesion and neurite outgrowth. Miura et al. (2001) suggest that the interaction between the lectin domain of brevican and cell surface SGGLs acts as a cell recognition system that promotes neuronal adhesion and neurite outgrowth.

Tenascin-C and Tenascin-R as Ligands of Brevican: Tenascin-C (TN-C) is a ligand for brain lecticans and binding site maps on the tenascin molecule to fibronectin type III repeats, which corresponds to the proteoglycan lectin-binding site on tenascin-R (TN-R). In the G3 domain, the C-type lectin is flanked by EGF repeats and a complement regulatory protein-like motif. In aggrecan, these are subject to alternative splicing. The mRNA for this splice variant was shown to be expressed in human chondrocytes. The alternative splicing in the aggrecan G3 domain may be a mechanism for modulating interactions and ECM assembly (Day et al. 2004).

The TN-R and the proteoglycans of the lectican family show an overlapping distribution in developing brain. In TN-R-deficient animals, the perineuronal nets tend to show a granular component within their lattice-like structure at early stages of development and the staining intensity for brevican was reduced in perineuronal nets, extremely low for hyaluronan and neurocan, and virtually no for phosphacan. It was indicated that the lack of TN-R initially and continuously disturbs the molecular scaffolding of extracellular matrix components in perineuronal nets (Brückner et al. 2000). Differential patterns of co-expression of proteoglycans, hyaluronan and TN-R in the individual regions and laminae of the hippocampal formation and the inhomogeneous composition of these components suggest that the extracellular matrix is specifically adapted to the functional domains of intrahippocampal connections and afferent fibre systems (Brückner et al. 2003). Zacharias and Rauch (2006) suggest that a complex network of protein-protein interactions within the brain extracellular matrix, as shown for TN-R and lecticans, is important for the fine-regulation of developmental processes such as microprocess formation along the neurite and neurite outgrowth.

Though the C-type lectin domain of brevican also binds TN-R, this interaction is mediated by a protein-protein interaction through the fibronectin type III domains 3–5 of TN-R, independent of any carbohydrate or sulfated amino acid. Interestingly, the lectin domains of versican and other lecticans also bind the same domain of TN-R by protein-protein interactions. In earlier study, the C-type lectin domain of versican was shown to bind TN-R and the interaction was mediated by a carbohydrate-protein interaction. Surface plasmon resonance analysis revealed that brevican lectin has at least a tenfold higher affinity than the other

lectican lectins. Reports suggest that C-type lectin domain can interact with fibronectin type III domains through protein-protein interactions, and that brevican is a physiological TN-R ligand in the adult brain (Aspberg et al. 1997; Hagihara et al. 1999; Lundell et al. 2004).

38.2 Neurocan

Neurocan is a multidomain hyaluronan-binding CS-PG with a 136-kDa core protein that is synthesized by neurons and binds to hyaluronic acid, whereas the astroglial proteoglycan phosphacan is an mRNA splice variant representing the entire extracellular portion of a receptor-type protein tyrosine phosphatase. The concentration of neurocan in brain increases during late embryonic development followed by a steep decline during early postnatal period together with hyaluronan. Neurocan also undergoes extensive proteolytic processing during the course of brain development (Miller et al. 1995). Mouse brain neurocan, but not brevican, is retained on a heparin affinity matrix (Feng et al. 2000). Due to its inhibition of neuronal adhesion and outgrowth in vitro and its expression pattern in vivo it was suggested to play an important role in axon guidance and neurite growth. Neurocan of brain is developmentally regulated with respect to its concentration, carbohydrate composition, sulfation, and localization. Although the possible neuroprotective involvement of CS-PG remains to be investigated, Mizuno et al. (2008) suggested that both the reactive astrocytes and the differential accumulation of CS-PGs may create a non-permissive microenvironment for neural regeneration in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS).

38.2.1 Cellular Sites of Synthesis

Neurocan mRNA was evident in neurons, including cerebellar granule cells and Purkinje cells, and in neurons of the hippocampal formation and cerebellar nuclei. The distribution of neurocan message is more wide spread, extending to the cortex, hippocampal formation, caudate putamen, and basal telencephalic neuroepithelium. Neurocan mRNA is present in both the ependymal and mantle layers of the spinal cord but not in the roof plate. The presence of neurocan mRNA in areas where the proteoglycan is not expressed suggests that the short open reading frame in the 5′-leader of neurocan may function as a cis-acting regulatory signal for the modulation of neurocan expression in the developing CNS (Engel et al. 1996). Ihara's epileptic rat (IER) is an animal model of temporal lobe epilepsy with mycrodysgenesis that exhibits abnormal migration of hippocampal neurons and recurrent spontaneous seizures. It was suggested that the insufficient expression of neurocan may affect the development of neuronal organization in the hippocampus, and that the remodeling of ECM in the dentate gyrus may contribute to the mossy fiber sprouting into the inner molecular layer (Kurazono et al. 2001).

38.2.2 Characterization

The composite sequence of overlapping cDNA clones is 5.2 kb long, including 1.3 kb of 3′-untranslated sequence and 76 bp of 5′-untranslated sequence. An ORF of 1257 amino acids encodes a protein with a molecular mass of 136 kDa containing ten peptide sequences present in the adult and/or early postnatal brain proteoglycans. The deduced amino acid sequence revealed a 22-amino acid signal peptide followed by an immunoglobulin domain, tandem repeats characteristic of the hyaluronic acid-binding region of aggregating proteoglycans, and an RGDS sequence. The C-terminal portion (amino acids 951–1215) has approximately 60% identity to regions in the C termini of the fibroblast and cartilage proteoglycans, versican and aggrecan, including two EGF-like domains, a lectin-like domain, and a complement regulatory protein-like sequence. The proteoglycan contains six potential N-glycosylation sites and 25 potential threonine O-glycosylation sites. In the adult form of the proteoglycan (which represents the C-terminal half of neurocan) a single 32-kDa chondroitin 4-sulfate chain is linked at serine-944, whereas three additional potential chondroitin sulfate attachment sites are present in the larger proteoglycan species. The 1D1 (one of the CS-PG) proteoglycan of adult brain, containing a 68-kDa core protein, is generated by a developmentally regulated in vivo proteolytic processing of 136-kDa species which is predominant in early postnatal brain (Rauch et al. 1992).

Neurocan gene of 25 kb contains the coding sequence for the mRNA on 15 exons. The exon-intron structure reflected the structural organization of neurocan, which is a multidomain protein belonging to proteoglycan family. All introns between conserved modular domains are phase I introns. Primer extension experiments indicate a transcriptional start point 28 bases downstream of a consensus TATA sequence. Analysis of 1 kb of 5′ flanking sequence revealed in addition to AP1, AP2, and SP1 consensus binding sites multiple E-box elements and a glucocorticoid responsive element. Single-strand conformation polymorphism mapped neurocan to chromosome 8 between the microsatellite markers D8Mit29 and D8Mit78. The multidomain structure and the preferential expression of neurocan in the brain suggest its involvement in neurological disorders (Rauch et al. 1995).

The coding sequence of human neurocan mRNA known as CS-PG3 was described by Prange et al. (1998). Sequence

homology searches indicated close homology to the mouse and rat neurocan. Neurocan mRNA is a brain-specific transcript of approx. 7.5 kb. A longer cDNA clone, GT-5 was mapped to the physical map of chromosome 19. Full coding sequence of the mRNA indicates a 3963 bp ORF corresponding to a 1321 amino acid protein, similar to protein in mouse and rat. The amino acid sequence of human neurocan shows 63% identity with both the mouse and rat sequences. The genomic structure of the gene spans approx. 41 kb, and is transcribed in the telomere to centromere orientation.

Two Proteoglycan Fragments of Neurocan: In adult rat brain, neurocan is completely cleaved into some proteoglycan fragments including the C-terminal half known as neurocan-C and an N-terminal fragment with a 130 kDa core glycoprotein (neurocan-130). Results suggested that not only the C-terminal half (CSPG-150) but also the N-terminal half (CSPG-130) of CSPG-220 existed in a CSPG form in rat brain. The contents of CSPG-130 and CSPG-150 in the rat brain reached maximum levels around the time of birth. Both CSPG-130 and 150 were observed when CSPG-220 was hardly detectable in extracts from the adult rat brain (Matsui et al. 1994). The two neurocan-derived CS-PGs are distributed in adult rat cerebrum. Neurocan-130 exhibits pericellular localization around a subset of neurons in addition to diffuse distribution in the neuropil where as, neurocan-C was distributed only diffusely in the neuropil. The neurocan-130 was mainly localized in the cytoplasm of glial cell processes, the so-called glial perineuronal net, encompassing the cell bodies of certain neurons. The presence of neurocan-130 in a limited number of glial cells may reflect some functional heterogeneity of the glia (Matsui et al. 1998). Under electron microscopy neurocan shows two globular domains interconnected by an extended flexible filament of 60–90 nm. Electron microscopic shapes indicated a mucin-like character for the central neurocan region (Retzler et al. 1996b). Neurocan has been shown to bind to the neural cell adhesion molecule N-CAM and to inhibit its homophilic interaction (Retzler et al. 1996a).

The full-length neurocan is detected in juvenile brains but not in adult brains. However, full-length neurocan transiently appeared in adult rat hippocampus when it was lesioned by kainate-induced seizures. The full-length neurocan was detectable also in the adult brain when it was exposed to mechanical incision or epileptic stimulation. The full-length neurocan transiently appeared in the peri-ischemic region of transient middle cerebral artery occlusion (tMCAO) in adult rat. The induction of neurocan expression by reactive astrocytes suggested that juvenile-type neurocan plays some roles in brain repair (Matsui et al. 2002; Deguchi et al. 2005).

Neurocan-Like and 6B4 Proteoglycan: The neurocan-like and 6B4 proteoglycan-like reactivities in rat embryo were investigated from E10.5–15.5. It is likely that neurocan serves as a barrier molecule to regulate the direction of axonal growth from the dorsal root ganglia. By contrast, results suggested possible functions of 6B4 proteoglycan in rat embryo (Katoh-Semba et al. 1998). Within first week postnatally, neurocan expression is strongly downregulated. But it is re-expressed in areas of axonal growth (sprouting) after brain injury as found in the denervated fascia dentata of the rat after entorhinal cortex lesion. In the denervated zone, neurocan-positive astrocytes were confirmed by electron microscopy. In contrast to the situation during development, astrocytes, but not neurons, express neurocan and enrich the ECM with this molecule. Similar to the situation during development it is suggested that neurocan acts to maintain the boundaries of the denervated fascia dentata after entorhinal cortex lesion (Haas et al. 1999).

38.2.3 Ligand Interactions

Neurocan can bind to various structural extracellular matrix components, such as hyaluronan, heparin, tenascin-C and tenascin-R, and the growth and mobility factors FGF-2, HB-GAM, and amphoterin. Neurocan can also interact with several cell surface molecules, such as N-CAM, L1/Ng-CAM, TAG-1/axonin-1, and an N-cadherin-binding N-acetyl-galactosamine-phosphoryl-transferase. In vitro studies have shown that neurocan is able to modulate the cell-binding and neurite outgrowth promoting activites of these molecules. Binding of phosphacan and neurocan to intact tenascin-C, and of phosphacan to the fibrinogen globe, is significantly increased in presence of calcium. These interactions seem to be mediated by the proteoglycan core proteins rather than through the glycosaminoglycan chains (Milev et al. 1997). A divalent cation-dependent interaction between the COOH-terminal domain of neurocan and those fibronectin type III repeats is substantially involved in the binding of neurocan to tenascin-C (Rauch et al. 1997). Neurocan like phosphacan is also high affinity ligand of amphoterin and HB-GAM (K_D = 0.3–8 nM), two heparin-binding proteins that are developmentally regulated in brain and functionally involved in neurite outgrowth (Milev et al. 1998a). Analysis of the molecular structures and substructures involved in homophilic and heterophilic interactions of these molecules and complementary loss-of-function mutations might shed some light on the roles played by neurocan and interacting molecules in the fine tuning of the nervous system (Rauch et al. 2001; Rauch 2004). Treatment with phosphacan and neurocan on poly-L-lysine (PL) significantly impaired both neuronal attachment and neurite extension, and both phosphacan and neurocan are repulsive substrata for adhesion and neurite regeneration of adult rat

dorsal root ganglion (DRG) neurons in vitro (Sango et al. 2003). Li et al. (2005) examined the formation of thalamocortical pathway in the cerebral neocortex of normal and reeler mutant mice. Results support the hypothesis that a heterophilic molecular interaction between L1 and neurocan is involved in determining the thalamocortical pathway within the neocortical anlage.

38.3 Brevican

Brevican has the smallest core protein among aggrecan/versican/neurocan family and is one of the most abundant CS-PG in the adult brain. Expression of brevican is highly specific in the brain and increases as the brain develops. Brevican may play a role in maintaining the extracellular environment of mature brain as a major constituent of the adult brain ECM. Because of smallest core protein, this novel proteoglycan has been named "brevican" (from the Latin word *brevis* meaning "short") (Fig. 38.1).

38.3.1 Characterization

Brevican carries chondroitin sulfate chains and, like other members of the family, contains a hyaluronic acid-binding domain in its N-terminal region, an EGF-like repeat, a lectin-like and a complement regulatory protein-like domain in its C-terminal region (see Fig. 37.4). In contrast, the central region of brevican is much shorter than that of aggrecan, versican, or neurocan, and shows little homology with these proteoglycans. Brevican core protein exists as a 145 kDa full-length form and a 80 kDa N terminally truncated form. A significant amount of brevican devoid of any glycosaminoglycan chains is present in the brain, indicating that brevican is a "part-time" proteoglycan (Yamada et al. 1994).

Two sets of overlapping Brevican cDNA clones from rat brain have been characterized that differ in their 3′-terminal regions. Each set was found to hybridize with two brain-specific transcripts of 3.3 and 3.6 kb. The 3.6-kb transcript encodes a polypeptide that exhibits 82% sequence identity with bovine brevican and is thought to be the rat ortholog of brevican. The polypeptide deduced from ORF of 3.3-kb transcript is truncated just carboxyl-terminal of the central domain of brevican and instead contains a putative glypiation signal. Both soluble and membrane-bound brevican isoforms exist. Isoforms of brevican are indeed glycosylphosphatidylinositol-anchored to the plasma membrane. Brevican is widely distributed in the brain and is localized extracellularly. During postnatal development, amounts of both soluble and phosphatidylinositol-specific phospholipase C-sensitive isoforms increase, suggesting a role for brevican in the terminally differentiating and the adult nervous system (Seidenbecher et al. 1995).

38.3.2 Murine Brevican Gene

The mouse genomic sequence of 13,700 nt revealed that the murine gene has a size of approximately 13 kb and contains the sequence of mRNA for the secreted brevican isoform on 14 exons. The exon-intron structure reflected the structural organization of the multidomain protein brevican. No consensus TATA sequence was found upstream of the first exon, and RNase protection experiments revealed multiple transcriptional start sites for the brevican gene. The first part of the sequence of intron 8 corresponded to an alternative brevican cDNA, coding for a GPI-linked isoform. Single strand conformation polymorphism analysis mapped the brevican gene (*Bcan*) to chromosome 3 between the microsatellite markers D3Mit22 and D3Mit11 (Rauch et al. 1997).

Site of Expression: In rat cerebellar cortex, brevican occurs predominantly in the protoplasmic islet in the internal granular layer after the third postnatal week. Brevican is localized in close association with the surface of astrocytes that form neuroglial sheaths of cerebellar glomeruli where incoming mossy fibers interact with dendrites and axons from resident neurons. Studies indicate that brevican is synthesized by astrocytes and retained on their surface by an interaction involving its core protein. Purified brevican inhibits neurite outgrowth from cerebellar granule neurons in vitro, an activity that requires chondroitin sulfate chains. The brevican present on the surface of neuroglial sheaths may be controlling the infiltration of axons and dendrites into maturing glomeruli (Yamada et al. 1997).

Ogawa et al. (2001) demonstrated that brevican is expressed by both oligodendrocytes and white matter astrocytes in the fimbria, but the expression of brevican in these two glial cell types is differently regulated during development. At P14, brevican immunoreactivity was observed throughout the fimbria, with particularly strong immunoreactivity in the developing interfascicular glial rows. In contrast, the expression in astrocytes started around P21 as oligodendrocytes began to down-regulate the expression. In the adult fimbria, brevican expression was restricted to astrocytes. The secreted brevican transcript was detectable in the pituitary of both male and female adult rats. In posterior lobe of pituitary, pituicytes were heavily labelled. In anterior and intermediate lobes of pituitary, signals for brevican transcripts were observed in cells of various sizes (Dong et al. 2004).

Brevican is expressed by neuronal and glial cells, and as a component of the perineuronal nets it decorates the surface of large neuronal somata and primary dendrites. One

brevican isoform harbors a glycosylphosphatidylinositol anchor attachment site and is indeed glypiated in stably transfected HEK293 cells as well as in oligodendrocyte precursor Oli-neu cells. The major isoform is secreted into the extracellular space, although a significant amount appears to be tightly attached to the cell membrane. Brevican is most prominent in the microsomal, light membrane and synaptosomal fractions of rat brain membrane preparations. The association with the particulate fraction is in part sensitive to chondroitinase ABC and phosphatidylinositol-specific phospholipase C treatment. Furthermore, brevican staining on the surface of hippocampal neurons in culture is diminished after hyaluronidase or chondroitinase ABC treatment. Results provide a mechanism by which perineuronal nets are anchored on neuronal surfaces (Seidenbecher et al. 2002). Brevican, TN-R and phosphacan are present at the nodes of Ranvier on myelinated axons with a particularly large diameter in CNS of rat brain.

Transcripts for Secreted and GPI-anchored Brevican Are Differentially Distributed in Rat Brain: In contrast to the other family members, brevican occurrs both as soluble isoforms secreted into the extracellular space and membrane-bound isoforms which are anchored to the cell surface via a glycosylphosphatidylinositol (GPI) moiety. Expression of both variants, which are encoded by two differentially processed transcripts from the same gene, is confined to the nervous system. Whereas the 3.6-kb transcript encoding secreted brevican displays a widespread distribution in grey matter structures, including cerebellar and cerebral cortex, hippocampus and thalamic nuclei with silver grains accumulating over neuronal cell bodies, the smaller transcript (3.3 kb) encoding GPI-anchored isoforms appears to be largely confined to white matter tracts and diffusely distributed glial cells. During ontogenetic development, both brevican transcripts are generally up-regulated. However, the expression of glypiated brevican is delayed by about 1 week, compared with the expression of the secreted isoform (Seidenbecher et al. 1998).

38.4 Hyaluronan: Proteoglycan Binding Link Protein

Hyaluronan (HA) is a ubiquitous component of extracellular matrices, and in several systems it plays a central role in regulating cellular proliferation and differentiation. Cell or tissue-specific functions of HA are likely to be mediated by cell or tissue-specific HA-binding proteins (Meyer-Puttlitz et al. 1995). The HA plays an important role in tissue reorganization in response to injury. In the mammalian CNS, HA is present throughout development and into adulthood. Link proteins are glycoproteins in cartilage that are involved in the stabilization of aggregates of proteoglycans and hyaluronic acid Link protein (LP), an extracellular matrix protein in cartilage, stabilizes aggregates of aggrecan and hyaluronan, giving cartilage its tensile strength and elasticity. targeted mutations in mice in the gene encoding LP (Crtl1) showed defects in cartilage development and delayed bone formation with short limbs and craniofacial anomalies. Thus LP is important for the formation of proteoglycan aggregates and normal organization of hypertrophic chondrocytes (Watanabe et al. 1999) (Fig. 37.1).

38.4.1 Cartilage link protein 1 and Brain Link Proteins

Spicer et al. (2003) described a vertebrate hyaluronan and proteoglycan binding link protein gene family (HAPLN), consisting of four members including cartilage link protein CRTL1. Human and mouse link proteins share 81–96% amino acid sequence identity. Two of the four link protein genes (HAPLN2 and HAPLN4) are restricted to the brain/CNS, while one HAPLN3 is widely expressed. Genomic structures revealed that all four HAPLN genes are similar in exon-intron organization and also similar in genomic organization to the 5′ exons for the CS-PG core protein genes. The HAPLN genes expressed by most tissues suggest the fundamental importance of the hyaluronan-dependent extracellular matrix to tissue architecture and function in vertebrates.

The CRTL1 stabilizes aggregates of aggrecan and hyaluronan in cartilage (Czipri et al. 2003). The aggrecan forms link protein-stabilized complexes with HA, via its N-terminal G1-domain that provides cartilage with its load bearing properties. Similar aggregates, in which other CS-PGs (i.e. versican, brevican, and neurocan) substitute for aggrecan, may contribute to the structural integrity of many other tissues including skin and brain (Seyfried et al. 2005). A human brain link protein-1 (BRAL1) is predominantly expressed in brain (Hirakawa et al. 2000). The predicted ORF of human *Bral1* encodes a polypeptide of 340 amino acids containing three protein modules: Ig-like fold and proteoglycan tandem repeat 1 and 2 domains, with an estimated mass of 38 kDa. Brain link protein-1 functions to stabilize the binding between hyaluronan and brevican. The deduced amino acid sequence of human BRAL1 exhibited 45% identity with human CRTL1. The immunoreactivity of BRAL1 was predominantly observed in myelinated fiber tracts in the adult brain and could be detected at P20 in the white matter of the developing cerebellum. Furthermore, BRAL1 colocalized with the versican V2 isoform at the nodes of Ranvier. BRAL1 may play a pivotal role in the

formation of the hyaluronan-associated matrix in the CNS that facilitates neuronal conduction by forming an ion diffusion barrier at the nodes (Oohashi et al. 2002).

The *Bral2* gene is predominantly expressed in brain. The *Bral2* mRNA expression is first detected at P20 and continued through adulthood. In situ hybridization revealed that BRAL2 is synthesized by these neurons themselves, especially by the GABAergic neurons in the cerebellar cortex. The colocalization and synergic importance of BRAL2 and brevican in the perineuronal nets is indicated by the analysis using wild-type and brevican-deficient mouse brain, which suggests that BRAL2 is involved in the formation of ECM contributing to perineuronal nets and facilitate the understanding of a functional role of these ECMs (Bekku et al. 2003).

38.4.2 Brain Enriched Hyaluronan Binding (BEHAB)/Brevican

While the functions of HA are mediated by HA-binding proteins, Hockfield S and co-workers reported the characterization of a cDNA with a high degree of sequence homology to members of the proteoglycan tandem repeat (PTR) family of HA-binding proteins. Unlike other HA-binding proteins, the expression of this cDNA is restricted to the CNS. Hockfield S and co-workers proposed the name BEHAB, Brain Enriched Hyaluronan Binding protein, for this gene. The expression of BEHAB mRNA is developmentally regulated. In the embryo, BEHAB is expressed at highest levels in mitotically active cells. The sequence of BEHAB suggests that the encoded protein is functionally important. The size and sequence of BEHAB are consistent with the possibility that it could serve a function like link protein, stabilizing interactions between HA and brain proteoglycans. In rat and cat brain, BEHAB is expressed at very high levels in ventricular zones throughout the neuraxis. Expression is first detected at embryonic day 15 (E15) in the spinal cord, and is detected at progressively more rostral levels at later ages (Jaworski et al. 1996). There exist several BEHAB/brevican isoforms, each of which may mediate different functions. BEHAB/brevican has been cloned from bovine, mouse, rat and human. Two isoforms have been reported: a full-length isoform that is secreted into ECM and a shorter isoform with a sequence that predicts a glycophosphatidylinositol (GPI) anchor. The BEHAB/brevican gene maps to human chromosome 1q31 (Gary et al. 2000).

Glial tumors, gliomas, are the most common primary intracranial tumors. Their distinct ability to invade the normal surrounding tissue makes them difficult to control and nearly impossible to completely remove surgically. The ECM can modulate, in part, the permissiveness of a tissue to cell movement. One ECM molecule that shows dramatic upregulation in gliomas is BEHAB/brevican. The BEHAB/brevican gene is consistently expressed by human glioma and is not expressed by tumors of nonglial origin (Gary and Hockfield 2000; Jaworski et al. 1996; Zhang et al. 1998). BEHAB/brevican expression is also upregulated during periods of increased glial cell motility in development and following brain injury. Experimental evidence suggests that in glioma, in addition to upregulation of BEHAB/brevican, proteolytic processing of the full-length protein also may contribute to invasion. Reports suggest that up-regulation and proteolytic cleavage of BEHAB/brevican increases significantly the aggressiveness of glial tumors. It will be important to determine the therapeutic potential of inhibiting BEHAB/brevican cleavage in gliomas (Nutt et al. 2001).

38.5 Proteolytic Cleavage of Brevican

BEHAB/brevican can be cleaved into an N-terminal fragment that contains a hyaluronan-binding domain (HABD) and a C-terminal fragment (Yamada et al. 1995). The BEHAB/brevican protein is cleaved in invasive human and rodent gliomas. Matthews et al. (2000) suggested a function for ADAMTS family members in BEHAB/brevican cleavage and glioma and indicated that inhibition of ADAMTS in glioma might provide a novel therapeutic strategy (Viapiano et al. 2008). ADAMTS (a disintegrin and metalloproteinase with thrombo- spondin motifs) proteases are complex secreted enzymes containing a prometalloprotease domain of the reprolysin type attached to an ancillary domain with a highly conserved structure that includes at least one thrombospondin type 1 repeat (Lemons et al. 2001). ADAMTS proteases are synthesized as zymogens, with constitutive proprotein convertase removal of the propeptide occurring prior to secretion. Their enzymatic specificity is heavily influenced by their ancillary domain, which plays a critical role in directing these enzymes to their substrates, the cell surface and the extracellular matrix. Known functions of ADAMTS proteases include (1) processing of procollagens N-proteinase; (2) cleavage of the matrix proteoglycans aggrecan, versican and brevican; (3) inhibition of angiogenesis; and (4) blood coagulation homoeostasis as the von Willebrand factor cleaving protease. ADAMTS-1, -4 and -5 are present in CNS, which are able to cleave the aggregating CS-PG, aggrecan, phosphacan, neurocan and brevican. ADAMTS-4 mRNA and protein expression was decreased. Changes in ADAMTS expression during the course of CNS inflammation may contribute to ECM degradation and disease progression (Cross et al. 2006). ADAMTS4 that selectively cleaves lecticans was detected in cultures of neurons, astrocytes and microglia (Hamel et al. 2005). The ADAMTS-induced cleavage of brevican in the

dentate outer molecular layer is closely associated with diminished synaptic density, and may, therefore, contribute to synaptic loss and/or reorganization in this region (Mayer et al. 2005; Porter et al. 2005)

In human glioblastomas ADAMTS4 and ADAMTS5 (aggrecanases 1 and 2) are expressed in considerable amounts. ADAMTS5 expression is confined to proliferating glioblastoma cells of surgical tumor sections and with lower intensity to astroglial cells in normal brain sections, as opposed to brevican. Selective cleavage of the Glu^{395}-Ser^{396} bond of brevican in adult brain tissues is thought to be important for glioma cell invasion. ADAMTS-4 has such an activity (Nakada et al. 2005). In vitro, glioblastoma-derived ADAMTS5 degrades recombinant human brevican to several smaller fragments. Results show that ADAMTS4 and 5 are upregulated on proliferating glioblastoma cells and these proteases may contribute to their invasive potential (Held-Feindt et al. 2006). ADAMTS-5 is capable of degrading brevican and is overexpressed in glioblastoma cells, and that ADAMTS-5 may play a role in glioma cell invasion through the cleavage of brevican (Nakada et al. 2005; Porter et al. 2005).

38.6 Proteoglycans in Sensory Organs

Various proteoglycans are expressed in ocular tissues. The TGF-β2 regulates the expression of proteoglycans in aqueous humor. The expression of CS in stroma was upregulated at early postnatal stages and reduced during development in rat eyes. Decorin is expressed in trabecular meshwork tissues. In retinal tissues, neurocan and phosphacan were expressed mainly in nerve fiber-rich layers during rat postnatal stages. Soluble extracellular proteoglycans in corneal and trabecular meshwork tissues contribute to the stromal transparency in the corneal tissues and the resistance of the aqueous humor outflow in trabecular meshwork tissues (Inatani and Tanihara 2002).

CS and HS proteoglycans are the major constituents expressed in and secreted by retinal tissue. Soluble HS proteoglycans are found in extracellular matrices of the basement membrane, whereas HS proteoglycans with their membrane-binding domain are localized primarily in the neurites of retinal neuronal cells, indicating their role as receptors for cytokines. The expression in the nerve fiber-rich layers of several CS proteoglycans, such as neurocan and phosphacan, is restricted in the nervous tissues, and is upregulated as retinal development proceeds, then decreases after maturation of the retina. In vitro data suggest that these proteoglycans regulate axon guidance and synapse formation during the development of nervous tissue. In contrast, in adult vertebrate retina, the IPM is a rich source of CS proteoglycans (Inatani and Tanihara 2002). Co-localization of the molecule with TN-R in the retina and optic nerve suggested a functional relationship between TN-R and phosphacan-related CS proteoglycan in vivo (Inatani et al. 2001; Xiao et al. 1997).

Aggrecan and versican were first seen at E16 in the optic nerve and retina of rat, whereas neurocan was not detected in the embryonic eye. At postnatal day P0, β-versican is largely confined to the inner plexiform layer whereas α-versican is also apparent in the neuroblastic layer. Both aggrecan and, much more weakly, neurocan immunoreactivity is present throughout the neonatal retina. Aggrecan and α-versican are also present throughout the optic nerve and disk, whereas β-versican and neurocan are confined to the laminar beams of the optic nerve (Popp et al. 2004). In mouse retinofugal pathway, phosphacan but not neurocan is likely the major carrier of the CS glycosaminoglycans that play crucial functions in axon divergence and age-related axon ordering in the mouse optic pathway. These two proteoglycans regulate axon growth and patterning not only through the sulfated sugars but also by interactions of the protein parts with guidance molecules on the optic axons (Leung et al. 2004).

Accumulation of neurocan in association with the retinal vasculature does not correlate with photoreceptor cell loss, because it was not observed in the rhodopsin mutant rats. During the earliest stages of the disease, accumulation of debris in the subretinal space in RCS rats with dystrophic retinas may be sufficient per se to initiate a cascade of metabolic changes that result in accumulation of neurocan. With time, the neurocan accumulated perivascularly may, by interaction with other matrix molecules, modulate at least some of the vascular alterations observed in rat animal model (Zhang et al. 2003). In transient retinal ischemia the intensity of the 220-kDa band as well as the 150-kDa band increased markedly after reperfusion. The neurocan expression by Müller cells suggests that this proteoglycan plays a role in the damage and repair processes in diseased retina (Inatani et al. 2000).

Neurocan was conspicuous in the medial and lateral superior olivary nuclei and much less stained in the cochlear nucleus and posterior colliculus. Different locations of CS-PG, including neurocan, may be associated with focal sites composed of neuronal surface, terminal boutons and ECM in the lower auditory tract of the adult dog (Atoji et al. 1997).

Neurocan was first detected in primary olfactory neurons at E11.5. Neurocan was expressed by primary olfactory axons as they extended toward the rostral pole of the telencephalon as well as by their arbors in glomeruli after they contacted the olfactory bulb. Being a strong promoter for neurite outgrowth neurocan supports the growth of primary olfactory axons through ECM. Phosphacan, unlike neurocan, was present within the mesenchyme surrounding the E11.5 and E12.5 nasal cavity. This expression decreased at E13.5, concomitant with a transient appearance of phosphacan in nerve fascicles. Within the embryonic olfactory bulb, phosphacan was localised to the external and internal

plexiform layers. The spatiotemporal expression patterns of neurocan and phosphacan indicate that these CS-PGs have diverse in situ roles, which are dependent on context-specific interactions with extracellular and cell adhesion molecules within the developing olfactory nerve pathway (Clarris et al. 2000). Glycosaminoglycan chains, such as CS and KS, and core proteins of the CS proteoglycan, neurocan and phosphacan, were barely detected in the migratory pathway from the olfactory placode. N-syndecan mRNA was localized in virtually all of migrating neurons as well as in cells of the olfactory epithelium and the vomeronasal organ. It is likely that a heterophilic interaction between NCAM and N-syndecan participates in the neuronal migration from the rat olfactory placode (Toba et al. 2002).

38.7 Functions of Proteoglycans in CNS

38.7.1 Functions of Chondroitin Sulphate Proteoglycans

In the adult, CS-PGs are generally thought to be inhibitory for tissue plasticity and to have a barrier function during regeneration (Bradbury et al. 2002). However, in the developing CNS, CS-PGs show a functional dualism: depending on the neuronal cell type and the way they are presented to neurons, both growth inhibitory and growth promoting effects have been described (Emerling and Lander 1996; Akita et al. 2004; Wu et al. 2004). The inhibitory properties of CS-PGs have mostly been attributed to their glycosaminoglycan (GAG) chains (Asher et al. 2002; Sivasankaran et al. 2004), however, the core protein may also act as an inhibitor (Monnier et al. 2003; Ughrin et al. 2003). In regions containing low levels of adhesion molecules, various CS-PGs may act as barriers to cell migration and axonal growth. In regions containing high levels of adhesion proteins, brain CS-PGs may still act to maintain certain boundaries while allowing selective axonal extension to proceed. There are numerous regions of overlap in the expression patterns of CS-PGs and adhesion molecules in vivo, and the relative levels of these molecules as well as the organization of the ECM may be important factors that regulate the rate of axonal growth locally. Differential expression of CS-PGs may be important for modulating cell adhesion as well as axonal growth and guidance during neural development, and continued expression may prevent these processes in normal mature nervous system as well as following brain injury (Margolis et al. 1996). The swift assembly and remodeling of ECMs have been associated with axonal guidance functions in the periphery and with the structural stabilization of myelinated fiber tracts and synaptic contacts in the maturating CNS. Particular interest has been focused on the putative role of CS-PG in suppressing CNS regeneration after lesions. The axon growth inhibitory properties of several of these CS-PGs in vitro and the partial recovery of structural plasticity in lesioned animals treated with chondroitin sulfate degrading enzymes in vivo have significantly contributed to the increased awareness of this long time neglected structure.

Little is known about the contribution of CS-PGs to regeneration failure in vivo at dorsal root entry zone (DREZ), a CNS region that blocks regeneration of sensory fibers following dorsal root injury without glial scar formation. It is suggested that the proteoglycans (Brevican, Neurocan, Versican V1, and Versican V2) are abundant in the DREZ at the time regenerating sensory fibers reach the PNS/CNS border and may therefore participate in growth-inhibition in this region (Beggah et al. 2005). Quaglia et al. (2008) demonstrated that (1) neurocan and/or brevican contribute to the non-permissive environment of the DREZ several weeks after lesion and that (2) delayed stimulation of the growth program of sensory neurons can facilitate regeneration across DREZ provided its growth-inhibitory properties are attenuated. Post-injury enhancement of the intrinsic growth capacity of sensory neurons combined with removal of inhibitory CS-PG may therefore help to restore sensory function and thus attenuate the chronic pain resulting from human brachial plexus injury.

In areas of denervation the role of CS-PGs is less clear, since they are enriched in zones of sprouting, i.e. zones of axonal growth. However, results reveal a temporal pattern in CS-PG mRNA expression in the denervated fascia dentata. This suggests specific biological functions for CS-PGs during the denervation-induced reorganization process: whereas the early increase in CS-PGs in the denervated zone could influence the pattern of sprouting, the late increase of aggrecan mRNA suggests a different role during the late phase of reorganization (Schäfer et al. 2008).

Functions of Sciatic Nerve-Derived Versican- and Other CS-PGs: Human sciatic nerves contained two chondroitin sulphate proteoglycans of M_r of 130 and 900 kDa. Following chondroitinase ABC treatment of 130 kDa proteoglycan, a core protein of ~45 kDa was shown to react with polyclonal antibodies against the chondroitin-dermatan sulphate proteoglycan decorin from human fibroblasts. Chondroitinase ABC treatment of 900 kDa proteoglycan yielded a core protein with a M_r of ~400 kDa. Observations suggest two proteoglycans from human sciatic nerve are immunochemically related to chondroitin sulphate proteoglycans versican and decorin that may contribute to successful regeneration in the peripheral nervous system of mammals. The versican- and decorin-like molecules are involved in cell-extracellular matrix interactions and inhibit adhesion of several cell lines, neonatal dorsal root ganglion neurons and Schwann cells.

Observations suggest that binding of the two proteoglycans to fibronectin is involved in the modulation of adhesion of cells to fibronectin (Braunewell et al. 1995).

38.7.2 Functions of Brevican and Neurocan in CNS

Perineuronal nets (PNN) are specialized ECM structures enwrapping CNS neurons, which are important regulators for neuronal and synaptic functions. Brevican is an integral component of PNN, which appear in hippocampal primary cultures. Brevican is primarily synthesized by co-cultured glial fibrillary acidic protein (GFAP-)-positive astrocytes and co-assembles with its interaction partners in PNN-like structures on neuronal somata and neurites. Both excitatory and inhibitory synapses are embedded into PNN. Furthermore, axon initial segments are strongly covered by a dense brevican coat. Altogether, mature primary cultures can form PNN (John et al. 2006). Digestions with chondroitinase ABC and hyaluronidase indicated that aggrecan, versican, neurocan, brevican, and phosphacan are retained in PNNs through binding to HA. A comparison of the brain and spinal cord ECM with respect to CS-PGs indicated that the PNNs in both parts of the CNS have the same composition (Deepa et al. 2006).

Mice lacking brevican gene are viable and fertile and have a normal life span. Brain anatomy was normal, although alterations in the expression of neurocan were detected. The PNN formed but appeared to be less prominent in mutant than in wild-type mice. Brevican-deficient mice showed significant deficits in the maintenance of hippocampal long-term potentiation (LTP). Detailed behavioral analysis revealed no significant deficits in learning and memory. Results indicated that brevican is not crucial for brain development but has restricted structural and functional roles (Brakebusch et al. 2002). A brevican deficiency resulted in a reorganization of the nodal matrices, which was characterized by the shift of tenascin-R (TN-R), and concomitantly phosphacan, from an axonal diameter-dependent association with nodes to an axonal diameter independent association. Bekku et al. (2009) revealed that brevican plays a crucial role in determining the specialization of the hyaluronan-binding nodal matrix assemblies in large diameter nodes. Brevican associates with astrocytes ensheathing cerebellar glomeruli and inhibits neurite outgrowth from granule neurons.

At molecular level, brevican promotes EGFR activation, increases the expression of cell adhesion molecules, and promotes the secretion of fibronectin and accumulation of fibronectin microfibrils on the cell surface. Moreover, the N-terminal cleavage product of brevican, but not the full-length protein, associates with fibronectin in cultured cells and in surgical samples of glioma. Results provide evidence of the cellular and molecular mechanisms that may underlie the motility-promoting role of brevican in primary brain tumors. In addition, these results underscore the important functional implications of brevican processing in glioma progression (Hu et al. 2008).

Neurocan-deficient mice are viable and fertile and have no obvious deficits in reproduction and general performance. Brain anatomy, morphology, and ultrastructure are similar to those of wild-type mice. Perineuronal nets surrounding neurons appear largely normal. Mild deficits in synaptic plasticity may exist, as maintenance of late-phase hippocampal long-term potentiation is reduced. Results indicate that neurocan has either a redundant or a more subtle function in the development of the brain (Zhou et al. 2001; Okamoto et al. 2001). The distal transected cords of infant rats are more permissive for axon extension than those of adults. Neurocan mRNA was up-regulated in the distal cord of adult rats shortly after transection, followed by a longer wide distribution of neurocan activity in both neurons and astrocytes. By contrast, upregulation of neurocan mRNA was not seen in infant rats, although transient expression of neurocan immunoreactivity was seen in neurons. Combined with the different regenerative capacity of infant and adult rats, it indicates that neurocan inhibits spinal cord regeneration (Qi et al. 2003).

Extracellular Matrix Alterations: During hippocampal development, neurocan is expressed throughout the alveus, neuropil layers, and parts of the dentate gyrus from E16 to P2. The CS-PG phosphacan is expressed primarily in the neuropil layers at postnatal stages. After E18, intense labeling of neurocan was observed in regions of the alveus surrounding L1-expressing axon fascicles. In vitro, axons from brain regions that project through the alveus during development would not grow across CS-PG substrata, in a concentration-dependent manner. In addition, hippocampal axons from dissociated neuron cultures only traveled across CS-PG substrata as fasciculated axon bundles. These results implicate CS-PG in the regulation of axon trajectory and fasciculation during hippocampal axon tract formation (Wilson and Snow 2000).

Watanabe et al. (1995) revealed that neurocan was distributed throughout the cerebral cortex of rat during early postnatal development but was absent from the centres of cortical barrels at the time of entry and arborization of thalamocortical axons. At this developmental stage, expression of neurocan mRNA was shown by in situ hybridization to be down-regulated in the barrel centres. It appears that neuronal stimuli through early thalamocortical fibres from the sensory periphery cause reduced expression of neurocan mRNA in neurocan-producing cells in the presumptive barrel centres.

Further, mice lacking neurocan, brevican, tenascin-R, and tenascin-C brain extracellular matrix molecules were found

to be viable and fertile. However, the brains of 1-month-old quadruple KO mice revealed increased levels of fibulin-1 and fibulin-2 with an unusual parenchymal deposition of these molecules. The quadruple KO mice also displayed obvious changes in the pattern of deposition of hyaluronan (Rauch et al. 2005).

Regulation of Cadherin and Integrin Function by Neurocan: Li et al. (2000) showed that the interaction of neurocan with its GalNAcPTase receptor coordinately inhibits both N-cadherin- and β1-integrin-mediated adhesion and neurite outgrowth. The inhibitory activity is localized to an NH_2-terminal fragment of neurocan containing an Ig loop and an HA-binding domain. The coordinate inhibition of cadherin and integrin function on interaction of neurocan with its receptor may prevent cell and neurite migration across boundaries.

The L1/Ng-CAM adhesion molecule of the Ig superfamily is implicated in neural processes including neuronal cell migration, axon outgrowth, learning, and memory formation. It has been suggested that neurocan can block homophilic binding. The sequences involved in neurocan binding are localized on the surface of the first Ig domain and largely overlap with the G-F-C β-strands proposed to interact with the fourth Ig domain during homophilic binding. It was found that the C-terminal portion of neurocan is sufficient to mediate binding to the first Ig domain of L1, and that the sushi domain cooperates with a glycosaminoglycan side chain in forming the binding site for L1 (Oleszewski et al. 2000).

38.7.3 Neurocan in Embryonic Chick Brain

Neurocan has been found in the embryonic avian heart and vasculature. In stage 11 quail embryos, neurocan was prominently expressed in the myocardium, dorsal mesocardium, heart-forming fields, splanchnic mesoderm, and vicinity of the extraembryonic vaculature, and at lower levels in the endocardium. Results suggest that neurocan may function as a barrier that regulates vascular patterning during development (Mishima and Hoffman 2003). Zanin et al. (1999) revealed the presence of aggrecan and versican in stages 12–21 chicken embryo hearts in distinctive spatial and temporal patterns. Versican is found in the myocardium and the myocardial basement membrane. In contrast, aggrecan is specifically colocalized with several groups of migrating cells including endocardial cushion tissue cells, epicardial cells, a mesenchymal cell population in the outflow tract that may be of neural crest origin, and a mesenchymal cell population in the inflow tract. The observations indicated that versican and aggrecan are expressed in unique patterns and that they play different roles in development.

In the chick embryo, aggrecan has a regionally specific and developmentally regulated expression profile during brain development. Aggrecan expression is first detected in chick brain on E7, increases from E7 to E13, declines markedly after E16, and is not evident in hatchling brains. The time course and pattern of aggrecan expression observed in ventricular zone cells suggested that it might play a role in gliogenesis. In aggrecan-deficient model (nanomelic chicks) expression and levels of neurocan and brevican are not affected, indicating a non-redundant role for these members of the aggrecan gene family. Results suggest that aggrecan functions in specification of a sub-set of glia precursors that might give rise to astrocytes in vivo (Schwartz and Domowicz 2004).

38.8 Chondroitin Sulfate Proteoglycans in CNS Injury Response

38.8.1 CS-PGs in CNS in Response to Injury

Injury to the CNS leads to permanent loss of function due to the inability of severed nerve fibers to regenerate back to their targets. Lack of CNS repair has been attributed to several causes, including to extrinsic growth-inhibitory molecules associated with myelin, and to ECM CS-PGs produced by activated glial cells post-injury (Filbin; 2003; Silver and Miller 2004; Zurn and Bandtlow 2006). Treatment with antibodies against the myelin inhibitor Nogo (Schnell and Schwab 1990), with a Nogo-66 receptor antagonist peptide (GrandPre et al. 2002), or with enzymes degrading CS-PGs (Bradbury et al. 2002; Barritt et al. 2006), has provided evidence for the growth-inhibitory function of these molecules. However, the limited ability to regenerate following injury is also due to an intrinsic low capacity of adult axons to grow. Interestingly, the growth capacity of central branches of sensory neurons located in the spinal cord can be increased if their peripheral branches are previously cut (conditioning lesion), i.e. if their axonal growth program is activated before spinal cord lesion (Richardson and Issa 1984; Neumann and Woolf 1999). Yet the timing of this peripheral lesion is critical since peripheral nerve priming delayed by 2 weeks post-lesion fails to promote growth of central sensory axons (Neumann and Woolf 1999). Only the combination of two priming lesions, one at the time of spinal cord injury, and one after 1 week, has been shown to facilitate growth in the same model (Neumann et al. 2005).

Experimental data now indicate that the expression of a number of different CS-PGs is increased following CNS injury. After spinal cord injury, neurocan, brevican, and versican increased within days in injured spinal cord

parenchyma surrounding the lesion site and peaked at 2 weeks. Neurocan and versican were persistently elevated for 4 weeks postinjury, and brevican expression persisted for at least 2 months. On the other hand, phosphacan labeling decreased in the same region immediately following injury but later recovered and then peaked after 2 months. Thus, the production of several CS-PG family members is differentially affected by spinal cord injury, overall establishing a CS-PG-rich matrix that persists for up to 2 months following injury. Optimization of strategies to reduce CS-PG expression to enhance regeneration may need to target several different family members over an extended period following injury (Jones et al. 2003a; b). Upregulation of hyalectans (lecticans) neurocan, versican and brevican, plus NG2 and phosphacan following injury have been shown to exhibit inhibitory effects on neurite outgrowth in vitro. It is likely therefore that the increased expression of these molecules contributes to the non-permissive nature of the glial scar. It is important to remember also that not only does the glial scar contain many different inhibitory molecules, but that these are the products of a number of different cells, including not just astrocytes, but also oligodendrocyte progenitor and meningeal cells. It is arguable that the latter two cell types make a greater contribution than astrocytes to the inhibitory environment of the injured CNS. Evaluation of total soluble CS-PGs 2 weeks after dorsal column lesion in the rat expressed after spinal cord injury (SC-I) demonstrated that NG2 is highly upregulated and is a major CS-PG species. NG2 expression is upregulated within 24 h of injury, peaks at 1 week, and remains elevated for at least an additional 7 weeks (Jones et al. 2002). Attempts have been made to alter the CS-PG component of the glial scar in the hope that this will facilitate improved axonal regeneration. Studies have reported an improved regenerative response following treatment of the injured CNS with chondroitinase ABC. The CS-PGs representing a significant source of inhibition within the injured CNS, studies indicate that successful CNS regeneration may be brought about by interventions which target these molecules and/or the cells which produce them(Morgenstern et al. 2002; Chan et al. 2007).

Axonal Regeneration Through Regions of CS-PG Deposition After Spinal Cord Injury: Jones et al. (2003b). examined growth responses of several classes of axons to this inhibitory environment in the presence of a cellular fibroblast bridge in a spinal cord lesion site and after a growth factor stimulus at the lesion site. Analysis showed dense labeling of NG2, brevican, neurocan, versican, and phosphacan at the host-lesion interface after SC-I. NG2 expression also increased after sciatic nerve injury, wherein axons successfully regenerate. Cellular sources of NG2 in SC-I and peripheral nerve lesion sites included Schwann cells and endothelial cells. Notably, these cellular lesion sites produced the cell adhesion molecules L1 and laminin. Thus, axons grow along substrates coexpressing both inhibitory and permissive molecules, suggesting that regeneration is successful when local permissive signals balance and exceed inhibitory signals (Jones et al. 2003b; Tang et al. 2003). Versican V2 protein levels, however, displayed an opposite trend, dropping below unlesioned spinal cord values at all time points studied (Tang et al. 2003).

Haddock et al. (2007) examined changes in brevican and phosphacan, following transient middle cerebral artery occlusion, a model of stroke in rat and their spatial relationship with ADAMTS-4. The co-localization of ADAMTS or its activity indicated a functional role for this matrix-protease pair in degeneration/regeneration processes that occur in stroke.

Effect of Hypoxic-ischemic Brain Injury in Perinatal Rats: CS-PG are involved in the pathologic process of hypoxia-ischemia (H-I) in the neonatal brain. Brevican is secreted by OLs and their progenitors prior to and during active myelination whereas NG2 is produced by early OL progenitors. In neonatal hippocampus of rat after H-I of unilateral carotid artery ligation and exposure to hypoxia, a cavitary infarct involving the ipsilateral parietal and temporal regions of cerebral cortex, hippocampus, and striatum of most rat pups was evident after H-I. The abundance of brevican was reduced in ipsilateral hippocampus after H-I and the total G1 proteolytic fragment of brevican was lower in the ipsilateral hippocampus and the level of a protease-generated brevican fragment was significantly diminished in OL-rich hippocampal fimbria. Hippocampal NG2 levels were also lower after H-I, but were not different from contralateral side at 14 days. The early events in the process could be involved in apoptotic cell death and/or tissue injury (Aya-ay et al. 2005). In contrast to injured adult CNS, the amount of neurocan was reduced after hypoxia in the neonatal hypoxic-ischemic cerebral hemisphere. The amounts of phosphacan and neuroglycan C were also reduced significantly 24 h after hypoxia at the right injured cortex compared to those at the left cortex. But phosphacan was conversely intensified both at 24 h and 8 days after hypoxia at the infarcted area. Hypoxic-ischemic insult may unmask phosphacan epitopes at the injured sites, resulting in intensified immunostaining. Because intensified immunostaining for neurocan and neuroglycan C was not observed, unmasking seems to be specific to phosphacan among these three CS-PG (Matsui et al. 2005).

Versican and brevican are expressed with distinct pathology in neonatal hypoxic-ischemic injury. Unique expression profiles for lecticans after neonatal H-I

suggested deposition of brevican at elevated rates in response to progressive gray matter injury, whereas diminished versican expression may be associated with deep cerebral white matter injury (Leonardo et al. 2008).

38.8.2 Glial Scar and CNS Repair

Injury to CNS results in the formation of the glial scar, a primarily astrocytic structure that represents an obstacle to regrowing axons. CS-PG are greatly upregulated in the glial scar. The glial scar, a primarily astrocytic structure bordering the infarct tissue inhibits axonal regeneration after stroke. In this environment, axon regeneration fails, and remyelination may also be unsuccessful. The glial reaction to injury recruits microglia, oligodendrocyte precursors, meningeal cells, astrocytes and stem cells. Damaged CNS also contains oligodendrocytes and myelin debris. Most of these cell types produce molecules that have been shown to be inhibitory to axon regeneration. Oligodendrocytes produce NI250, myelin-associated glycoprotein (MAG), and tenascin-R, oligodendrocyte precursors produce NG2 DSD-1/phosphacan and versican, astrocytes produce tenascin, brevican, and neurocan, and can be stimulated to produce NG2, meningeal cells produce NG2 and other proteoglycans, and activated microglia produce free radicals, nitric oxide, and arachidonic acid derivatives. Most of these molecules participate in rendering the damaged CNS inhibitory for axon regeneration. Demyelinated plaques in multiple sclerosis consist mostly of scar-type astrocytes and naked axons. The extent to which the astrocytosis is responsible for blocking remyelination is not established, but astrocytes inhibit the migration of both oligodendrocyte precursors and Schwann cells which must restrict their access to demyelinated axon (Fawcett and Asher 1999; Buss et al. 2009).

Various reports indicate that Neurocan is up-regulated in the scar region after stroke. Neurocan, expressed in the CNS, exerts a repulsive effect on growing cerebellar axons. Immunocytochemistry revealed neurocan to be deposited on the substrate around and under astrocytes but not on the cells. Astrocytes therefore lack the means to retain neurocan at the cell surface. Findings raise the possibility that neurocan interferes with axonal regeneration after CNS injury. In the spinal cord under various types of injury, reactive gliosis emerges in the lesion accompanied by CS-PG up-regulation. Several types of CS-PG core proteins and their side chains have been shown to inhibit axonal regeneration in vitro and in vivo. The bone morphogenetic proteins (BMPs) stimulate glial scar formation in demyelinating lesions of adult spinal cord. BMP4 and BMP7 increase rapidly at the site of demyelination, and astrocytes surrounding the lesion increase expression of phosphorylated Smad1/5/8. Thus, local increase in BMPs at the site of a demyelinating lesion causes upregulation of gliosis, glial scar formation, and heightened expression of CS-PGs such as neurocan and aggrecan that may inhibit remyelination (Fuller et al. 2007). Bone marrow stromal cells (BMSCs) reduce the thickness of glial scar wall and facilitate axonal remodeling in the ischemic boundary zone. Analysis showed that reactive astrocytes were the primary source of neurocan, and BMSC-treated animals had significantly lower neurocan and higher growth associated protein 43 expression in the penumbral region compared with control rats. Neurocan gene expression was significantly down-regulated in rats receiving BMSC transplantation. Shen et al. (2008) suggest that BMSCs promote axonal regeneration by reducing neurocan expression in peri-infarct astrocytes.

38.9 Other Proteoglycans in CNS

In addition to lecticans, phosphacan, NG2, and testicans as proteoglycans are expressed in CNS (Schnepp et al. 2005). The NG2 chondroitin sulfate proteoglycan, a structurally unique, integral membrane proteoglycan, is found on the surfaces of several different types of immature cells. NG2 is associated with multipotential glial precursor cells (O2A progenitor cells), chondroblasts of the developing cartilage, brain capillary endothelial cells, aortic smooth muscle cells, skeletal myoblasts and human melanoma cells (Levine and Nishiyama 1996). Neuroglycan C (NGC), a brain-specific transmembrane proteoglycan, is thought to bear not only CS but also N- and O-linked oligosaccharides on its core protein. The structure of carbohydrate moiety of NGC is developmentally regulated, and differs from those of neurocan and phosphacan. The developmentally-regulated structural change of carbohydrates on NGC may be partly implicated in the modulation of neuronal cell recognition during brain development (Shuo et al. 2004). Brain-specific CS-PGs, including neurocan, phosphacan/receptor-type protein-tyrosine phosphatase beta, and neuroglycan C, have been detected in the CNS. These CS-PGs are involved in the proliferation of neural stem cells as a group of cell microenvironmental factors (Ida et al. 2006). Xiao et al. (1997) isolated a CS-PG from mouse brain with a fragment of ECM tenascin-R that comprises the amino-terminal cysteine-rich stretch and the 4.5 EGF-like repeats. The 173-kDa core protein of phosphacan is synthesized by glia and represents an extracellular variant of the receptor-type protein tyrosine phosphatase RPTP ζ/β. Keratan sulfate-containing glycoforms of phosphacan (designated phosphacan-KS) are also present in brain. In early postnatal rat cerebellum, neurocan, phosphacan, and phosphacan-KS

show the overlapping localization with tenascin, an extracellular matrix protein that modulates cell adhesion and migration. Phosphacan and neurocan are involved in the modulation of cell adhesion and neurite outgrowth during neural development and regeneration (Alliel et al. 2004; Meyer-Puttlitz et al. 1996).

38.10 Regulation of Proteoglycans in CNS

38.10.1 Growth Factors and Cytokine Regulate CS-PGs by Astrocytes

After injury to adult CNS, numerous cytokines and growth factors are released that contribute to reactive gliosis and ECM production. The presence of TGF-β1 and EGF greatly increase the production of several CS-PGs by astrocytes. Treatment of astrocytes with other EGF-receptor (ErbB1) ligands produced increases in CS-PG production similar to those observed with EGF. Treatment of astrocytes, however, with heregulin, which signals through other members of the EGF-receptor family (ErbB2, ErbB3, ErbB4), did not induce CS-PG upregulation. The specificity of activation through the ErbB1 receptor indicated the presence of multiple core proteins containing 4-sulfated or 6-sulfated chondroitin. Further analyses showed that treatment of astrocytes with EGF increased phosphacan expression, whereas treatment with TGF-β1 increased neurocan expression. These results elucidate some of the injury-induced growth factors that regulate the expression of CS-PGs which could be targeted in modulate CS-PG production after injury to the CNS (Smith and Strunz 2005).

ECM alterations in CNS of multiple sclerosis (MS) patients result from blood–brain barrier breakdown, release and activation of proteases, and synthesis of ECM components. In active MS plaque edges, CNS lecticans (versican, aggrecan, and neurocan) and dermatan sulfate PG were increased in association with astrocytosis; in active plaque centers they were decreased in the ECM and accumulated in foamy macrophages, suggesting that these ECM PGs are injured and phagocytosed along with myelin. Results indicate that ECM PG alterations are specific, temporally dynamic, and widespread in MS patients and may play critical roles in lesion pathogenesis and CNS dysfunction (Sobel and Ahmed 2001).

Glutamate activation of excitatory amino acid receptors induces the synthesis and release of PGs with neurite-promoting activity from hippocampal neurones. Both cerebroglycan (CBG), a glycosylphatidylinositol-anchored heparan sulphate PG, and neurocan are expressed in hippocampal neurones. Exposure of hippocampal neurones to 100 μM glutamate resulted in an increase in CBG mRNA levels and an increase in axonal and dendritic length. The increase in CBG mRNA levels following glutamate exposure was mediated via both N-methyl-D-aspartate and metabotropic receptor activation (Wang and Dow 1997).

38.10.2 Decorin Suppresses PGs Expression

Decorin transcripts express in brain on postnatal day 3 followed by a slow decline to the lower adult level. Postnatal decorin expression was observed in the grey matter of neocortex, hippocampus and thalamus, in myelinated fibre tracts and in several mesenchymal tissues (blood vessels, pia mater and the choroid plexus). In the neocortex, decorin is expressed in a specific laminar pattern with intense staining of the cortical plate and its derivatives, which differs remarkably from the distributions observed for other proteoglycans (Miller et al. 1995). Decorin seems to serve yet unknown functions in the developing rat brain parenchyma in addition to its well-established role as a constituent of the mesenchymal ECM (Kappler et al. 1998).

Though the inhibitory CS-PGs and myelin-associated molecules are major impediments to axon regeneration within CNS, decorin infusion can suppress the levels of multiple inhibitory CS-PGs and promote axon growth across spinal cord injuries. Decorin treatment of acute spinal cord injury and cultured adult spinal cord microglia can increase plasminogen/plasmin synthesis and induced 10- and 17-fold increases in plasminogen and plasmin protein levels, respectively, within sites of injury. In addition to potentially degrading multiple axon growth inhibitory components of the glial scar, plasmin is known to play major roles in activating neurotrophins and promoting CNS plasticity (Davies et al. 2004, 2006). The formation of misaligned scar tissue by a variety of cell types expressing multiple axon growth inhibitory proteoglycans presents a physical and molecular barrier to axon regeneration after adult spinal cord injuries. Decorin reduces astrogliosis and basal lamina formation in acute cerebral cortex stab injuries. Decorin pretreatment of meningial fibroblasts in vitro resulted in increase in neurite outgrowth from co-cultured adult sensory neurons and suppression of NG2 immunoreactivity. In addition to suppressing inhibitory scar formation, decorin can directly boost the ability of neurons to extend axons within CS-PG or myelin rich environments. The ability of decorin to promote axon growth across acute spinal cord injuries via a coordinated suppression of inflammation, CS-PG expression and astroglial scar formation make decorin treatment a promising component of future spinal cord regeneration strategies (Minor et al. 2008).

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Part XIII

C-Type Lectins: Emerging Groups of C-Type Lectins

Regenerating (Reg) Gene Family

39

G.S. Gupta

39.1 The Regenerating Gene Family

Regenerating (Reg) gene is a multigene super-family grouped into four subclasses, types I, II, III, and IV based on the primary structures of the encoded proteins and represents the products of small secretory proteins, which can function as acute phase reactants, lectins, antiapoptotic factors or growth factors for pancreatic β-cells, neural cells and epithelial cells in the digestive system. Among C-type lectin gene superfamily, the Reg-related genes are classified in group VII and encode small secreted proteins with a single carbohydrate recognition domain linked to a short N-terminal peptide (Drickamer 1999; Zhang et al. 2003a). The genes coding these proteins are tandemly clustered on chromosome 2p12 in humans and may have arisen from the same ancestral gene by gene duplication. The expression of this group of proteins is controlled by complex mechanisms, some members being constitutively expressed in certain tissues while, in others, they require activation by several factors. These members have several apparently unrelated biological effects, depending on the member studied and the target cell. insoluble fibrils at physiological pH (Iovanna and Dagorn 2005).

39.2 Regenerating (*Reg*) Gene Products

Reg and Reg-related genes constitute a multigene family, the Reg family. Identified members of the human *Reg* gene family include Reg Iα, Reg Iβ, Reg IIIα (HIP/PAP), Reg IIIβ and Reg IV (Chakraborty et al. 1995; Dieckgraefe et al. 2002; Hartupee et al. 2001; Lasserre et al. 1994; Laurine et al. 2005). A glycoprotein expressed in exocrine pancreas (where it has been called PSP/LIT) and endocrine pancreas (where it has been called the Reg protein) is encoded by *Reg* gene which maps to 2p12. The *Reg* gene characterized in the exocrine pancreas has been found to be expressed in regenerating islets of 90% depancreatized rats and not in normal islets. In humans, it was identified only in the exocrine pancreas. The Reg gene is known to be involved in the growth of not only pancreatic β-cells, but also epithelial cells of the GI tract and carcinoma of its lineage. The expression of the REG gene is closely related to the infiltrating property of gastric carcinoma, and may be a prognostic indicator of differentiated adenocarcinoma of the stomach (Rosty et al. 2002; Yonemura et al. 2003).

Reg gene, first isolated from a rat regenerating islet cDNA library, (Terazono et al. 1988) and Reg-related genes constitute a multigene family (Reg Family), which consists of four subtypes (type I, II, III, and IV) based on the primary structures of the encoded proteins (Watanabe et al. 1994) that ameliorates the diabetes of 90% depancreatized rats and non-obese diabetic mice. The Reg protein family consists solely of a M_r 16-kDa CTLD and N-terminal secretion signal. Despite having a canonical C-type lectin fold, Reg proteins lack conserved sequences that support Ca^{2+}-dependent carbohydrate binding in other C-type lectins (Drickamer 1999). The human REG family genes have a common gene structure with 6 exons and 5 introns, and encode homologous 158–175-aa secretory proteins. The human REG family genes on chromosome 2, except for REG IV on chromosome 1, were mapped to a contiguous 140 kb region of the human chromosome 2p12. The gene order from centromere to telomere was 5′ HIP/PAP-III′-5′ RS 3′-3′ REG Iα 5′-5′ REG Iβ 3′-3′ REG-III 5′. The human REG gene family is constituted from an ancestor gene by gene duplication and forms a gene cluster on the region (Nata et al. 2004). Okamoto grouped the members of the family, Reg and Reg-related genes from human, rat and mouse, into three subclasses, types I, II, and III (Okamoto 1999). Stephanova et al. determined that the three rat PAP genes and the related Reg gene (REGL, regenerating islet-derived-like/pancreatic stone protein-like/pancreatic thread protein-like) were all located at 4q33–q34 (Stephanova et al. 1996). The mouse Reg family genes were mapped to a contiguous 75 kb region in chromosome 6, including Reg I, Reg II, Reg IIIα, Reg IIIβ, Reg IIIγ, and Reg IIIδ (Abe et al. 2000). Reg IIIδ was expressed predominantly in exocrine pancreas, whereas both Reg I and Reg II

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Table 39.1 Reg proteins, length of amino acids and chromosome localization in different species (Zhang et al. 2003a)

Super family memeber	Species	Length of amino acids	Chromosome localization	Reference(s)[a]
Reg I	Mouse Reg I	165	6	Abe et al. 2000; Unno et al. 1993
			12	
	Rat Reg	165	4q33-q34	Stephanova et al. 1996
	Human Reg I and II	166	2p12	Miyashita et al. 1995; Perfetti et al. 1994
	Human PSP/PTP	166	2p12	Miyashita et al. 1995; Dusetti et al. 1994a
Reg II	Mouse Reg II	173	6	Abe et al. 2000; Unno et al. 1993
			3	
Reg III	Rat PAP-I, PAP-II, Peptide- 23	175	4q33-q34	Stephanova et al. 1996
	Mouse Reg-III-α; Reg IIIβ	175	6	Abe et al. 2000; Narushima et al. 1997
	Mouse Reg-III-γ	174	6	Abe et al. 2000; Narushima et al. 1997
	Human HIP	175	2p12	Miyashita et al. 1995
	Bovine PTP	175	11	NCBI[b]
Reg IV	Human Reg IV	158	1q12-q21	Hartupee et al. 2001
	Mouse Reg IV	157	3	NCBI[c]

[a]References represent chromosomal localization
[b]Accession NP_991356
[c]Accession NP_080604

were expressed in hyperplastic islets and Reg IIIα, Reg IIIβ and Reg IIIγ were expressed strongly in the intestinal tract and weakly in pancreas (Table 39.1).

Although Reg IV was not found in the same chromosome as other members of human Reg gene and Reg-related gene (Table 39.1), it shared some common features with other members such as: sequence homology, tissue expression profiles, and exon-intron junction genomic organization (Hartupee et al. 2001). Several Reg III proteins are highly expressed in the pancreas and small intestine, including mouse Reg-IIIγ and human HIP/PAP (hepatointestinal pancreatic/pancreatitis associated protein) (Cash et al. 2006a; Christa et al. 1996) and in the pancreas and GI tract in pathological conditions (Iovanna et al. 1991; Okamoto 1999; Abe et al. 2000). Pancreatitis-associated protein (PAP)-I and -II are members of Reg-III subclass and encoded by gene PAP-I and PAP-II, respectively. PAP-I is also known as peptide 23 in the rat, and regenerating (Reg) islet-derived Reg-IIIβ in mouse, and PAP-II known as Reg-III in the rat and Reg-III-α in the mouse (Narushima et al. 1997). Thus, four types of Reg gene family have been identified. Among these, a total of 17 members have been discovered in mammals across human, pig, mouse, bovine and rat species. Table 39.1 lists some important members of Reg family (Schiesser et al. 2001; Zenilman et al. 1996). Hartupee et al. also suggested that a mouse homologue of Reg IV likely existed (Hartupee et al. 2001) and substantiated (Bishnupuri et al. 2010). While rat Reg cDNA had a single open reading frame that encoded a 165-amino acid protein with a 21-amino acid signal peptide, the human REG cDNA encoded a 166-amino acid protein with a 22-amino acid signal peptide and showed 68% homology to that of rat Reg protein. While some members of the family (Reg 1 and islet neogenesis-associated protein, i.e. INGAP) have been implicated in β-cell replication and/or neogenesis, the roles of the other members have yet to be characterized (Planas et al. 2006).

39.3 Reg 1

39.3.1 Tissue Expression

Reports suggest that the expression of Reg mRNAs and of the corresponding protein(s) was restricted to exocrine tissue irrespective of age, sex, and presence of insulitis and/or diabetes. Moreover, Reg remains localized in acinar cells in the two opposite situations of (a) cyclophosphamide-treated males in a prediabetic stage presenting a high level of both insulin and Reg mRNAs, and (b) the overtly diabetic females with no insulin but a high level of Reg mRNA (Sanchez et al. 2000). The Reg gene was more significantly increased in female mice than in male mice, and in both cases, the expression was not influenced by age. Nondiabetic female mice had a significantly higher expression of the gene than diabetic female mice. Overexpression of the Reg

gene was found in male mice treated with cyclophosphamide, an agent known to be a potent inducer of diabetes in male non-obese diabetic (NOD) mice (Baeza et al. 1997; Baeza et al. 2001). Bimmler et al. (2004) supported the concept that PSP/Reg and PAP are coordinately regulated secretory stress proteins (SSP). The cystic fibrosis (CF) mouse pancreas had constitutively elevated expression of the Reg/PAP cell stress genes, which are suggested to be involved in protection or recovery from pancreatic injury. The severity of caerulein-induced pancreatitis was not ameliorated in the CF mouse even though the Reg/PAP stress genes were already highly upregulated. While Reg/PAP may be protective they may also have a negative effect during pancreatitis due to their anti-apoptotic activity, which has been shown to increase the severity of pancreatitis (Norkina et al. 2006).

The two nonallelic Reg genes and the two insulin genes are expressed differentially during early embryogenesis. The differential expression of Reg-I and -II suggests that they may be induced by different and independent stimuli and have distinct functions (Perfetti et al. 1996). Correlation between Reg and insulin gene expression does not exist in the fetal pancreas during the developmental period but, on the contrary, such a correlation was present in the adult pancreas (Moriscot et al. 1996). Scattered distribution of Reg protein is observed in pancreatic islet cells of streptozotocin (STZ)-treated rats. The increased Reg gene expression in neonatal STZ-treated rat pancreas is a useful model for studying the relationship between Type II (Non-Insulin Dependent) Diabetes (NIDDM) and β cell Regeneration or Reg gene protein.

39.3.2 Gastric Mucosal Cells

Reg mRNA and its product are distributed in the basal part of the oxyntic mucosa and expressed mainly in enterochromaffin-like (ECL) cells. Levels of both Reg mRNA and its product are markedly increased during the healing process of water immersion-induced gastric lesions (Asahara et al. 1996). Although Reg protein is reported to have a trophic effect on gastric epithelial cells, its involvement in human gastric diseases was studied by Fukui et al. (2003). Both gastrin and gastric mucosal inflammation enhance Reg gene expression in the fundic mucosa in rats. The Reg gene is associated with hypergastrinemia and fundic mucosal inflammation and may be involved in *H. pylori*-induced gastritis. The expression of Reg I is controlled through separate promoter elements by gastrin and *Helicobacter* (Bernard-Perrone et al. 1999; Steele et al. 2007). A link between Reg protein and *H. pylori* infection may help explain the molecular mechanisms underlying *H. pylori*-associated diseases, including gastric cancer (Yoshino et al. 2005). During the healing course of gastric erosion, Reg expression is highly increased in ECL cells surrounding the ulcer crater, suggesting its role as a regulator of gastric mucosal regeneration. During healing, the gene expression of several proinflammatory cytokines and Reg was markedly augmented. Among the proinflammatory cytokines, CINC-2 β is the only cytokine in which augmented expression preceded the increase of Reg gene expression. CINC-2 β, expressed in damaged gastric mucosa, stimulates the production of Reg protein in ECL cells via CXCR-2 and may be involved in the accelerated healing of injured gastric mucosa (Kazumori et al. 2000; Fukuhara et al. 2010). REG expression has been observed in various tumors including gastric carcinoma (Dhar et al. 2004; Miyaoka et al. 2004). Gastrin regulates Reg mRNA abundance in human corpus. Mutations of Reg that prevent secretion are associated with ECL cell carcinoids, suggesting a function as an autocrine or paracrine tumor suppressor (Higham et al. 1999).

39.3.3 Ectopic Expression

Ectopic expression of the *Reg* gene occurs in some human colonic and rectal tumors, suggesting that enhanced Reg expression may be related to the proliferative state of tumor cells. Regenerating protein may act not only as a regulator of gastric epithelial cell proliferation but also as a modifier of other multiple physiologic functions (Kazumori et al. 2002). Reg-1 protein is expressed in human hearts obtained from autopsied patients who died of myocardial infarction. In view of emerging evidence of Reg for tissue regeneration in a variety of tissues/organs, it is proposed that the damaged heart may be a target for Reg action and that Reg may protect against acute heart stress (Kiji et al. 2005). While hepatocyte and cholangiocyte proliferation was suppressed, hepatic stem cells and/or hepatic progenitor cells were activated. Reg I was significantly upregulated in liver of the 2-AAF/PH rat model, accompanied by the formation of bile ductules during liver regeneration. The presence of Reg I in normal testis is weak. But its strong expression in the testis cancer suggests its potential role in normal and neoplastic germ cell proliferation (Mauro et al. 2008). Reg I may act through IL-6 to exert effects on squamous esophageal cancer cell biology. Transfecting TE-5 and TE-9 cells with Reg Iα and -Iβ led to significantly increased expression of IL-6 mRNA and protein, but with little or no effect on expression of IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17A, IFN-γ, TNF-α, GC- or TGF-β1. The elevated IL-6 expression in REG Iα transfectants was silenced by siRNA-mediated knockdown (Usami et al. 2010).

Ectopic expression of Reg also was observed in ductal cell carcinomas. In ductal cell carcinomas, expression of Reg immunoreactivity was considered as one of phenotypic heterogeneity, as seen in AAT, lysozyme, and CMG immunoreactivity (Kimura et al. 1992). The diminution in pancreatic β-cell mass caused by subcutaneous implantation of an insulinoma is associated with reduced Reg gene expression and that the increase in β-cell replication after resection of the tumor is preceded by return of Reg gene expression toward normal. *Reg I* expression in untreated endoscopic biopsy specimens may provide a basis for new treatments of locally advanced thoracic squamous cell esophageal cancers (Motoyama et al. 2006). *Reg I*-knockout mice reveal the role of Reg-1 in the regulation of cell growth that is required in generation and maintenance of the villous structure of small intestine (Ose et al. 2007). Reg I appears to enhance the chemo- and radiosensitivity of squamous esophageal cancer cells, which suggests that it may be a useful target for improved and more individualized treatments for patients with esophageal squamous cell carcinoma (Hayashi et al. 2008).

39.3.4 Reg Protein

Unno et al. (1993) isolated two mouse distinct cDNAs and genes, one of which was a mouse homologue to rat and human Reg gene, the other a novel type of Reg gene. They were designated Reg I and Reg II, respectively. The two proteins encoded by these genes share 76% amino acid sequence identity with each other. Both genes span about 3 kbp, and the genomic organization of six exons and five introns is conserved between them. Chromosomal mapping studies indicated that the Reg I gene is localized on mouse chromosome 12, whereas the Reg II gene is localized on chromosome 3. Both Reg I and Reg II mRNAs are detected in the normal pancreas and hyperplastic islets of aurothioglucose-treated mice, but not in the normal islets. It is remarkable that in the gallbladder Reg I is expressed, but Reg II is not (Unno et al. 1993).

Moriizumi et al. (1994) isolated a novel human gene and cDNA encoding a member of the Reg I proteins, Reg Iβ. The gene encodes a 166-amino acid protein which has 22 amino acid substitutions in comparison with previously isolated human Reg protein, Reg Iα. While Reg Iβ was expressed only in pancreas, RegI α was expressed in kidney and stomach as well as in pancreas. The human REG gene has a high degree of similarity to the rat Reg gene. A REG-related sequence (REGL) is also located in 2p12 and expressed in the pancreas and physically mapped within a 100-kb genomic region (Bartoli et al. 1995; Perfetti et al. 1994). Reg may be involved in the expansion of β-cell mass during regeneration as well as in the maintenance of normal β-cell function. Sequence comparisons of Reg and Reg 1 suggested similar exon-intron organisation. The proteins encoded by Reg and Reg 1 comprise 166 amino acids and differ by 22 amino acids only (Bartoli et al. 1995). The 5′-Regulatory region −304/−237 of rat *Reg I* gene encoding a growth stimulating factor for pancreatic β-cells contained positive cis-acting elements. Gel shift assays showed the formation of a specific complex with the −256/−237 oligonucleotide (Miyashita et al. 1995).

39.3.5 *REGIA (Reg Iα)* Gene in Human Pancreas

[Synonym names for Reg1A protein are: pancreatic stone protein; pancreatic thread protein; Islet of Langerhans regenerating protein; regenerating protein I α; Islet cells regeneration factor; ICRF; regenerating islet-derived 1 α].

In human, four REG family genes, i.e., REG 1α, Reg Iβ, REG-related sequence (RS) and HIP/PAP, have been isolated and characterized but only Reg Iα protein has been isolated from human pancreatic secretion. A discoordinate expression of the two *REG* genes was found with a higher level of Reg Iα mRNA in fetus and a higher level of Reg Iβ in adult. In addition, while Reg Iα mRNA level was correlated with the expression of genes encoding exocrine proteins in adults, Reg Iβ mRNA level presented no correlation with any ductular, endocrine, or exocrine gene expression. In human pancreatic cell lines only Reg Iβ gene and protein expressed. These results suggest that two Reg genes and proteins play different roles in human pancreas (Sanchez et al. 2001). Reg Iα is a growth factor known to affect pancreatic islet β cells. Since tropical calcific pancreatitis (TCP) is known to have a variable genetic basis, reports do not suggest the interaction between mutations in the susceptibility genes: serine protease inhibitor Kazal 1 (*SPINK1*) and *CTSB* (a gene for cathepsin B) with REG 1α polymorphisms (Mahurkar et al. 2007).

Function of REGIA (Reg Iα) in Pancreas: Acinar cells can transdifferentiate into other pancreatic-derived cells. In these cells, Reg I overexpression is linked to acinar cell differentiation, whereas inhibition of Reg I leads to β cell and possibly ductal phenotype. Human and rat Reg I proteins, and recombinant protein are mitogenic to primary cultures of β- and ductal cells. Reg I expression in acinar cells is important in maintaining pancreatic cell lineage, and when decreased, cells can dedifferentiate and move toward becoming other pancreatic cells (Sanchez et al. 2004, 2009). Pancreatic-derived cells exposed to Reg I grow by activation of signal transduction pathways involving the mitogen-activated protein kinase phosphatases and cyclins, with concomitant induction of mitogen-activated protein kinase phosphatase (MKP-1). However, high intracellular levels of Reg I lead to decreased growth, likely via a binding to

and inactivation of MKP-1. Inhibition of cell growth, and possible induction of apoptosis, may lead to differentiation of these cells to other cell types (Mueller et al. 2008; Zenilman et al. 1998; Levine et al. 2000). Induction of Reg I and its receptor may be important for recovery from acute pancreatitis (Bluth et al. 2006)

The administration of Reg protein can be used as a therapeutic approach for diabetes mellitus (Watanabe et al. 1994), although the serum Reg protein level is not a marker for progression of type I diabetes (Christofilis et al. 1999). Infact, Pdx1-Cre-mediated pancreas inactivation of IGF-I gene [in pancreatic-specific IGF-I gene-deficient (PID) mice] results in increased β-cell mass and significant protection against both type 1 and type 2 diabetes, where multiple Reg family genes (*Reg-2, -3α,* and *-3β*,) were significantly upregulated in pancreas. Interestingly, Reg family genes were also activated after streptozotocin-induced β-cell damage and diabetes (wild-type T1D mice) when islet cells were undergoing regeneration and Reg proteins increased in exocrine as well as endocrine pancreas, suggesting their potential role in β-cell neogenesis in PID or T1D mice. Reg proteins (Reg 1 and islet neogenesis-associated protein) were also shown to promote islet cell replication and neogenesis (Lu et al. 2006). The administration of poly (ADP-ribose) synthetase/polymerase (PARP) inhibitors such as nicotinamide to 90% depancreatized rats induces islet regeneration. Reg protein induces β-cell replication via the Reg receptor and ameliorates experimental diabetes. Studies suggest that poly(ADP-ribose) polymerase binds Reg promoter and regulates the transcription by autopoly (ADP-ribosyl)ation (Akiyama et al. 2001). Depletion of Reg I, associated with the pathogenesis of impaired glucose tolerance of pancreatitis-associated diabetes, Bluth et al. (2008) support that replacement therapy could be useful in such patients.

REG Iα gene expression and its promoter activity were enhanced by IFN-γ and IL-6. Reg Iα protein promoted cell growth and cell resistance to H_2O_2-induced apoptosis in AGS cells. REG Iα gene is inducible by cytokine stimulation and its gene product may function as a mitogenic and/or an antiapoptotic factor in the development of early gastric cancer (Sekikawa et al. 2005). The IL-6-responsive element was located within the sequence from −142 to −134 of the REG Iα promoter region. Reg Iα protein mediated the antiapoptotic effects of STAT3 signaling in gastric cancer cells by enhancing Akt activation, Bad phosphorylation and Bcl-xL expression. The expression of Reg Iα protein was significantly correlated with that of p-STAT3 in gastric cancer tissues. It appeared that Reg Iα protein plays a pivotal role in anti-apoptosis in gastric tumorigenesis under STAT3 activation (Sekikawa et al. 2008). The enhanced expression of IL-22 in infiltrating inflammatory cells and concommitant enhancement of Reg Iα in the inflamed epithelium indicated the role of IL-22/REG Iα axis in ulcerative colitis (UC). The IL-22-responsive element was located between −142 and −134 in the REG Iα promoter region. REG Iα protein may have a pathophysiological role as a biological mediator for immune cell-derived IL-22 in the UC mucosa (Sekikawa et al. 2010).

Role in Pathology: PAP and Reg Iα are up-regulated during the pancreas regeneration. The PAP expression represents a subset of low-grade, low-stage human hepatocellular carcinomas (HCC) with frequent β-catenin mutation and hence more favorable prognosis, whereas further genetic or epigenetic alterations, such as p53 mutation and Reg 1α expression, lead to more advanced HCCs (Yuan et al. 2005). Three genes encoding related proteins, PAP, Reg Iα and Reg Iβ, are over-expressed in cancer (Rechreche et al. 1999). Human Reg I protein is ectopically expressed in colorectal mucosa at the transition zone of colorectal cancer, and occasionally within the tumor itself. Although ectopic Reg I expression in colorectal epithelia is not a marker for the presence of carcinoma, it may be a sensitive marker for mucosa at risk for development of neoplasia (Zenilman et al. 1997). High levels of REG 1α expression within tumors are an independent predictor of poor prognosis in patients with breast cancer (Sasaki et al. 2008). Reg 1A is a molecular marker of prognostic value and is associated with peritoneal carcinomatosis in colorectal cancer (Astrosini et al. 2008). High levels of REG 1A expression by tumor cells are an independent predictor of a poor prognosis in patients with NSCLC (Minamiya et al. 2008). Stage Ta/T1 urothelial carcinoma of the bladder (Ta/T1 BC) has a marked tendency to reoccur. Expression of REG 1A is an independent predictor of recurrence in Ta/T1 BC (Geng et al. 2009). Reg Iα protein may play a role in the development of gastric cancers (Fukui et al. 2004).

Reg I-expressing cells are present in the bile ductules and increased during regeneration. Reg I is significantly upregulated in the liver of the 2-acetylaminofluorene-PH rat model, accompanied by the formation of bile ductules during liver regeneration (Wang et al. 2009). Reg Iα protein, which was rarely expressed in ductal epithelial cells of normal minor salivary gland (MSG), was overexpressed in patients with Sjögren's syndrome (SS). Reg Iα protein may play a role in the regeneration of ductal epithelial cells in the MSGs of patients with SS (Kimura et al. 2009). REG expresses in Barrett's esophagus. Expression of Reg Iα was more frequently observed in patients who showed squamous re-epithelialization of Barrett's esophagus at biopsy sites (Chinuki et al. 2008). Yamauchi et al. (2009) demonstrated that PPARγ-agonist thiazolidinediones (TZDs) inhibited cell proliferation and Reg Iα protein/mRNA expression in gastrointestinal cancer through a PPARγ-dependent pathway. TZDs may, therefore, be a candidate for novel anti-cancer

drugs for patients with gastrointestinal cancer expressing both REG Iα and PPARγ.

39.3.6 REGIB (Reg Iβ) Gene in Human Pancreas

Human REG Iβ gene encodes a protein secreted by the exocrine pancreas that is highly similar to the Reg1A (Reg 1α) protein. Human Reg I β, also known as secretory pancreatic stone protein 2 and lithostathine 1 β, is a type I subclass member of Reg family. The Reg 1β protein is associated with islet cell regeneration and diabetogenesis, and may be involved in pancreatic lithogenesis. Reg family members REG1A, REGL, PAP and this gene are tandemly clustered on chromosome 2p12 and may have arisen from the same ancestral gene by gene duplication.

Pancreatic juice in vertebrates contains a group of 16-kDa proteins which is composed of the protein species such as pancreatic stone protein (PSP). PSP is a 16-kDa acidic protein with an isoelectric point in the range of pH 5.5–6. A truncated form of this protein was originally isolated from calcium carbonate stones surgically removed from the main pancreatic duct of humans with chronic pancreatitis. PSP is a secretory protein that is related to C-type lectins.

39.4 Pancreatic Stone Protein/Lithostathine (PSP/LIT)

[Alternative names**:** Lithostathine-1-β*;* Regenerating islet-derived protein 1-β/Regenerating protein I β (REG-1β); Pancreatic stone protein 2 (PSP-2)]

39.4.1 Characterization

Reg protein was first found in pancreatic stones. It was named Pancreatic Stone Protein (PSP) and later renamed lithostathine (LIT), as it was assumed to prevent stone formation. The protein was found in regenerating endocrine pancreas and was named Reg (for Regenerating) protein. The functional human Reg gene is a single copy gene, spans approximately 3.0 kbp, and is composed of six exons and five introns. TATA box and CCAAT box-like sequences are located at 27 and 100 bp upstream from the transcriptional initiation site. The human Reg mRNA was detected predominantly in the pancreas, and at lower levels in the gastric mucosa and the kidney. Furthermore, the Reg gene was found to be expressed ectopically in colon and rectal tumors. Immunoblot analysis demonstrated several molecular forms (15–18 kDa) of the Reg protein in the pancreas. The 166-amino acid sequence encoded by the human Reg gene contains the 144-amino acid sequence of pancreatic stone protein determined by De Caro et al. (1989) and the partially determined 45-amino acid sequence of pancreatic thread protein (Gross et al. 1985), indicating that the Reg protein, pancreatic stone protein, and pancreatic thread protein are simply different names for a single protein deriving from the Reg gene (Watanabe et al. 1990). The 144 amino acid protein is O-glycosylated on Thr-5. The glycan chain is variable in length and in charge. The PSP/LIT 3-D structural organization is of the C-lectin type, even though CRD of PSP'LIT is unlikely to have any functional calcium-binding site. However, the PSP/LIT binds Ca^{2+} with 1:1 stoichiometry (Lee et al. 2003). EPR studies, using divalent vanadyl (VO^{2+}) ion as a paramagnetic substitute for Ca^{2+} also showed that VO^{2+} binds to PSP/LIT with a metal:protein binding stoichiometry of 1:1 and that VO^{2+} competes with Ca^{2+} in binding to PSP/LIT. Mutations of a cluster of acidic residues on the molecular surface resulted in almost complete loss (95–100%) of binding of Ca^{2+} and VO^{2+}, showing that these residues are critical for calcium binding by PSP/LIT (Lee et al. 2003). The Arg^{11}-Ile^{12} bond is readily cleaved by trypsin and the resulting C-terminal polypeptide precipitates at physiological pH and tends to form fibrils.

The rat PSP/LIT gene was characterized over 2.7 kbp of gene sequence and 2.43 kbp of 5′-flanking sequence. The lithosathine sequence spanned over six exons. The promoter region contained the TATAAA and CCAAT consensus sequences 30 and 107 bp upstream of the cap site, respectively. Furthermore, a tract of (TG)22 repeat, with potential Z-DNA conformation, was found at position-1081 (Dusetti et al. 1993). The rat cDNA encoding a protein homologous to Reg encodes a protein, designated Reg-2 and shows 60%, 78% and 61% similarities with the reported amino acid sequences of the rat, bovine and human proteins, respectively (Kamimura et al. 1992). Using in situ hybridization, the three rat PAP, and the related REG genes mappped in the same chromosomes region, namely 4q33—>q39. This rat chromosome region is thus homologous to the human 2p12 region, which also contains the PAP gene, the REG1A gene, and a REG-related gene (REGL) suggesting the tandem organization of Reg/lithostathin genes (Gharib et al. 1993; Stephanova et al. 1996).

Reg-I (PSP) and Reg-III (PAP) are induced after the onset of acute pancreatitis, and both have been proposed as potential markers of pancreatitis. After induction of pancreatitis, serum levels of Reg I and III protein differ significantly. PSP/Reg is up-regulated in blood after trauma and its level is related to the severity of inflammation. The serum PSP/Reg-protein concentration may reflect pancreatic damage, especially in acute pancreatitis, and may be a sensitive a marker for such damage as elastase-1, although false positivity was apparent inrenal failure and in some patients with hepatic

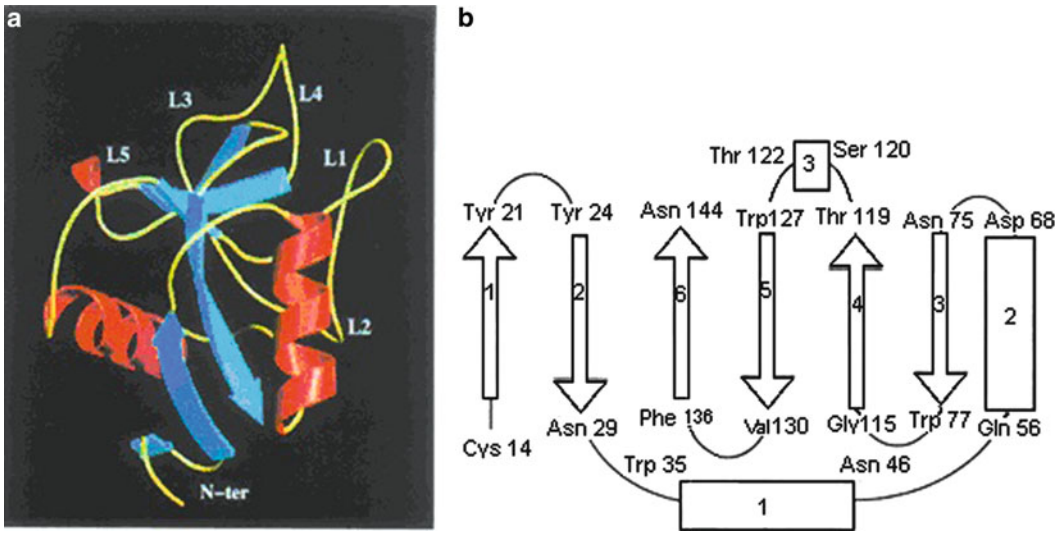

Fig. 39.1 Structure of hPSP/hLIT. The protein folds into one globular domain that consists of three α helices (*Boxed*) and six β-strands, the latter forming two antiparallel β-sheets (**a**). The sequential connectivity follows the scheme β1-β2-αl-α2-β3-β4-α3-β5-β6 (**b**). (**b**) shows schematic diagram of hLIT polypeptide topology. The β-strands are depicted as *arrows*, with the *arrowheads* indicating the direction of the chain. α-Helices are shown as *rectangles* (Reprinted by permission from Macmillan Publishers Ltd: EMBO J, Bertrand et al. © 1996)

dysfunction or digestive system malignancies (Satomura et al. 1995). Furthermore, PSP/Reg binds to and activates neutrophils. Therefore, PSP/Reg and Reg-III/PAP are acute-phase proteins that could serve as a marker for disease severity and posttraumatic complications (Keel et al. 2009; Zenilman et al. 2000). PSP/LIT/Reg mRNA was strongly induced by serum from rats with acute pancreatitis (SAP) in AR-42J cells. Treatment with interleukins (IL) IL-1 or IL-6 or dexamethasone alone was ineffective. Combination of IL-6 with dexamethasone resulted in strong induction of the PSP/LIT/Reg gene, but the further addition of IL-1 to the mixture reduced induction. Treatment with tumor TNFα or IFNγ induced PSP/LIT/Reg mRNA expression. Findings suggest that expression of the PSP/LIT/Reg mRNA during acute pancreatitis could be mediated by specific combinations of cytokines and/or glucocorticoids.

39.4.2 3D-Structure

Crystals of human PSP/LIT grown in PEG 4000 as the precipitating agent, belong to the hexagonal space group $P6_1$ (or its enantiomorph $P6_5$) and diffract to 1.55 A resolution. There is one molecule in the asymmetric unit and the crystals have 39% solvent (Pignol et al. 1995).

The C-terminal part of hPSP/hLIT is homologous with the animal C-type lectins showing the highest percentage of homology with the rat cartilage proteoglycan and the human IgE receptor (Petersen 1988). Amino acid similarities are distributed over regions corresponding to the consensus CRD and two of the three disulfide bridges in hPSP/hLIT are conserved in the C-type lectin sequence motif (Drickamer 1999). Studies on the three-dimensional structure of hPSP/hLIT confirmed that hPSP/hLIT belongs to the family of C-type lectins. Overall protein structure of hPSP/hLIT has the shape of a heart with dimensions 45 × 30 × 25 A (Fig. 39.1a) (Bertrand et al. 1996). The protein folds into one globular domain that consists of three α helices and six 5-strands, the latter forming two antiparallel β-sheets. The sequential connectivity follows the scheme β1-β2-αl-α2-β3-β4-α3-β5-β6 (Fig. 39.1b). The well-defined N-terminal residue of the structure participates in an intramolecular disulfide bond (Cysl4-Cys25) forming a loop. This loop contains a short β-strand (β1; residues 19–21) which forms an anti-parallel β1-sheet with strands β2 and β6. The two major helices αl and α2, of respectively three and four helical turns, are oriented perpendicular to one another framing the three β-strands (β1, β2 and β6) of the lower half of the molecule (Fig. 39.1a). The Cys42- Cys140 disulfide bridge connects αl to α6. The upper half of the molecule includes the other three β-strands (β3, β4 and β5). Two strands of this second antiparallel β-sheet, β4 and β5, are separated by a single helical turn (α3) and are connected at one extremity by the Cys 115-Cys 132 disulfide bridge. In addition to the secondary structure elements, the structure also contains several extended loops and stretches of non-regular secondary structure which represent >60% of the molecule.

39.4.3 Secretory Forms of PSP

Secretory forms of pancreatic stone protein (sPSP, Mr 17,500-22,000) have been purified from human pancreatic juice. Secretory forms of PSP are inhibitors of CaCO3 crystal

growth. Proteins homologous to human sPSP are present in other mammalian species and may act as stabilizers of Ca^{2+}-supersaturated pancreatic juice (Bernard et al. 1991). In mature pancreas, expression of Reg/sPSP gene occurs primarily in acinar cells. The gene product, which encodes a secretory protein inhibiting CaCO3 crystal growth in juice, is unlikely to play a specific role in islet regeneration (Rouquier et al. 1991). A cDNA encoding pre-sPSP revealed that it comprised all but the 5′ end of sPSP mRNA, which was obtained by sequencing the first exon of the sPSP gene. The complete mRNA sequence is 775 nt long, including 5′- and 3′-noncoding regions of 80 and 197 nucleotides, respectively, attached to a poly(A) tail of approximately 125 nt. It encodes a preprotein of 166 amino acids, including a prepeptide of 22 amino acids. PSP-S gene expression is specifically reduced in chronic calcifying pancreatitis (CCP) patients (Giorgi et al. 1989). Ovocleidin 17, a major protein of the chicken eggshell calcified layer shows a C-type lectin domain with a sequence similariy of 30% to that of PSP and lectins and anticoagulant proteins from snake venom (Mann and Siedler 1999).

Serum PSP showed an isoelectric point (pH 9) similar to that reported for the pancreatic thread protein. Schmiegel et al. (1990) did not support the etiopathogenic role postulated for pancreatic stone protein in chronic pancreatitis and chronic calcifying pancreatitis by other investigators. The PSP in human calculi derives from human pancreatic juice (PSP S2-5) through the tryptic cleavage of the Arg-^{11}-Ile-^{12} bond (De Caro et al. 1989; Rouimi et al. 1987). EM studies revealed that the stone protein was markedly present in the zymogen granules and condensing vacuoles of the normal pancreatic acinar cells, the label was found in the acinar and ductal lumen. In chronic pancreatitis, the localization of PSP/LIT, when it occurred, was extremely weak in the acinar cells. No PSP/LIT was specifically characterized in hepatocytes, gastric mucosa, and enterocytes. However, a weak but specific reaction was found in the secretory granules of Paneth cells (Lechene de la Porte et al. 1986).

De Reggi et al. (1995) isolated the major oligosaccharide chains of human pancreatic PSP/LIT and determined their sequences by means of NMR. There were 11 different glycoforms and seven of them were sequenced. They all were from the same site of glycosylation (Thr5) and displayed the same core 2 structure: GlcNAc(β 1–6)[Gal(β 1–3)]GalNAc α-. They ranged in size from 4 to 9 sugar residues. Elongation was found to proceed from a common tetrasaccharidic core: Gal(β 1–4)GlcNAc(β 1–6)[Gal(β 1–3)]GalNAc-ol through N-acetyllactosamine units. The non-reducing ends of some oligosaccharides carry the antigenic determinant H, with presence of external Fuc linked only in (α 1–2) to Gal. All the glycans, except one, carry a sialic acid in (α 2–3) linkage to Gal, with one disialylated form which displays a supplementary (α 2–6) linkage. These findings are consistent with the polymorphism of the protein, either in its native form or after enzymic processing.

39.4.4 Functions of PSP/LIT

39.4.4.1 As Inhibitor of Crystal Growth

Calcium carbonate crystals are observed in the juice of patients suffering with chronic calcifying pancreatitis (CCP), a lithogenic disorder characterized by the presence of stones, which obstruct pancreatic ducts and remain scarce or absent in juice from healthy individuals (De Caro et al. 1988). Several lines of evidence indicate that the inhibition of calcium carbonate crystal nucleation and growth in human pancreatic juice is exerted by human lithostathine (hPSP/hLIT); patients suffering from CCP have an abnormally low concentration of hPSP/hLIT (Bernard et al. 1995). The hPSP/hLIT binds to the surface of calcium carbonate crystals, and provides inhibitory effects (Bernard et al. 1992). Inhibitory effects of hPSP/hLIT can be seen with the entire protein, which shows an activity 100-fold greater (Geider et al. 1996; Gerbaud et al. 2000) than that of 11-residue N-terminal peptide obtained after cleavage with trypsin (Bernard et al. 1992). Consequently, it is likely that both the N-terminal peptide and the C-terminal domain bind to calcium carbonate crystals; a conclusion reinforced by the fact that C-terminal proteolytic fragment (residues 12–144) is a major component of pancreatic stones (De Caro et al. 1988).

As pancreatic juice is supersaturated with respect to calcium carbonate, it was hypothesized that PSP/LIT stabilizes pancreatic juice and calcite precipitation is prevented by PSP/LIT; the decreased PSP levels could be a key factor in the growth of calcium carbonate crystals and stone development during the course of chronic calcifying pancreatitis (CCP) (Multigner et al. 1985). The secretary PSP (sPSP) could play that role, since an activity inhibiting the nucleation and growth in vitro of CaCO3 crystals was found in pancreatic juice, associated with these proteins. Moreover, sPSP concentration was significantly lower in the pancreatic juice of patients with CCP than in control patients. While aiming at discovering how peptides inhibit calcium salt crystal growth, Gerbaud et al. (2000) showed that the peptide backbone governed the binding more than did the lateral chains. The ability of peptides to inhibit crystal growth is essentially based on backbone flexibility. Proteins homologous to sPSP were also found in the dog, rat, swine, monkey and ox. They constitute a new family of pancreatic secretory proteins, whose biological role would be to maintain pancreatic juice in a stable state towards CaCO3 (De Caro et al. 1988).

The inhibition property of crystal nucleation and growth property of hPSP/hLIT is shared by antifreeze proteins (AFPs) in fish. The AFPs are synthesized and secreted in the liver of marine fishes to provide protection from freezing environments and are classified into three groups (Davies and Hew 1990); type I (helical peptides, M_r 3–5 K), type II (cysteine-rich, M_r 14 K), and type III (predominantly

β-sheet, M_r 6–7 K). The type II AFPs are homologous to hPSP/hLIT, showing sequence similarities distributed over both the N- and C-terminal domains (Ewart et al. 1992; Ng and Hew 1992). PSP/LIT is cleaved by trace amounts of trypsin, resulting in a C-terminal polypeptide and an N-terminal undecapeptide. The N-terminal undecapeptide has been identified as the active site of PSP/LIT regarding crystal inhibition. Thus, it has been assumed to inhibit calcium carbonate precipitation and therefore to prevent stone formation in the pancreatic ducts. This function is, however, debatable (Bimmler et al. 1997) since most of these studies have been carried out in vitro. De Reggi (1998) showed conclusively that PSP/LIT does not inhibit calcium carbonate nucleation and crystal growth. Based on the findings it seemed unlikely that PSP/LIT is a physiologically relevant calcite crystal inhibitor (De Reggi et al. 1998).

39.4.4.2 Role in Regeneration of Islets

For several years it was believed that PSP served as an inhibitor of calcium carbonate precipitation in pancreatic juice, and it was proposed that its name should be changed to "lithostathine" (LIT) (Sarles et al. 1990). However, it was later shown that PSP has no more crystal inhibitory activity than several of the pancreatic digestive enzymes (Bimmler et al. 1997; De Reggi et al. 1998). Studies demonstrated that the expression of PSP/LIT protein is increased during regeneration of islets after nicotinamide treatment and partial pancreatectomy (Terazono et al. 1990; Unno et al. 1993). These observations led to suggest that PSP/LIT may be a protein involved in regeneration (Watanabe et al. 1994) and may act as a growth mediator stimulating the proliferation of β-cells. Tissue culture studies implied a mitogenic activity of PSP/LIT on the growth of various cell types (Zenilman et al. 1996; Fukui et al. 1998), and application of PSP/LIT was observed to partially ameliorate diabetes in NOD mice (Gross et al. 1998; Kobayashi et al. 2000). PSP/LIT is a bifunctional protein which might be involved in the control of the bacterial ecosystem in the intestine. PSP/LIT is not expected to present sugar- or calcium-binding properties (Patard et al. 1996).

39.4.4.3 PSP/LIT in Alzheimer's Disease

PSP/LIT is a found precipitated in the form of fibrils in Alzheimer's disease (AD). Recombinant PSP/LIT is essentially monomeric at acidic pH while it aggregates at physiological pH. Electron microscopic studies of aggregates showed an apparently unorganized structure of numerous monomers which tend to precipitate forming regular unbranched fibrils. Aggregated forms seemed to occur prior to the apparition of fibrils. In addition, these fibrils resulted from a proteolytic mechanism due to a specific cleavage of the Arg^{11}-Ile^{12} peptide bond. It is deduced that the NH_2-terminal undecapeptide of PSP/LIT normally impedes fiber formation but not aggregation. A theoretical model explaining the formation of amyloid plaques in neurodegenerative diseases or stones in lithiasis starting from PSP/LIT has been described (Cerini et al. 1999). PSP/LIT and PAP were significantly increased in the cerebrospinal fluid of patients with AD at the very early stages of AD, and their level remained elevated during the course of the AD. Because PSP/LIT undergoes an autolytic cleavage leading to its precipitation and the formation of fibrils, it is likely that it may be involved in amyloidosis and tangles by allowing heterogeneous precipitation of other proteins (Duplan et al. 2001).

Symptoms of pancreaticobiliary maljunction/choledochal cysts are caused by the obstruction of bile and pancreatic ducts due to protein plugs compacted in the common channel. PSP is reported to be a key protein to form protein plugs in chronic pancreatitis (Chen et al. 2005; Ochiai et al. 2004). PSP expression is not increased because of a protein-deficient diet. After a temporary increase in PSP levels due to a carbohydrate-deficient high-protein diet, there were no signs of a diet-dependent regulation of this protein, which may differ from that of other pancreatic secretory proteins. These findings contradict earlier reports that had drawn conclusions based solely on mRNA levels (Bimmler et al. 1999). Both chronic ethanol consumption and dietary protein deficiency increase the capacity of the pancreatic acinar cell to synthesize PSP/LIT (Apte et al. 1996). Alternatively, activated trypsin cleaves soluble PSP and creates insoluble PSP.

39.5 Pancreatic Thread Protein

39.5.1 Pancreatic Thread Proteins (PTPs)

PTPs are acinar cell products and members of the regenerating gene (Reg) family. PTP/Reg protein was mitogenic to both β-cell and ductal cell lines but not to mature, nondividing islets. This supports the hypothesis that PTP/Reg protein is mediator of β-cell growth and may be involved in modulating the duct-to-islet axis (Zenilman et al. 1996). PTP forms double helical threads in the neutral pH range after purification, undergoing freely reversible, pH-dependent globule-fibril transformation. PTP forms filamentous bundles reminiscent of the paired helical filaments of Alzheimer's disease in vitro. A major 0.9-kb as well as several minor transcripts of PTP have been identified in human pancreas. An excess of serum PTP may filter to CSF through blood brain barrier (Blennow et al. 1995). There were significantly higher levels of PTP mRNA in brains with AD compared with aged controls, with increased amounts of 1.2-, 0.6-, and 0.4-kb transcripts (de la Monte et al. 1990; Ozturk et al. 1989). The experimentally derived

secondary structure of human PTP (HPTP) consists of a significant proportion of β-sheets and β-turns and lesser amounts of α-helical structures. The β-sheet component presumably plays an important role in the pH-dependent globule-fibril transformation of HPTP leading to antiparallel β-sheet structure in the aggregated state. The secondary structure of HPTP and its globule-fibril transformation lend credence to the belief that AD may be viewed as a conformational disease (Renugopalakrishnan et al. 1999).

39.5.2 Neuronal Thread Proteins

Neuronal thread proteins (NTP) are phosphoproteins which are expressed during neuritic sprouting. The 15–18-kDa NTP cluster is associated with development and neuronal differentiation, whereas the 21-kDa and 39–42-kDa species are overexpressed in AD, correlating with neurodegenerative sprouting and synaptic disconnection. Both human and experimental (rat) focal cerebral infarcts revealed up-regulation of NTP gene expression in perifocal neurons (de la Monte et al. 1996a). In AD, high levels of NTP immunoreactivity were detected in neuronal perikarya, neuropil fibers, and white matter fibers (axons). NTP accumulates in cortical neurons and colocalizes with phospho-tau-immunoreactive cytoskeletal lesions that correlate with dementia. NTPs overexpression in relation to paired helical filament-associated neurodegenerative lesions in AD has been indicated (de la Monte et al. 1997). In view of the rapid phosphorylation, the accumulation of NTP in AD cortical neuronal perikarya suggests a problem related to post-translational processing and transport of NTP molecules in AD neurodegeneration (de la Monte et al. 1996b). Primary human primitive neuroectodermal tumor (PNETs), malignant astrocytomas, and several human PNET and glioblastoma cell lines also express thread protein immunoreactivity. However, in addition to the 21-kDa species, there are ~8, 17 and ~14-kDa thread protein-immunoreactive molecules expressed in both PNET and glioblastoma cell lines. Glycosylated residues were not detected in either the PNET- or glioblastoma-derived thread proteins. Studies suggest that there are several distinct neuronal and glial derived thread proteins expressed in the central nervous system and that their levels of expression may be modulated with cell growth (Xu et al. 1993).

Insulin, insulin-like growth factor, type 1 (IGF-1), and nerve growth factor modulate NTP gene expression during neuronal differentiation in PNET cell lines. Insulin effected neuronal differentiation and modulation of NTP gene expression in PNET cells utilizes a signal transduction cascade that requires tyrosyl phosphorylation of IRS-1 (Xu et al. 1995b). Ethanol may inhibit NTP expression associated with CNS neuronal differentiation by uncoupling the IRS-1-mediated insulin signal transduction pathway (Xu et al. 1995a). Studies indicate a functional role for NTP in relation to the turnover or processing of neuronal cytoskeletal proteins, attributes that may be modulated by insulin/IGF-1-mediated signaling (de la Monte et al. 2003). Further studies suggest that reduced survival in neurons that over-express AD7c-NTP may be mediated by impaired insulin/IGF-1 (de la Monte and Wands 2004). Brains with AD, AD + Parkinson's disease (PD), and AD + Down's syndrome (DN) contain significantly higher densities of NTP immunoreactive neurons and more frequent immunostaining of neuropil and white matter fibers compared with PD dementia (PDD) and aged controls which had few or no AD lesions.

39.5.3 Pancreatic Proteins Form Fibrillar Structures upon Tryptic Activation

Graf et al. (2001) studied the structural/functional consequences of trypsin activation on 16-kDa proteins with respect to the kinetics of conversion from soluble to insoluble protein forms and the kinetics of assembly of protein subunits into polymerized thread structures. Trypsin activation of recombinant stress proteins or counterparts (PSP/Reg, PAP-I and PAP-III) resulted in conversion of 16-kDa soluble proteins into 14-kDa soluble isoforms [called pancreatic thread protein and pancreatitis-associated thread protein (PATP), respectively] that rapidly polymerize into sedimenting structures. Activated thread proteins show long lived resistance to a wide spectrum of proteases contained in pancreatic juice, including serine proteases and metalloproteinases. In contrast, PAP-II, following activation with trypsin or pancreatic juice, did not form insoluble structures and was rapidly digested by pancreatic proteases. SEM and TEM demonstrated that activated thread proteins polymerized into highly organized fibrillar structures with helical configurations. Through bundling, branching, and extension processes, these fibrillar structures formed dense matrices that span large topological surfaces (Fig. 39.2) (Graf et al. 2001). Such studies suggested that PSP/Reg and PAP-I and III isoforms consist of a family of highly regulated soluble secretory stress proteins, which, upon trypsin activation, convert into a family of insoluble helical thread proteins. Dense extracellular matrices, composed of helical thread proteins organized into higher ordered matrix structures, may serve physiological functions within luminal compartments in the exocrine pancreas.

39.5.3.1 PSP/LIT in Alzheimer's Disease

The term 'cerebral proteopathies' has been proposed to designate all brain diseases the hallmarks of which are the misfolding and subsequent aggregation of proteins (Walker and LeVineH 2000). The most characteristic feature of many neuron-degenerative diseases is the formation of fibrillar

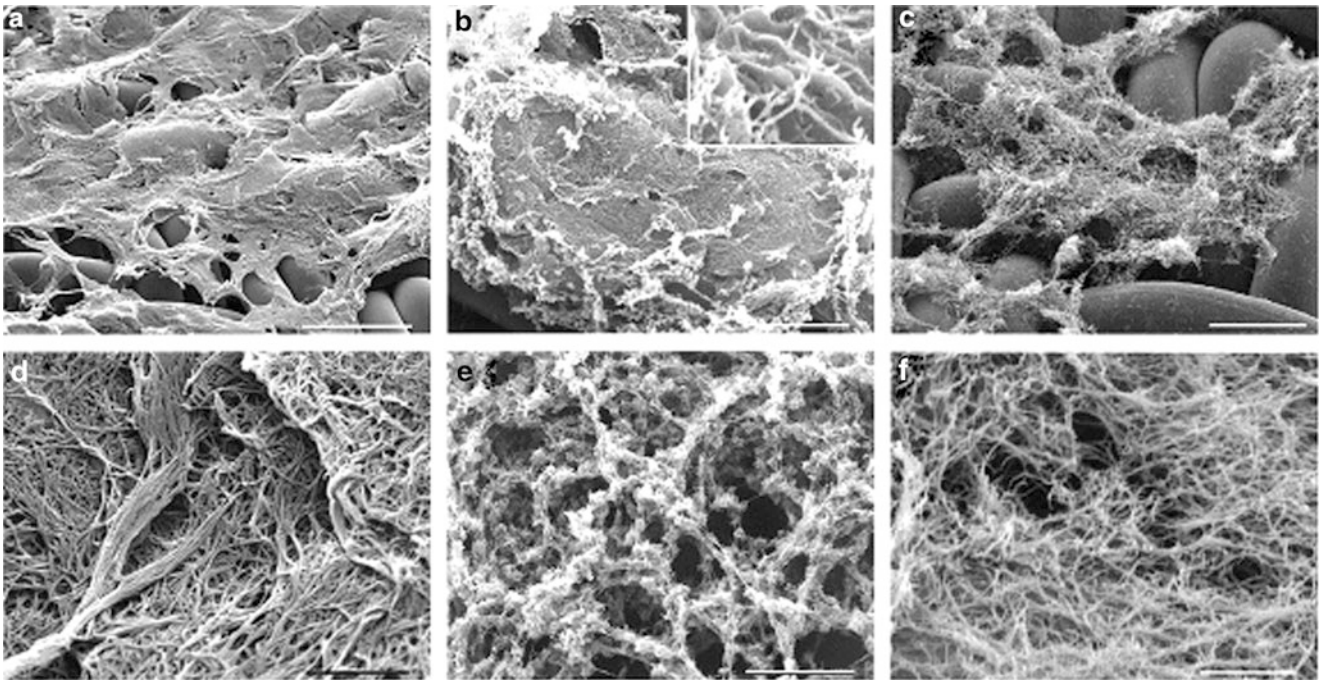

Fig. 39.2 Trypsin activation of purified recombinant secretory stress proteins generates a matrix of highly organized fibrils. Scanning electron microscopy of PTP and PATP generated in vitro. The micrographs demonstrate fibrous networks following activation of PAP-I, PAP-III and PSP/reg with trypsin. (**a**) and (**d**) show the matrix obtained with PATP I (**a**, bar 70 mm; **d**, 3 mm). (**b**) and (**e**) show the matrix obtained with PATP III (**b**, 20 mm; **e**, 7 mm). (**c**) and (**f**) show the matrix obtained with PTP (**c**, 40 mm; **f**, 4 mm). Inset in (**b**) gives a higher magnification micrograph showing individual fibrils that emerge from the dense matrix. (**a**) and (**c**), part of the plastic mesh of the pouch can be seen (Reprinted with permission from Graf et al. 2001© American Society for Biochemistry and Molecular Biology)

aggregates rather than the type of protein involved. Several neuron-degenerative diseases, such as AD are characterized by fibrillar deposits of proteins or peptides in brain. Many of these deposits are characterized by properties similar to those of starch and hence were called amyloid structures. Among proteins that form clinical fibril deposits, PSP/LIT readily polymerizes into fibrils after self-proteolysis of its N-terminal undecapeptide (Cerini et al. 1999). Autocatalytic cleavage leads PSP/LIT to the formation of quadruple-helical fibrils (QHF-litho) that are present in AD, and overexpressed during the very early stages of the disease before clinical signs appear (Duplan et al. 2001). PSP/LIT does not appear to change its native-like, globular structure during fibril formation. Therefore, it was thought that PSP/LIT constitutes an important protein in the deposition of polypeptides in vivo in relation to neurodegenerative diseases

Grégoire et al. (2001) studied 3D structure of hPSP/hLIT protofibrils using atomic force microscopy. These aggregates consisted of a network of protofibrils, each of which had a twisted appearance. Electron microscopy analysis showed that this twisted protofibril has a quadruple helical structure. Three-dimensional X-ray structural data and the results of biochemical experiments demonstrated that when forming a protofibril, hPSP/hLIT was first assembled via lateral hydrophobic interactions into a tetramer. Each tetramer then linked up with another tetramer as the result of longitudinal electrostatic interactions. All these results were used to build a structural model for the PSP/LIT protofibril called the quadruple-helical filament (QHF-litho) (Laurine et al. 2003). Ho et al. (2006) investigated the structural properties of recombinant hPAP lacking N-terminal propeptide, both in the soluble form and as fibrillar aggregates. Based on the solution structure analysis and the TANGO prediction, peptide hPAP84–116 was synthesized and found to form fibrils. The mechanism of fibril formation by hPAP has been compared with that of hPSP/hLIT. Over-all, studies support the idea that PSP/LIT may play a role in the ethiology of AD and hPSP/hLIT strongly resembles the prion protein in its dramatic proteolysis and amyloid proteins in its ability to form fibrils.

39.6 REG-II/REG-2/PAP-I

The 16 kDa Regenerating islet derived-2 (REG-2) has also been referred to as Islet of Langerhans regenerating protein 2, Lithostathine-2, Pancreatic stone protein 2, or Pancreatic thread protein 2. The rat cDNA for this gene has been cloned by Kamimura et al. (1992). Reg-2 is the

murine homologue of the human secreted HIP/PAP C-type lectin. Reg-2, related to the protein encoded by the REG gene, is identical with PAP-1 (pancreatitis-associated protein-1) and a β-cell-derived autoantigen in non-obese diabetic mice. The autoimmune response against this protein may convert a regenerative into an islet-destructive process accelerating development of type 1 diabetes (Gurr et al. 2007; Liu et al. 2008). Reg-2 is a likely mouse exocrine pancreas cytoprotective protein whose expression is regulated by keratin filament organization and phosphorylation (Zhong et al. 2007).

Motor neurons are the only adult mammalian neurons of the CNS to regenerate after injury. This ability is dependent on the environment of the peripheral nerve and an intrinsic capacity of motor neurons for regrowth. The 16 kDa Reg-2, that is expressed solely in regenerating and developing rat motor and sensory neurons, is a potent Schwann cell mitogen in vitro. In vivo, Reg-2 protein is transported along regrowing axons and inhibition of Reg-2 signaling significantly retards the regeneration of Reg-2-containing axons. During development, Reg-2 production by motor and sensory neurons is regulated by contact with peripheral targets. Strong candidates for peripheral factors regulating Reg-2 production are cytokines of leukemia inhibitory factor (LIF)/CNTF family, because Reg-2 is not expressed in developing motor or sensory neurons of mice carrying a targeted disruption of LIF receptor gene (Livesey et al. 1997). Purified Reg-2 can itself act as an autocrine/paracrine neurotrophic factor for a subpopulation of motoneurons, by stimulating a survival pathway involving phosphatidylinositol-3-kinase, Akt kinase and NF-kB and therefore Reg-2 expression is a necessary step in the CNTF survival pathway in late embryonic spinal cord (Nishimune et al. 2000; Fang et al. 2010).

Reg-2 is normally not expressed by dorsal root ganglion (DRG) cells but Reg-2 is rapidly upregulated in DRG cells after sciatic nerve transection and after 24 h recovery is expressed almost exclusively in small-diameter neurons (Averill et al. 2002). Subsequently, expression shifts from small to large neurons (Nishimune et al. 2000). Reg-2 is also upregulated by DRG neurons in inflammation with a very unusual expression pattern. In a rat model of monoarthritis, Reg-2 immunoreactivity was detected in DRG neurons at 1 day. In addition, the presence of Reg-2 in central axon terminals implicates Reg-2 as a possible modulator of second order dorsal horn cells (Averill et al. 2008).

Analysis on gene-expression made in cardiomyocytes during myocarditis, revealed that the Reg-2/PAP-I mRNA level is most markedly increased in cardiomyocytes rather than in noncardiomyocytes. Reg-2/PAP-I mRNA was approximately 2000-fold greater in cardiomyocytes under active myocarditis than in normal. Moreover, Reg-2/PAP-I protein and other Reg/PAP family gene expressions were remarkably increased in EAM hearts. In addition, IL-6 expression was significantly related to Reg-2/PAP-I. Therefore, the Reg/PAP family, which was found to dramatically increase is suspected to play an important role in myocarditis (Watanabe et al. 2008).

To assess the role of Reg-2, Lieu et al. (2006) used Reg-$2^{-/-}$mice in a model of fulminant hepatitis induced by Fas and in the post-hepatectomy Regeneration and demonstrated that Reg-2 deficiency enhanced liver sensitivity to Fas-induced oxidative stress and delayed liver regeneration with persistent TNFα/IL6/STAT3 signaling. In contrast, overexpression of human HIP/PAP promoted liver resistance to Fas and accelerated liver regeneration with early activation/deactivation of STAT3. Reg-2/HIP/PAP-I is therefore a critical mitogenic and antiapoptotic factor for the liver (Lieu et al. 2006).

Reg-2 expression was found to be upregulated in pancreatic islets both during diabetes development and as a result of adjuvant treatment in diabetic NOD mice and in C57BL/6 mice made diabetic by streptozotocin treatment. The upregulation of Reg-2 by adjuvant treatment was independent of signaling through MyD88 and IL-6. Upregulation of Reg-2 was also observed in the pancreas of diabetic mice undergoing β cell regenerative therapy with exendin-4 or with islet neogenesis-associated protein. Adjuvant immunotherapy regulates T1D in diabetic mice and induces Reg-2-mediated regeneration of β cells (Huszarik et al. 2010). In contrast to pancreatic islet neogenesis-associated protein [INGAP, Reg3δ] promote the growth or regeneration of the endocrine islet cells, Reg-2 overexpression did not protect acinar cells against caerulein-induced acute pancreatitis, indicating clear subtype specificities of the Reg family of proteins (Li et al. 2010). Pancreas-specific ablation of IGF-I in mice induced an over-expression of Reg-2 and Reg3β in the pancreas and protected them from streptozotocin (Stz)-induced β-cell damage. This indicated that Reg-2 protects insulin-producing cells against Stz-induced apoptosis by interfering with its cytotoxic signaling upstream of the intrinsic proapoptotic events by preventing its ability to inactivate JNK (Liu et al. 2010).

39.7 Reg-III

39.7.1 Murine *Reg-IIIα, Reg-IIIβ, Reg-III γ,* and *Reg-IIIδ* Genes

Human type III REG gene is divided into six exons spanning about 3 kb, and encodes a 175 aa protein with 85% homology with HIP/PAP. REG-III was expressed predominantly in pancreas and testis, but not in small intestine, whereas HIP/PAP was expressed strongly in pancreas and small intestine.

IL-6 responsive elements existed in the 5′-upstream region of human REG-III gene indicating that human REG-III gene might be induced during acute pancreatitis (Nata et al. 2004). The murine cDNAs type III Reg, Reg-IIIα, Reg-IIIβ and Reg-IIIγ encode 175-, 175- and 174- aa proteins, respectively, with 60–70% homology. All three genes are composed of six exons and five introns spanning approx. 3 kb, and exhibit distinctive structural features unique for members of the Reg gene family. All mouse Reg genes, Reg-IIIα, Reg-IIIβ, Reg-IIIγ, Reg-I and Reg-II, are assigned to the adjacent site of chromosome 6C. The Reg family genes were mapped to a contiguous 75 kb region of mouse genome according to the following order: 5′-Reg-IIIβ-Reg-IIIα-Reg II-Reg I-Reg-IIIγ-3′. Reg-IIIα, Reg-IIIβ and Reg-IIIγ were expressed weakly in pancreas, strongly in intestinal tract, but not in hyperplastic islets, whereas both Reg-I and Reg-II were expressed in hyperplastic islets (Narushima et al. 1997). REG3α is normally expressed in pancreatic acinar and endocrine cells. Transfection assays suggest that REG3α stimulates β-cell replication, by activating Akt kinase and increasing the levels of cyclin D1/CDK4 (Cui et al. 2009).

Abe et al. (2000) sequenced the 6.8 kb interspace fragment between Reg-IIIβ and Reg-IIIα and encountered a novel type III Reg gene, Reg-IIIδ. Reg-IIIδ gene is divided into six exons spanning about 3 kb, and encodes a 175 amino acid protein with 40–52% identity with the other five mouse Reg proteins. Reg-IIIδ was expressed predominantly in exocrine pancreas, but not in normal islets, hyperplastic islets, intestine or colon, whereas both Reg I and Reg II were expressed in hyperplastic islets and Reg-IIIα, Reg-IIIβ and Reg-IIIγ expressed strongly in the intestinal tract. In mouse, type I, type II and type III Reg genes (i.e. *Reg-I, Reg-II*, and *Reg-III* gene) have also been isolated.

The rat PAP-III gene spans over 2.5 kb sequences along with a 1.7 kb of 5′-flanking sequence. The PAP-III coding sequence spanned over six exons. There were striking similarities between PAP-III and PAP-I and II genes, in genomic organization as well as in promoter sequences. Moreover, the rat PAP-III gene was mapped to chromosome 4 which coincides with that of PAP-I and II genes. The three genes are derived from same ancestral gene by duplication. Expression of the PAP-III gene was compared with that of PAPs I and II (Dusetti et al. 1995). Rat cDNA and a regeneration-promoting gene encodes a 174-amino-acid (aa) Reg-III protein with a 25-aa signal peptide. Reg-III was expressed in regenerating pancreatic islets, but not in normal islets (Suzuki et al. 1994). Analysis of the open reading frame of a cDNA indicated that the deduced protein from the mRNA was a polypeptide of 174 amino acids, unexpectedly similar to that of rat PAP-II/Reg-III (Honda et al. 2002). However, the length of the identified mRNA (1,467 bp) was longer than that of rat PAP-II mRNA (885 bp), because the elongated mRNA was generated through a different polyadenylation site in the same gene. The elongated mRNA after acute pancreatitis was strongly induced in the restricted early phase, in comparison with the original mRNA. It was suggested that the elongated mRNA affects the function of PAP-II/Reg-III protein because the elongated mRNA with long three; untranslated regions is known to be involved in the translation efficiency. The identified mRNA may play an important role in the progression of pancreatitis (Honda et al. 2002). Proteomic approach identified that rat Reg-III could be functionally associated with Reg I (Shin et al. 2005).

39.7.2 Human Reg-III or HIP/PAP

Reg-III was strongly induced in gut epithelial cells following bacterial reconstitution, as well as in the colitis initiated by DSS. The mRNA expression of HIP/PAP was enhanced in colonic epithelial cells of patients with inflammatory bowel disease (IBD). Reg-III mRNA expression was localized in the epithelial cells including goblet cells and columnar cells in mice; on the other hand, HIP/PAP-expressing cells were correlated with Paneth cell metaplasia in human colon. Epithelial expression of Reg-III or HIP/PAP was induced under mucosal inflammation initiated by exposure to commensal bacteria or DSS as well as inflamed IBD colon (Dieckgraefe et al. 2002). *Salmonella* infection increased ileal mucosal PAP/Reg-III protein levels in enterocytes located at the crypt-villus junction. Increased colonization and translocation of *Salmonella* was associated with higher ileal mucosal PAP/Reg-III levels and secretion of this protein in feces. PAP/Reg-III protein is increased in enterocytes of the ileal mucosa during *Salmonella* infection and is associated with infection severity. Fecal PAP/Reg-III might be used as a new and non-invasive infection marker (van Ampting et al. 2009).

REG-IIIα and REG-1α are activated in many human hepatocellular carcinomas (HCC), REG-IIIα is down-regulated in most primary human gastric cancer cells. In gastric cancer REG-IIIα might be useful in the diagnosis of cancer (Choi et al. 2007). Two genes involved in HCC belong to REG family. They encode the regenerating islet-derived 3 α (REG-IIIα/HIP/PAP/REG-III) and 1α (REG-1α) proteins, both involved in liver and pancreatic regeneration and proliferation. REG-IIIα is a target of β-catenin signaling in Huh7 hepatoma cells. The upregulation of REG-IIIα and REG-Iα expression is significantly correlated to the β-catenin status in HCC and hepatoblastomas. The Wnt/β-catenin signaling pathway is activated in HCC. Evidence shows that both genes are downstream targets of the Wnt pathway during liver tumorigenesis (Cavard et al. 2006). Results predict that the REGα and REGγ inserts play

virtually no role in oligomerization or in proteasome activation. By contrast, removal of REGβ insert reduces binding of this subunit and REGα/REGβ oligomers to proteasomes. However, findings showed that REG inserts are not required for binding and activating the proteasome (Zhang et al. 1998).

Reg-III proteins bind their bacterial targets via interactions with cell wall peptidoglycan but lack the canonical sequences that support CRD in other C-type lectins. HIP/PAP recognizes the peptidoglycan carbohydrate backbone in a calcium-independent manner via a conserved "EPN" motif that is critical for bacterial killing. While EPN sequences govern calcium-dependent carbohydrate recognition in other C-type lectins, the unusual location and calcium-independent functionality of the HIP/PAP EPN motif suggest that this sequence is a versatile functional module that can support both calcium-dependent and calcium-independent carbohydrate binding. Further, HIP/PAP binding affinity for carbohydrate ligands depends on carbohydrate chain length, supporting a binding model in which HIP/PAP molecules "bind and jump" along the extended polysaccharide chains of peptidoglycan, reducing dissociation rates and increasing binding affinity. The dynamic recognition of highly clustered carbohydrate epitopes in native peptidoglycan is an essential mechanism governing high-affinity interactions between HIP/PAP and the bacterial cell wall.

39.8 Rat PAPs

39.8.1 Three Forms of PAP in Rat

Rat PAP-I, PAP-II, and PAP-III are members of a multigenic family of proteins expressed in several tissues. Rat PAP appears in pancreatic juice after induction of prancreatic inflammation. In acute pancreatitis in rats, PAP was first observed 6 h after induction of pancreatitis with taurocholate or cerulein, reached maximal levels in zymogen granules and in pancreatic tissue during the acute phase (48 h), and disappeared during recovery (day 5). It was never detected in spleen, liver, kidney, heart, or lung. The PAP-I is therefore an acute-phase protein that differs from other proteins of that family because of its exocrine nature. PAP-I is expressed in the pancreas in relation to the severity of cerulein-induced pancreatitis (Keim et al. 1991; Keim et al. 1994). PAP-I was shown to be antiapoptotic, mitogenic, and anti-inflammatory and can promote cell adhesion to the extracellular matrix. PSP/LIT/RegIα can be mitogenic. Because polymerization might regulate activity, the ability of rat PAP-I was examined to interact with itself (homodimerization), PAP-II, PAP-III, and PSP/LIT/RegIα (heterodimerization). PAP-I interacted significantly with all members of the PAP protein family, homodimerization showing the strongest interaction as judged by the β-galactosidase test (Bodeker et al. 1999). A change in gene expression of PAP reflects acute inflammatory changes in the pancreas most sensitively (Funakoshi et al. 1995).

In vitro, dexamethasone and IL-6 induced a marked transcription of PAP-I, II and III genes in AR42J cells. In vivo, pancreas mRNA levels of PAP-I, II or III increased by 2.6-fold, 1.9-fold, and 1.3-fold respectively after dexamethasone treatment. Histopathologic evaluation revealed less inflammation and necrosis in pancreata obtained from dexamethasone treated animals. Thus, dexamethasone significantly decreases the severity of pancreatitis. The protective mechanism of dexamethasone may be via upregulating PAP gene expression during injury (Kandil et al. 2006).

The PAP-I and III**,** but not PAP-II mRNAs are constitutively expressed in the small intestine of rats. Between day 20 of gestation and day 21 of age, PAP mRNAs could barely be detected. Their concentrations increased dramatically from day 21 to day 45 of age and remained constant thereafter. Rats adapted to a diet with low carbohydrate content showed a significant decrease in PAP mRNA concentrations. Gene expression of PAP-I and III mRNAs is regulated in a coordinate manner in the rat small intestine during development and on nutritional and hormonal manipulations (Sansonetti et al. 1995).

Rat PAP mRNA is barely detectable in normal pancreas and overexpressed during acute pancreatitis (Iovanna et al. 1991). Rat PAP mRNA was constitutively expressed in duodenum, jejunum, and ileum, at similar levels as in pancreas during the acute phase of pancreatitis. A weak expression was also detected in several other tissues. Serum from rats with acute pancreatitis (SAP) induced the expression of PAP mRNA in AR-42J cells. in a dose-dependent manner. It suggests that SAP contains factors responsible for the PAP mRNA expression and that the cis-acting elements are localized within the 1.2 kbp upstream region of the transcription initiation site (Dusetti et al. 1994b). Free radicals are involved in the pathogenesis of acute pancreatitis, during which PAP-I is overexpressed. PAP-I expression could be induced by oxidative stress and could affect apoptosis. It appears that during oxidative stress, PAP-I might be part of a mechanism of pancreatic cell protection against apoptosis (Ortiz et al. 1998).

The mRNA level of a PAP-like protein was found to be elevated in the ileal Peyer's patch of lambs during the early phase of scrapie infection. The ovine PAP-like protein cDNA encodes a putative 178 amino acid protein with a signal peptide and a C-lectin binding domain. REG/PAP protein's deduced amino acid sequences were conserved. The overall amino acid identity between the ovine PAP-like protein and

bovine, human and rat REG/PAP proteins varied from 23% to 85%. The expression of the ovine PAP-like protein mRNA was restricted to the ileal and jejunal Peyer's patches. The data provided will offer the possibility to search for a link between this PAP-like protein and early events in the development of scrapie (Skretting et al. 2006).

Although induction of PAP family genes has been reported in peripheral nerve injury models, the expression of PAP in CNS after traumatic brain injury (TBI) induced by weight drop was examined by Ampo et al. (2009). There was a significant upregulation of PAP-I and PAP-III mRNA in the injured cortex beginning at 1 day after TBI. PAP-I and PAP-III staining was localized in a subpopulation of neurons in the peri-injured region. Expression of both PAP-I and PAP-III mRNA was observed following a transient increase in inflammatory cytokines, including TNF-α, IL-6, and IL-1β mRNA. It appears that expression of PAP family members in response to traumatic and inflammatory stimuli are not restricted to the pancreas, intestine, and peripheral nervous system, but are likely a more general cellular response, including the CNS in rat (Ampo et al. 2009). Studies suggest that the Reg family of proteins is protective in acute pancreatitis (Viterbo et al. 2009).

39.8.2 PAP-I/Reg-2 Protein (or HIP, p23)

The rat PAP gene was characterized over 3.2 kb of gene sequence and 1.2 kb of 5′-flanking sequence. Several potential regulatory elements were identified in the promoter region, including a pancreas-specific consensus sequence. The PAP coding sequence spanned over six exons. The first three exons encoded the 5′-untranslated region of the mRNA, the signal peptide, and 39 amino acids of the NH2-terminal end of the mature protein, respectively. The other three exons encoded a domain of the protein with significant homology to the CRD of animal lectins (Dusetti et al. 1993). Analysis of the rat PAP-I promoter indicated that the region between nt −180 and −81 possessed silencer activity in cells that did not express PAP-I. Transient transfection assays revealed that the sequence with silencer activity was located within the rep27 region (position −180/ −153). Suppressor activity was observed when rep27 was inserted upstream from the core PAP-I promoter, in both orientations. Results suggest that the rep27 cis-acting element contributes to the tissue specific expression of PAP-I gene (Ortiz et al. 1997). The promoter of the PAP-I gene represents a potential candidate to drive expression of therapeutic molecules to the diseased pancreas. Studies show that (1) a recombinant adenovirus containing a fragment of the PAP-I promoter allows specific targeting of a reporter gene to the mouse pancreas and (2) expression of the reporter gene in pancreas is induced during acute pancreatitis (Dusetti et al. 1997).

A correlation between PAP-I mRNA levels and glutathione levels seems to exist in the mouse pancreas (Fu et al. 1996). In experimental pancreatitis a correlation was found between the severity of pancreatitis and the amount of PAP in pancreatic homogenates (Keim and Löffler 1986). Consumption of alcohol for short term does not alter serum PAP values during 56 h after drinking. However, longterm drinking induced at least a 10-fold increase in serum PAP. Nordback et al. (1995) support the suggestion that heavy long term drinking often induces subclinical pancreatic damage, but not clinical pancreatitis (Nordback et al. 1995).

Oxidative stress has an important role in the pathogenesis of pancreatitis. PAP-1 is a protein secreted upon induction of acute pancreatitis. As a result, oxidative stress induced PAP-1 mRNA expression in AR42J cells in a time-dependent manner. Cell viability decreased with the concentration of glucose oxides delivered to the cells that had received glucose. Oxidative stress-induced PAP-1 expression was augmented in cells transfected with PAP-1**S** cDNA. PAP-1 induction by oxidative stress decreased in the cells transfected with PAP-1 **AS** cDNA. PAP-1 may be a defensive gene for oxidative stress-induced cell death of pancreatic acinar cells (Lim et al. 2009).

Induction of PAP-I gene was also described in liver during hepatocarcinogenesis. It was noted that expression of PAP-I in hepatocarcinoma (HCC) occurred through inactivation of its silencer element and was not concomitant in all malignant cells. On that basis, PAP-I was assayed in serum from patients with chronic hepatitis, liver cirrhosis or hepatocarcinoma. PAP-I levels were normal in chronic active or persistent hepatitis, significantly higher in cirrhosis and strongly elevated in hepatocarcinoma. Because those clinical entities often develop in that sequence, serum PAP-I appeared as a potential marker of hepatocarcinoma development (Dusetti et al. 1996). However PAP assay can only be recommended in cases of justified suspicion of HCC with negative α-fetoprotein (Montalto et al. 1998). PAP-I was not found in normal melanocytes, melanoma tumors, and melanoma cell lines, even after stress induction. Exogenous PAP-I can modify the adhesion and motility of normal and transformed melanocytes, suggesting a potential interaction with melanoma invasivity (Valery et al. 2001).

39.8.3 PAP-II/PAP2

Frigerio et al. (1993b) cloned two overlapping cDNAs encoding a protein structurally related to PAP. The second PAP, which was called PAP-II, was of same size as the original PAP (PAP-I) and showed 74.3% amino acid homology. PAP-II mRNA concentration increased within 6 h following induction of pancreatitis, reached maximal levels

(>200 times control values) at 24–48 h, and decreased thereafter, similar to PAP-I. However, PAP-II mRNA could not be detected in the intestinal tract or in other tissues. PAP-II genomic DNA fragment was characterized over 2.7 kb of gene sequence and 1.9 kb of 5′ flanking sequence. The PAP-II coding sequence spanned six exons separated by five introns. Several potential Regulatory elements were identified in the promoter region, including two glucocorticoid-response elements and one IL-6-response element. Grønborg et al. (2004) reported a protein that is 85% identical to HIP/PAP and was designated as PAP-II. Two transcripts are generated from PAP-II gene in rat pancreas by alternative splicing in the 5′ untranslated region as shown by the existence of two forms of PAP-II mRNA with identical coding sequence but a different 5′-untranslated region. We demonstrate that this is the result of a differential splicing. (Vasseur et al. 1995).

NR8383 macrophages which were cultured in the presence of PAP2 aggregated and exhibited increased expression of IL-1, IL-6, TNF-α, and IL-10. Chemical inhibition of the NFkB pathway abolished cytokine production and PAP facilitated nuclear translocation of NF-kB and phosphorylation of IkBα inhibitory protein suggesting that PAP2 signaling involves this pathway. Similar findings were observed with primary macrophages derived from lung, peritoneum, and blood but not spleen. Furthermore, PAP2 activity was inhibited by the presence of serum, inhibition which was overcome with increased PAP2. These results demonstrate a new function for PAP2: it stimulates macrophage activity and likely modulates the inflammatory environment of pancreatitis (Viterbo et al. 2008b).

In rats, each of the three PAP-I isoforms has independent immunologic function on macrophages. PAP2 up-regulates inflammatory cytokines in macrophages in a dose-dependent manner and acts through NF-kB mechanisms. Truncation of the first 25 residues on the N terminus of PAP2 did not affect protein activity whereas truncation of the last 30 residues of the C terminus of PAP2 completely inactivated the function of PAP2. Additionally, reduction of three disulfide bonds proved to be important for the activity of this protein. Further investigation revealed two invariant disulfide bonds were important for activity of PAP2 while the disulfide bond that is observed in long-form C-type lectin proteins was not essential for activity. Further, preincubation with select rPAP2 mutant proteins affect translocation of this transcription factor into the nucleus (Viterbo et al. 2008a).

39.8.4 PAP-III

A third member of the rat PAP gene family was described by Frigerio et al. (1993a). The encoded protein, designated PAP-III, shows 66% and 63% identity with the rat PAP-I and II, respectively. The PAP-III gene is constitutively expressed in the small intestine and in the pancreas with acute pancreatitis, but not in the healthy pancreas. In vitro experiments revealed that PAP-III possessed a strong macrophage chemoattractant activity that was comparable with that of monocyte chemoattractant protein-1. PAP-III is involved in peripheral nerve regeneration and provides new insights into Schwann cell-macrophage interactions and therapeutic interventions (Namikawa et al. 2006). The ovulatory process in mammals involves gross physiological events in the ovary that cause transient deterioration of the ovarian connective tissue and rupture of the apical walls of mature follicles. A study revealed that the ovulatory events included induction of mRNA for PAP-III. In situ hybridization indicated that PAP-III mRNA expression was limited mainly to the hilar Region of the ovarian stroma, with most of the signal emanating from endothelial cells that lined the inner walls of blood vessels, and from small secondary follicles. Ovarian transcription of PAP-III mRNA was moderately dependent on ovarian progesterone synthesis. In conclusion, the present evidence of an increase in PAP-III gene expression in gonadotropin-stimulated ovaries provides further evidence that the ovulatory process is comparable to an inflammatory reaction (Yoshioka et al. 2002).

PAP-III is expressed with increased frequency in the bladder urothelium in a rat cystitis model and associated with bladder inflammation and implicates PAP-I in the abnormal sensation in cystitis (Takahara et al. 2008). Reg-III kills Gram-positive bacteria and plays a vital role in antimicrobial protection of the mammalian gut. Reg-III proteins bind their bacterial targets via interactions with cell wall peptidoglycan but lack the canonical sequences that support calcium-dependent carbohydrate binding in other C-type lectins.

Reg-IIIβ is found in many areas of the body and shown to play an important role in both the development and regeneration of subsets of motor neurons. The Reg-IIIβ expressed by motor neurons is both an obligatory intermediate in the downstream signaling of the leukemia inhibitory factor/ciliary neurotrophic factor (CNTF) family of cytokines, maintaining the integrity of motor neurons during development, as well as a powerful influence on Schwann cell growth during regeneration of the peripheral nerve. In mice, Reg-IIIβ positive motor neurons are concentrated in cranial motor nuclei that are involved in the patterning of swallowing and suckling. Suckling was impaired in Reg-IIIβ KO mice and correlated this with a significant delay in myelination of the hypoglossal nerve. In summary, Reg-IIIβ has an important role in the developmental fine-tuning of neonatal motor behaviors mediating the response to peripherally derived cytokines and growth factors and regulating the myelination of motor axons (Tebar et al. 2008).

39.9 Functions of PAP

39.9.1 PAP: A Multifunctional Protein

The fact that PAP is secreted by pancreatic acinar cells into the pancreatic juice initially suggested a role for this protein in pancreatic juice homeostasis. Pancreatic juice is supersaturated in $CaCO_3$ and, in the absence of physiological inhibitors this salt will precipitate in crystal formation. In vitro, the rat HIP/PAP has been reported to prevent cell death in neuronal primary cultures (Nishimune et al. 2000) and pancreatic cells (Malka et al. 2000), promote the growth of epithelial intestinal cells (Moucadel et al. 2001), and stimulate DNA synthesis in Schwann cells (Livesey et al. 1997). The hamster homologue to HIP/PAP, called INGAP peptide (section 39.12), has been shown to enhance nerve outgrowth from explanted dorsal root ganglia (Tam et al. 2004). In vivo, HIP/PAP can function as an acute phase reactant in human pancreatitis (Orelle et al. 1992). Reports also suggest that HIP/PAP could regulate both viability and proliferation in hepatocytes. Human HIP/PAP expressed in hepatocytes exhibits mitogenic and an antiapoptotic properties in primary cultures and liver regeneration after partial hepatectomy is stimulated in HIP/PAP transgenic mice (Simon et al. 2003). HIP/PAP accelerates liver regeneration and protects against acetaminophen injury in mice (Lieu et al. 2005). Despite the obvious interest of these observations, it is difficult to link these effects with the enormous amount of PAP released by pancreas during acute pancreatitis or cellular stress.

39.9.2 PAP in Bacterial Aggregation

Evidence from a large number of studies seemed to indicate that PAP had a variety of activities, which included anti-inflammatory, anti-apoptotic, proliferative, and antibacterial effects (Closa et al. 2007; De Reggi and Gharib 2001; Graf et al. 2006; Iovanna and Dagorn 2005). With respect to the antimicrobial activity, PAP and PSP were shown to induce bacterial aggregation without inhibiting growth, and trypsin appeared to stimulate the effect of PSP (Iovanna et al. 1991, 1993). Cash et al. (2006a) demonstrated that PAP can bind to the peptidoglycan layer of Gram positive bacteria and exert a direct bactericidal effect. PAP also bound to chitin and the mannose polymer mannan, indicating that PAP recognizes carbohydrate patterns (Cash et al. 2006a, b). Cash et al. (2006a) proposed that PAP-I is part of the innate immune system and its intestinal expression plays an important role in the maintenance of the intestinal bacterial flora (Cash et al. 2006a). By analogy, in the pancreas PAP-I is probably upregulated during acute panceatitis to prevent bacterial infection.

PAP and its paralog PSP induce bacterial aggregation. It is proposed that insoluble PAP might be the biologically active form. PAP has been shown to bind to the peptidoglycan of Gram positive bacteria and to exert a direct bactericidal effect. Medveczky et al. (2009) showed that N-terminal cleavage of PAP by trypsin at the Arg^{37}-Ile^{38} peptide bond or by elastase at the Ser^{35}-Ala^{36} peptide bond is a prerequisite for binding to the peptidoglycan of the Gram positive bacterium *Bacillus subtilis*. Trypsin-mediated processing of PAP resulted in the formation of the characteristic insoluble PAP species, whereas elastase-processed PAP remained soluble. N-terminally processed PAP induced rapid aggregation of *Bacillus subtilis* without significant bacterial killing. Thus, N-terminal processing is necessary for the peptidoglycan binding and bacterial aggregating activity of PAP and that trypsin-processed and elastase-processed forms are functionally equivalent. The observations also extend the complement of proteases capable of PAP processing, which now includes trypsins, pancreatic elastases and bacterial zinc metalloproteases of the thermolysin type.

It has been demonstrated that Reg-IIIγ and HIP/PAP are directly bactericidal for Gram-positive bacteria at μM concentrations (Mukherjee et al. 2009), revealing a unique biological function for mammalian lectins. The bactericidal activities of Reg-IIIγ and HIP/PAP depend on binding to cell wall peptidoglycan, a polymer of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) cross-linked by short peptides. This finding identified Reg-III lectins as a distinct class of peptidoglycan binding proteins. However, given the lack of canonical carbohydrate binding motifs in Reg-III proteins, the molecular basis for lectin-mediated peptidoglycan recognition remains unknown. Furthermore, Reg-III lectins are secreted into the intestinal lumen where there are abundant soluble peptidoglycan fragments derived from the resident microbiota through shedding or enzymatic degradation of the bacterial cell wall. Thus, it remains unclear how Reg-III lectins selectively bind to bacterial surfaces without competitive inhibition by soluble peptidoglycan fragments in the luminal environment.

39.9.3 PAP-I: An Anti-inflammatory Cytokine

PAP-I has been implicated in the endogenous regulation of inflammation. In addition to its role in acute pancreatitis, PAP-I has also been associated with inflammatory diseases, such as Crohn's disease. During acute pancreatitis, PAP-I may contribute to stress response to control bacterial proliferation (Iovanna et al. 1991). In pancreatic AR4-2J cells, PAP-I is one of the effectors for the TNF-α-induced

apoptosis inhibition (Malka et al. 2000). Although PAP-II is first isolated as a pancreatic secretory protein that contributes to pancreatic regeneration, it is up-regulated during the acute phase of pancreatitis and likely modulates the inflammatory environment of pancreatitis (Iovanna et al. 1991; Cavard et al. 2006; Choi et al. 2007; Dusetti et al. 1994; Lasserre et al. 1992; Viterbo et al. 2008). PAP-II is found to inhibit TNF-α-mediated inflammatory responses (Vasseur et al. 2004; Gironella et al. 2005). In the exocrine pancreas, PAP-I is associated with pancreatic acinar cell and protects cells from oxidative stress and TNF-α-induced pancreatic stress (Malka et al. 2000; Ortiz et al. 1998). Moreover, expression of PAP-II is also increased in gut epithelial cells in human inflammatory bowel disease (Ogawa et al. 2003).

PAP2 mediates the expression of inflammatory cytokines in macrophages through the NF-kB pathway (Viterbo et al. 2008). It was shown that both antisense gene knockdown and Ab neutralization of PAP2 in rats with experimental acute pancreatitis caused a significant increase in disease severity (Zhang et al. 2004). These findings corroborate other studies which showed protective roles served by PAP proteins during tissue injury (Vasseur et al. 2004; Kandil et al. 2006; Gironella et al. 2005). Therefore, PAP proteins are key regulators of inflammation and their absence causes a dysregulated inflammatory process. Truncation of the first 25 residues on the N terminus of PAP2 did not affect protein activity whereas truncation of the last 30 residues of the C terminus of PAP2 completely inactivated the function of PAP2. In addition, reduction of three disulfide bonds proved to be important for the activity of this protein (Viterbo et al. 2008).

Several reports suggest that Reg proteins could also be functional in the nervous system and expressed in the brain during development and Alzheimer's disease (Watanabe et al. 1994; de la Monte et al. 1990) (section 39.5.3.1). PAP-I may contribute to the signaling pathway of ciliary neurotrophic factor and is involved in the regeneration and survival of motor neurons (Livesey et al. 1997; Nishimune et al. 2000). The expression of PAP-I has been found to be induced in urinary tract afferent neurons following cyclophosphamide-induced cystitis (Takahara et al. 2008), suggesting its potential role in the abnormal sensation in cystitis. Moreover, the PAP-I expression in isolectin B4 (IB4)-positive small DRG neurons is up-regulated and followed by a dynamic shift from small to large DRG neurons after peripheral nerve injury (Averill et al. 2002). Increased expression of PAP-I also occurs in neurons following traumatic brain injury (Ampo et al. 2009). These data indicate that PAP-I expression in response to injury and inflammation could be a general response in the pancreas, intestine, and both peripheral and central nervous systems. However, it remains unclear whether PAP-II is involved in the response of primary sensory neurons to the stimulations of peripheral inflammation and nerve injury. A recent study shows that the expression of PAP-II is strongly induced in DRG neurons following peripheral tissue inflammation and nerve injury, suggesting an involvement of PAP-II in the signal processing of the spinal sensory pathways in chronic pain states. PAP-II may play potential roles in the modulation of spinal sensory pathways in pathological pain states (He et al. 2010).

While PAP has been shown to be anti-bacterial and anti-apoptotic in vitro, its definitive biological function in vivo is not clear. In vivo evidence indicate that PAP mediates significant protection against pancreatic injury. PAP may exert its protective function by suppressing local pancreatic as well as systemic inflammation during acute pancreatitis (Heller et al. 1999; Zhang et al. 2004). During pancreatitis, PAP released by the pancreas could mediate lung inflammation through induction of hepatic TNFα expression and subsequent increase in circulating TNFα (Folch-Puy et al. 2003). Since, PAP-I and IL-10 responses share several features, Folch-Puy et al. (2006) assessed their expression and involvement of JAK/STAT and NF-kB signaling pathways in the suppression of inflammation mediated by PAP using pancreatic acinar cell line (AR42J). PAP-I inhibits the inflammatory response by blocking NF-kB activation through a STAT3-dependent mechanism. Important functional similarities to the anti-inflammatory cytokine IL-10 suggested that PAP-I could play a role similar to that of IL-10 in epithelial cells (Folch-Puy et al. 2006).

PAP-I is able to activate the expression of the anti-inflammatory factor: suppressor of cytokine signaling (SOCS)3 through the JAK/STAT3-dependent pathway. The JAK/STAT3/SOCS3 pathway seems to be a common point between PAP and several cytokines. Therefore, it is reasonable to propose that PAP-I is an anti-inflammatory cytokine (Closa et al. 2007). A model of caerulein-induced pancreatitis was used to compare the outcome of pancreatitis in PAP/HIP$^{-/-}$ and wild-type mice**.** PAP can be strongly induced by IL-6 and IL-10 and IL-10-related cytokines through a STAT3-mediated pathway. The expression of PAP itself appears to be induced in pancreatic acinar cells by the presence of PAP in the medium. This is also related to the activation of STAT3 pathway since at least two functional STAT-responsible elements have been reported in the promoter of the PAP gene (Dusetti et al. 1995b). This self-induction suggests the existence of a positive feedback mechanism in pancreatic acinar cells via a PAP receptor and a cross-talk with other cytokines (Closa et al. 2007).

Arginine-induced pancreatic acinar cell injury has been reported in vivo. Arginine inhibits the proliferation of pancreatic aciner AR4-2J cells. The anti-proliferation by arginine was due to an increase in apoptosis. Studies suggest that arginine induces apoptosis and PAP gene expression in

pancreatic acinar cells and that PAP might inhibit the induction of apoptosis (Motoo et al. 2000). The anti-apoptotic and anti-inflammatory functions described in vitro for PAP/HIP have physiological relevance in the pancreas in vivo during caerulein-induced pancreatitis (Gironella et al. 2007). Assuming a protective role of PAP, Li et al. (2009) showed that PAP had no significant effect on proliferation and migration on human pancreatic stellate cell (PSCs). Cell-associated fibrillar collagen types I and III and fibronectin increased after addition of PAP to PSCs. PAP diminished the expression of MMP-1 and −2 and TIMP-1 and −2 and their concentrations in PSC supernatants. Studies offer new insights into the biological functions of PAP, which may play an important role in wound healing response and cell-matrix interactions (Li et al. 2009). The RII α regulatory subunit of cAMP-dependent protein kinase (PKA) is a binding partner of HIP/PAP. The HIP/PAP co-immunoprecipitates with RIIα in HIP/PAP expressing cells. Increase in basal PKA activity in HIP/PAP expressing cells suggests that HIP/PAP may alter PKA signaling (Demaugre et al. 2004).

Ferrés-Masó et al. (2009) described the intracellular pathways triggered by PAP-I in a pancreatic acinar cells and showed that PAP-I increased the transactivation activity of *PAP-I* and the binding on its promoter of the nuclear factors C/EBPβ, P-CREB, P-ELK1, EGR1, STAT3, and ETS2, which are downstream targets of MAPK signaling. p44/42, p38, and JNK MAPKs activity increased after PAP-I treatment. In addition, pharmacological inhibition of these kinases markedly inhibited the induction of PAP-I mRNA. These results indicated that the mechanism of PAP-I action involves the activation of the MAPK superfamily (Ferrés-Masó et al. 2009).

39.10 Panceatitis Associated Protein(PAP)/Hepatocarcinoma-Intestine Pancreas (HIP)

39.10.1 HIP Similarity to PAP

Lasserre et al. (1994) identified a gene (named HIP) the expression of which is markedly increased in 25% of human primary liver cancers. HIP mRNA expression is tissue specific since it is restricted to pancreas and small intestine. Pleiotropic biological activities have been ascribed to this protein, but little is known about the function of HIP/PAP in the liver. HIP protein consists in a signal peptide linked to a CRD, typical of C-type lectins without other binding domains. The analysis of HIP/PAP gene indicates that the HIP/PAP CRD is encoded by four exons, a pattern shared with all members of this group of proteins. This common intron-exon organization indicates an ancient divergence of the free CRD-lectin group from other groups of C-type lectins. Lasserre et al. (1994) provided evidence for the localization of HIP/PAP on chromosome 2, suggesting previous duplication of HIP/PAP and the related Reg Iα and Reg Iβ genes from the same ancestral gene. The sequence of the 5′ upstream region of the HIP gene shows several potential regulatory elements which might account for the enhanced expression of the gene during pancreatic inflammation and liver carcinogenesis (Lasserre et al. 1994). In normal liver, the protein is undetectable in normal mature hepatocytes and found only in some ductular cells, representing potential hepatic progenitor cells. Itoh et al. (1995) especially focussed on the 5′-flanking region, which spans about 3 kb and is composed of six exon. Exon 1 encodes the 5′-noncoding sequence and exon 2 consists of three miniexons, 2a, 2b and 2c; the common exon 2c encodes the sequence including the start codon. Analysis revealed the presence of at least three different types 5′-ends of human PAP/HIP transcripts which were derived from alternative use of 5′-exons. Although all three types of transcripts were expressed in both normal small intestine and pancreas, their gene expression was increased ectopically in gastric cancer, hepatocellular cancer and pancreatic acinar cell carcinoma. Furthermore, significant differences among the transcript types were detected between normal and tumor tissues, and especially between gastric and hepatocellular cancers, suggesting that PAP/HIP expression may vary with differences in 5′-alternative splicing.

Simon et al. (2003) proposed that HIP/PAP acts as a hepatic cytokine that combines mitogenic and anti-apoptotic functions through the PKA signaling pathway and consequently acts as a growth factor in vivo to enhance liver regeneration. The HIP/PAP (=human Reg-2) encoding gene is activated in primary liver cancers. The involvement of HIP/PAP has been suggested in the pathophysiology of interstitial cystitis (IC) patients, because, the urinary HIP/PAP levels were significantly higher and the HIP/PAP expression in the bladder urothelium was more frequently apparent in IC patients (Makino et al. 2010). The rHIP regulatory sequence is a potent liver tumor-specific promoter for the transfer of therapeutic genes, and AdrHIP-NIS-mediated ^{131}I therapy is a valuable option for the treatment of multinodular HCC (Herve et al. 2008).

To test whether HIP/PAP-I is a target of islet-directed autoimmunity, Gurr et al. (2002) measured splenic T-cell responses against HIP/PAP in NOD mice. Differential cloning of Reg from islets of a type 1 diabetic patient and the response of Reg to the cytokine IL-6 suggests that HIP/PAP becomes overexpressed in human diabetic islets because of the local inflammatory response and. HIP/PAP acts as a T-cell autoantigen in NOD mice. Therefore, autoimmunity to HIP/PAP might create a vicious cycle, accelerating the immune process leading to diabetes (Gurr et al. 2002; Shervani et al. 2004).

Laurine et al. (2005) focused on two proteins, REG Iα and newly identified PAP-IB, belonging to more closely related FI and FII families, respectively. REG Iα and PAP-IB share 50% sequence identity. PAP-IB was expressed almost only in pancreas, unlike REG Iα, whose expression was ubiquitous. In addition, the two proteins displayed distinctive surface charge distribution, which may lead to different ligands binding. In spite of their common fold that should result in closely related functions, REG Iα and PAP-IB are a good example of duplication and divergence, probably with the acquisition of new functions, thus participating in the evolution of the protein repertoire.

39.10.2 Characterization of HIP/PAP

Pancreatitis-associated protein hepatocarcinoma-intestine-pancreas (PAP/HIP) is 16-kDa basic protein with an isoelectric point in the range of pH 6.5–7.6. Although most species contain a single PAP form, rat contains three isoforms, PAP-I, PAP-II, and PAP-III, transcribed from three separate genes (Iovanna et al. 1991; Frigerio et al. 1993a, b). PAP levels increase in pancreatic juice during experimental and clinical pancreatitis (Keim et al. 1992). Although showing an acute phase response under conditions of pancreatic disease, the function of PAP is not clear. Several studies have demonstrated that PSP and PAP both act as acute phase reactants in pancreatic juice under a variety of conditions including acute pancreatitis (Keim et al. 1994), post-weaning period (Bimmler et al. 1999). Trypsin cleavage of PSP and PAP has resulted in the appearance of precipitated proteins believed to represent insoluble thread structures in humans (Gross et al. 1985a) and cows (Gross et al. 1985b). It is difficult to understand how precipitation properties could serve a useful function in pancreatic physiology.

The PAP coding sequence spans over six exons and the putative CRD is encoded by exons IV, V, and VI. This gene organization suggests that PAP belongs to C-type lectins, which have evolved from the same carbohydrate-recognition domain ancestral precursor through a different process. It is interesting to note that PAP is the smallest protein reported among the C-type lectins. In fact, it comprises a single CRD linked to a signal peptide whereas other C-type lectins contain the sugar-binding consensus combined with a variety of other protein domains which confer specific functions of lectins. In contrast, PAP does not have additional functional domains. One PAP gene (HIP/PAP) was described by Keim et al. as a 16 kDa secretory protein in rat pancreatic juice which appeared upon induction of pancreatic inflammation and represented up to 5% of total protein (Keim et al. 1991). The human ortholog was isolated in 1992 from pancreatic juice of diabetic patients who underwent combined kidney and pancreas transplantation (Keim et al. 1992). In these patients the donor pancreas developed acute pancreatitis and levels of PAP-I in the juice reached up to 7.5% of the total secretory protein. Because PAP was hardly detectable in the normal pancreatic juice, but became upregulated in inflammation, it was considered a unique acute phase protein, which was targeted to the exocrine secretions. Subsequent cDNA cloning revealed that PAP exhibited homology to the carbohydrate binding region of Ca^{2+}-dependent (C-type) lectins and thus PAP belongs to a larger secretory protein family which contain only a single C-type lectin domain (Iovanna et al. 1991; Orelle et al. 1992). Members of this protein family, which includes pancreatic stone protein (PSP), have been described in excellent reviews (Closa et al. 2007; De Reggi and Gharib 2001; Graf et al. 2006; Iovanna and Dagorn 2005). PAP expression was also detected in the intestines and other extrapancreatic tissues, but the physiological function of PAP in the pancreas or elsewhere has remained contentious.

Graf et al. (2001) demonstrated that trypsin cleaved all three isoforms of PAP at N-terminus after Arg^{37} (Arg^{11} in the mature PAP protein), which in turn forms insoluble fibrils and the processed PAP became resistant to proteases and formed insoluble, fibrillar structures (Graf et al. 2001; Schiesser et al. 2001). Specific PAP-I isoforms have been shown to be normal constituents of intestinal paneth cells (McKie et al. 1996) and, more importantly, serve to maintain gut microbial integrity. PAP proteins also serve important roles within the nervous system, being involved in motor neuron regeneration (Namikawa et al. 2005). Coupling its diffuse expression pattern with the interspecies conservation of this group of proteins supports important functional roles for this network of proteins. In addition, HIP/PAP-I is a promising candidate for the prevention and treatment of liver failure (Christa et al. 2000; Lieu et al. 2005).

The secretory human PAP was structurally related to rat PAP and had the same size as rat PAP. It showed 71% amino acid identity, the six half-cystines being in identical positions. Domains of the proteins showing homologies with calcium-dependent lectins were also conserved. In addition, expression in pancreas of the genes encoding the human protein and rat PAP showed similar characteristics (Orelle et al. 1992). The human PAP synthesis increases during inflammation and its use as biological marker of acute pancreatitis has been siggested. The human PAP gene spans 2748 bp and contains six exons interrupted by five introns. The gene has a typical promoter containing the sequences TATAAA and CCAAT 28 and 52 bp upstream of the cap site, respectively. The human PAP gene was mapped to chromosome 2p12. This localization coincides with that of the Reg/PSP/LIT gene, which encodes a pancreatic secretory protein structurally related to PAP (Dusetti et al. 1994a).

39.10.3 PAP Ligands (PAP Interactions)

HIP/PAP protein has a very high affinity for D-lactose, but insignificantly to α-D-glucopyroanose, α-L-fucose, α-D-galactopyranose, or N-acetyl-β-D-glucosamine (Christa et al. 1994). It promotes rat hepatocyte adhesion in primary culture, and interacts with the proteins of the extracellular matrix (Christa et al. 1996). NMR studies showed that HIP/PAP recognizes the peptidoglycan carbohydrate backbone in a calcium-independent manner via a conserved "EPN" motif that is critical for bacterial killing. While EPN sequences govern calcium-dependent carbohydrate recognition in other C-type lectins (Drickamer 1999), the unusual location and calcium-independent functionality of the HIP/PAP EPN motif suggest that this sequence is a versatile functional module that can support both calcium-dependent and calcium-independent carbohydrate binding. Further, it was shown that HIP/PAP binding affinity for carbohydrate ligands depends on carbohydrate chain length, supporting a binding model in which HIP/PAP molecules "bind and jump" along the extended polysaccharide chains of peptidoglycan, reducing dissociation rates and increasing binding affinity (Fig. 39.3). Lehotzky et al. (2010) proposed that dynamic recognition of highly clustered carbohydrate epitopes in native peptidoglycan is an essential mechanism governing high-affinity interactions between HIP/PAP and the bacterial cell wall. HIP/PAP protein binds laminin and acts as an adhesion molecule for hepatocytes (Christa et al. 1999).

39.10.4 Crystal Structue of human PAP (hPAP)

Crystals of hPAP belong to the orthorhombic space group P2(1)2(1)2(1), with unit-cell parameters a = 30.73, b = 49.35, c = 92.15 A and one molecule in the asymmetric unit (Abergel et al. 1999). hPAP folds into an α + β structure and consists of two α-helices and eight β-strands cross-linked by three disulfide bridges packed into a heart-like shape, similar to that of the C-type lectin domain (Fig. 39.4). However, hPAP was found to have a unique distribution of charged residues on the surface. Nine of the eleven acidic residues (Asp^{37}, Asp^{39}, Glu^{58}, Asp^{82}, Glu^{88}, Glu^{92}, Glu^{95}, Asp^{100}, and Glu^{149}) were found to be clustered on one side, whereas 12 of the 13 basic residues (Arg^{13}, Lys^{16}, Lys^{19}, Lys^{33}, Lys^{44}, Arg^{45}, Lys^{67}, Arg^{109}, Arg^{125}, Arg^{131}, Lys^{133}, and Arg^{140}) were located on the other side. This surface charge distribution results in a highly polarized protein molecule, suggesting strong electrostatic interactions between molecules. In addition, several hydrophobic residues, such as residues Leu^{28}–Pro^{32} and Val^{101}–Trp^{107}, were found to be highly exposed and to constitute a continuous hydrophobic surface. The hydrophobic loop Val^{101}–Trp^{107} is located between the negatively charged and positively charged surfaces and is especially interesting, because the TANGO program predicts that this region in hPAP shows high propensity for β aggregation. Because hPAP forms fibrils with a native-like structure, it is possible that some conformational rearrangements in the loop regions that do not perturb the native secondary structure may drive the assembly of protein into fibril (Ho et al. 2006).

39.10.5 Expression of PAP

HIP/PAP is rapidly overexpressed during the acute phase of pancreatitis (Iovanna et al. 1994). Motor neurons are the only adult mammalian neurons of the CNS that regenerate after injury. The gene corresponding to human HIP/PAP gene in rat, called Reg-2 or rat PAP-I, has been found to be expressed in regenerating motor neurons, and its product exhibits mitogen-like activity in vitro on Schwann cells (Livesey et al. 1997). In the exocrine pancreas, HIP/PAP expression pattern is consistent with that of an acute phase reactant; however, this does not account for its expression in the islets of Langerhans of endocrine pancreas. HIP/PAP was expressed in normal subjects in the intestine (Paneth and neuroendocrine cells), and the pancreas (acinar pancreatic cells and islets of Langerhans), whereas in the liver, its expression was triggered in the event of primary liver cancer (Christa et al. 1996). Lasserre et al. (1992) discovered HIP/PAP by differential screening of a human hepatocarcinoma, and showed its frequent overexpression in liver tumors and regenerative hepatic/ductular diseases (Christa et al. 1999). In human, HIP/PAP is expressed in HCC and increased in serum, suggesting its potential importance in human liver carcinogenesis (Dusetti et al. 1996). During early stages of chemical hepatocarcinogenesis in rat (Petersen et al. 1998), oval cell proliferation was observed during hepatocarcinogenesis in woodchuck virus carriers and in human livers from patients with different pathological conditions (Christa et al. 1999). HIP/PAP mRNA is abundantly expressed in tumoral but not in nontumoral/normal livers. HIP/PAP gene expression is increased in 25% of HCC. HIP/PAP expression is not restricted to hepatocellular carcinoma, but also detected in cholangiocarcinoma cells as well as in reactive non-malignant bile ductules. Altogether, reports suggest that HIP/PAP protein may be implicated in hepatocytic and cholangiolar differentiation and proliferation (Christa et al. 1999).

PAP-I Is Upregulated During Acute and Chronic Pancreatitis: Early after its discovery it became clear that acinar cells of the pancreas are the main source of hPAP in pathological situations. In experimental model of acute pancreatitis in rat, Morisset et al. (1997) found the induction of PAP and its localization in zymogen granules. Bodeker and co-

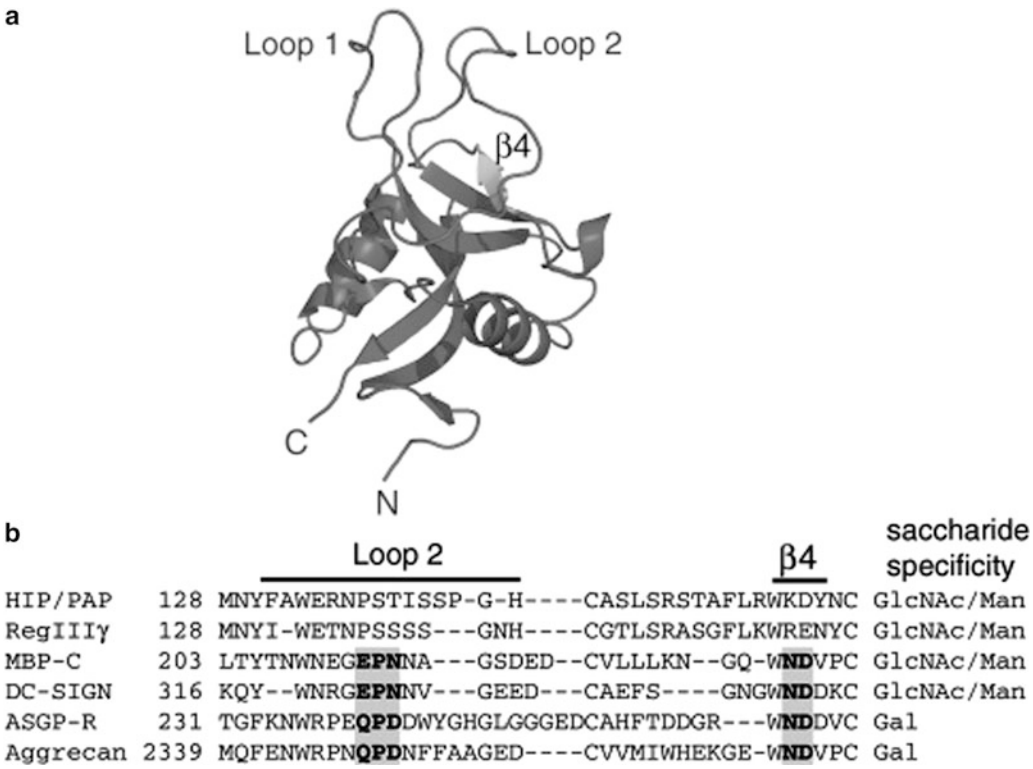

Fig. 39.3 Absence of canonical C-type lectin-carbohydrate binding motifs in Reg-III and HIP/PAP lectins: (**a**) NMR structure of HIP/PAP (PDB code 2GO0) (Ho et al. 2006: Fig. 39.4), with *Loops 1* and *2* of the *long loop* region and the β4 strand marked. (**b**) Alignment of the *long loop* region of Reg-III family members with other C-type lectin family members. MBP-C and DC-SIGN harbor *Loop 2* EPN motifs that govern sugar ligand binding and confer selectivity for mannose (Man) and GlcNAc (Drickamer 1992). Asialoglycoprotein receptor (ASGP-R) and aggrecan contain *Loop 2* QPD motifs that confer selectivity for Gal and GalNAc. Despite their selective binding to GlcNAc and Man polysaccharides RegIII lectins lack the *Loop 2* EPN motif (Cash et al. 2006a) (Adapted with permission from Lehotzky et al. 2010 © National Academy of Sciences)

workers (1998) described the pattern of PAP up-regulation in exocrine pancreas during the progression of the disease. The acute-phase response of pancreas seems to be a powerful emergency defense mechanism against further pancreatic aggression, as shown by the improved survival of the animals (Fiedler et al. 1998). PAP, induced in acute pancreatitis, was decreased by antisense-mediated gene knockdown of PAP gene expression and worsened pancreatitis (Lin et al. 2008).

PAP-I is not only expressed during acute pancreatitis but also in pancreatic adenocarcinoma, gastric carcinoma, hepatocellular carcinoma, and colorectal carcinoma. Expression in carcinoma might be another characteristic of PAP. Elevation of PAP in patients with pancreatic cancer is not merely explained by concomitant pancreatitis, but seems to be due to increased PAP production by the cancer cells and is also correlated to tumor load as expressed by the UICC stages (Cerwenka et al. 2001). PAP was overexpressed in 79% of pancreatic ductal adenocarcinoma, 19%of chronic pancreatitis, and 29% of mucinous cystadenoma. PAP was found in malignant ductular structures in pancreatic carcinomas as well as in benign proliferating ductules and acinar cells in chronic pancreatitis. It was not expressed in normal pancreas. The incidence of PAP overexpression was significantly higher in pancreatic cancer than in the other pancreatic diseases. PAP overexpression was significantly correlated with nodal involvement, distant metastasis, and short survival in pancreatic cancer. The overexpression of PAP in human pancreatic ductal adenocarcinoma indicates tumor aggressiveness (Xie et al. 2003). Functional differences between arteries and veins are based upon differences in gene expression. The most prominent difference was PAP-I, expressed 64-fold higher in vena cava versus aorta (Szasz et al. 2009).

TNF-α contributes to the development of acute pancreatitis. Because TNF-α is involved in the control of apoptosis and antiapoptotic mechanisms are mediated by NF-kB and MAP kinases, PAP-I is one of the effectors of apoptosis inhibition (Malka et al. 2000). The endotoxemia, which is caused by a bacterial infection, can exacerbate acute pancreatitis, whereas PAP has the ability to induce bacterial aggregation and supposed to protect the tissue from infection during inflammation. Studies suggest that PAP-I mRNA might be modulated by endotoxemia, independent of

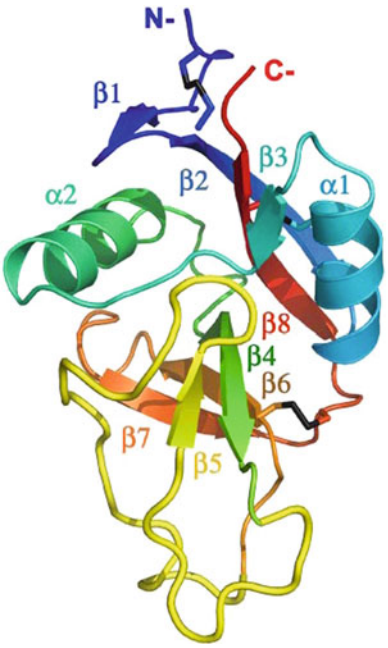

Fig. 39.4 NMR solution structure of hPAP at pH 4.0. Ribbon representation shows native hPAP. The tertiary fold of hPAP resembles that of the C-type lectin domain, which consists of two α-helices and eight β-strands cross-linked by three disulfide bridges (colored in *black*). The N and C termini are indicated. The region from Thr^{84} to Ser^{116} are highlighted in *yellow* (Adapted with permission from Ho et al. 2006 © The American Society for Biochemistry and Molecular Biology)

cerulein-pancreatitis. There were no strong correlations between PAP-I mRNA expression and the severity of pancreatitis (Wang et al. 2001).

PAP in Human Intestinal Tract: Members of the Reg family play regulatory roles in various endocrine cell populations and that their expression in endocrine cells is lineage-specific Analysis shows the presence of a transcript in the jejunum that has the same electrophoretic mobility as the pancreatic mRNA. No signal was detected in colon, however. In addition, PAP revealed the presence of a protein of 16,000 Da (as in pancreatic juice) in the homogenate of jejunum, but not of the colon. Positive immunoreactivity was observed on Paneth cells and in some goblet cells located in jejunum at the bottom of the crypts (Masciotra et al. 1995). In both adult and fetal normal tissues, HIP/PAP expression was detected only in endocrine cells of the small intestine, ascending colon, and pancreas (Hervieu et al. 2006).

Expression of PAP-I is altered in patients with celiac disease, where active phase of the disease is accompanied by an increased serum concentration of PAP (Carroccio et al. 1997). Increased serum level of PAP was diagnosed at ileal location in active CD with a sensitivity of 60%, a specificity of 94%, a positive predictive value of 84% and a negative predictive value of 81%. Elevated serum PAP is significantly associated with disease activity and ileal location (Desjeux et al. 2002). Clinical data suggest that pancreatitis could be an extraintestinal manifestation of inflammatory bowel disease. However, no experimental data support such a clinical relationship. Increased PAP mRNA has been reported in active inflammatory bowel disease (IBD). PAP-I is synthesised by Paneth cells and is overexpressed in colonic tissue of active IBD. PAP inhibits NF-kB activation and downregulates cytokine production and adhesion molecule expression in inflamed tissue. It may represent an anti-inflammatory mechanism and new therapeutic strategy in IBD (Gironella et al. 2005). PAP overexpression in pancreas demonstrates that inflammatory stress early occurs in the mouse pancreas during the course of TNBS-induced colitis. The concomitant pancreatic overexpression of IL-1B supported a pancreatic inflammatory mechanism mediated by cytokines (Barthet et al. 2003). Morphological pancreatic damage is also induced by the inhalation of cigarette smoke, which is likely to be mediated by alterations of acinar cell function (Wittel et al. 2006). PAP-I is also known as a marker for cystic fibrosis. PAP-I is increased in most neonates with cystic fibrosis and could be used for cystic fibrosis screening. Its combination with immunoreactive trypsinogen looks promising (Sarles et al. 1999). There was no evidence for polymorphism of the PAP gene in patients with hereditary or alcoholic pancreatitis. But the expression of the PAP in both groups of patients is related to the degree of cellular damage of the pancreas (Keim et al. 1999).

39.10.6 Similarity of PAP to Peptide 23 from Pituitary

Peptide 23 is a protein secreted by rat pituitary cells in primary culture. Although the secretion of this protein is stimulated by GH-releasing hormone and inhibited by somatostatin, the N-terminal amino acid sequence of peptide 23 shows no homology to rat GH. The cDNA of peptide 23 contained 777 nt and encoded a 175-amino acid protein with a 26-amino acid putative signal peptide with a calculated mol wt of mature protein (16,613 Da). Northern blot analysis revealed a major mRNA species of about 0.9 kb and a minor species of about 1.7 kb in cultured rat anterior pituitary cells. In rats, peptide 23 was most abundant in pancreas and GI tract. Sequence search revealed complete sequence identity between peptide 23 cDNA and PAP cDNA, 73% homology with HHC cDNA, and 55% homology with rat and human Reg cDNAs, expressed in regenerating pancreatic islets. Cloning of peptide-23 c-DNA revealed that it is identical to PAP. A dramatic increase in the expression of both genes was seen at the time of weaning in the third week postpartum. Peptide-23/PAP mRNA is most abundant in the ileum, whereas PSP/Reg is maximally expressed in the pancreas and duodenum. GRF modulates peptide-23/PAP expression

in GI tract in a similar fashion to that previously reported for pituitary cells in primary culture (Chakraborty et al. 1995c; Katsumata et al. 1995). Later studies demonstrated that peptide 23/PAP, previously thought to be of pituitary origin, is widely expressed in GI tract and that it is rapidly removed from the circulation by the kidney and by tissues which express peptide 23/PAP. Peptide 23/PAP is also expressed in rat uterus where estrogen may act as a physiological regulator of peptide 23 (Chakraborty et al. 1995a). Human fetal and adult tissues express at least one of the different transcripts of the PAP/Reg family, suggesting that the regulation of these homologous genes is coordinately controlled (Bartoli et al. 1998). The protein, present in very high concentration in pancreatic secretion, has been detected in brain lesions in AD and Down syndrome and in regenerating rat pancreatic islets (Cai et al. 1990).

39.10.7 Serum PAP: An Indicator of Pancreatic Function

Measuring serum PAP-I in acute pancreatitis has proved valuable in monitoring the course of the disease and the recovery of the patient. However, at admission PAP did not distinguish severe from mild acute pancreatitis better than C reactive protein; the measurement of PAP did not give appreciable diagnostic advantage in the early phase of acute pancreatitis (Kemppainen et al. 1996; Pezzilli et al. 1997). The serum PAP levels were significantly elevated in the patients with gastric, colorectal, biliary tract, hepatocellular, or pancreatic cancers compared with the healthy subjects. The increase of serum PAP levels in patients with gastrointestinal cancers reflects an ectopic expression of PAP-I. In cancer cells, the increased serum levels of PAP are correlated with the disease severity (Motoo et al. 1998, 1999). The PAP mRNA expression and serum PAP levels are closely related to neoplastic proliferative activity in patients with colorectal carcinoma (Cao et al. 2009). Serum PAP levels strongly correlate with creatinine clearance measurements. In patients with a pancreas-kidney transplantation, PAP may prove a useful biological and histological marker of pancreatic graft rejection (van der Pijl et al. 1997). Human PAP could be detected in pancreatic juice from patients with pancreatic diseases. Determination of PAP in pancreatic juice might be helpful for early detection of pancreatic injury (Motoo et al. 2001).

39.11 Reg IV (RELP)

Hartupee et al. (2001) isolated two cDNAs which encode a novel member of this multigene family. Based on primary sequence homology, tissue expression profiles, and shared exon-intron junction genomic organization, gene was assigned to the regenerating gene family as *Reg IV*. *Reg IV* has a highly restricted tissue expression pattern, with prominent expression in mucosal cells of GI tract. Namikawa et al. (2005) identified and sequenced a type-IV Reg gene in rats. Reg family members are mediators among injured neurons and glial cells that may play pivotal roles during nerve regeneration. Unlike other C-type lectins, human Reg IV binds to polysaccharides, mannan, and heparin in the absence of calcium. To elucidate the structural basis for carbohydrate recognition by NMR, mutant protein with Pro91 replaced by Ser (hReg IV-P91S) showed that the structural property and carbohydrate binding ability of hRegIV-P91S are almost identical with those of wild-type protein. The solution structure of hRegIV-P91S adopts a typical fold of C-type lectin. Based on NMR studies, two calcium-independent mannan-binding sites were proposed. One site is similar to the calcium-independent sugar-binding site on human Reg-III and Langerin. Interestingly, the other site is adjacent to the conserved calcium-dependent site 2 at position typical of C-type lectins (Ho et al. 2010)

39.11.1 Tissue Expression

Reg IV gene expresses in human colon, small intestine, stomach and pancreas, in rat spleen and colon and constitutively expressed in neuroendocrine cells of the intestinal mucosa. A potential role for Reg IV protein has been indicated in spleen and colon cell growth (StarceviÄć-Klasan et al. 2008). Heiskala et al. (2006) reported a robust de novo expression of Reg IV (RELP) in the neoplastic goblet cells of appendiceal mucinous cystadenomas and in the epithelial implants of pseudomyxoma peritonei (PMP). Reg IV serves as a marker for appendiceal mucinous cystadenomas and PMP, and may contribute to the pathogenesis of these disorders (Heiskala et al. 2006). Reg IV mRNA expression is significantly up-regulated by mucosal injury from active Crohn's disease or ulcerative colitis (UC). Reg IV mRNA was strongly expressed in inflamed epithelium and in dysplasias and cancerous lesions in UC tissues. Reg IV gene induction promoted cell growth and conferred resistance to H_2O_2-induced apoptosis in human colon cancer (DLD1) cells. Therefore, Reg IV gene is inducible by growth factors and may function as a growth promoting and/or an antiapoptotic factor in the pathophysiology of UC (Nanakin et al. 2007).

Intestinal neuroendocrine neoplasms showed co-expression of Reg IV and Hath1. Lung small-cell carcinoma and gastric mucocellular carcinoma expressed only Reg IV. Pancreatic islet-derived tumors, pheochromocytomas, and paragangliomas expressed only Hath1. The dissimilar expression patterns suggest that the proteins belong to different signaling pathways and are activated at different stages of neuroendocrine differentiation. Local Reg IV

expression may be influenced by the growth factors bFGF and HGF and/or their receptors CD138 and c-met, which were found to co-localize with Reg IV in intestinal neuroendocrine tumors (Heiskala et al. 2010). Reg IV might accelerate cell growth and disease progression of adenoid cystic carcinomas (ACCs) in salivary glands s (Sasahira et al. 2008). Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma (Oue et al. 2005). Bishnupuri et al. (2006a) examined Reg gene expression and associated changes in anti-apoptotic genes in an animal model of GI tumorigenesis and showed that dys-regulation of Reg genes occurs early in tumorigenesis. Furthermore, increased expression of Reg genes, specifically Reg IV contribute to adenoma formation and lead to increased resistance to apoptotic cell death in CRC (Bishnupuri et al. 2006a). Serum olfactomedin 4 (GW112, hGC-1) in combination with Reg IV is a highly sensitive biomarker for gastric cancer patients (Yoshida and Yasui 2009).

Levels of Reg IV protein in peritoneal lavage fluids increased in Reg IV-transfectants inoculated mice, but decreased in Reg IV-knockdown cell inoculated mice. It appears that Reg IV might accelerate peritoneal metastasis in gastric cancer and that Reg IV in lavage might be a good marker for peritoneal metastasis (Kuniyasu et al. 2009). The serum Reg IV concentration in presurgical gastric cancer patients is significantly elevated even at stage I and represents a novel biomarker for gastric cancer (Mitani et al. 2007). Reg IV is involved in gallbladder carcinoma carcinogenesis through intestinal metaplasia and is associated with relatively favorable prognosis in patients after surgery. The serum level of Reg IV may be of use or indicative of neoplasia (Tamura et al. 2009).

39.11.1.1 Colorectal Cancer (CRC)

Regenerating IV gene is overexpressed in HT-29 drug-resistant cells and more strongly expressed in 71% of colorectal tumors (in particular in mucinous carcinomas) than in normal colon tissues. Reg IV overexpression may be an early event in CRC carcinogenesis. A high level of RegIV and RegIV CRD expression was demonstrated in RegIV and RegIV CRD-transfected cells. RegIV enhances LoVo cell migration and invasion, and its CRD domain is critical for these effects (Guo et al. 2010). The comparison of Reg IV expression with that of other REG genes, regenerating Iα (Reg Iα), Reg Iβ and PAP, highlights its predominant expression in colorectal tumors (Violette et al. 2003). The over-expression of Reg IV in CRCs was significantly lower and inversely correlated with poor differentiation and venous invasion. Serum levels of Reg IV may predict CRC recurrence in the liver (Li et al. 2010; Oue et al. 2007). The expression of Reg IV in CRCs was positively linked to MUC2 and phosphorylation of the EGFR on Tyr^{1068}, suggesting that Reg IV may be a useful marker for intestinal type mucinous carcinoma and a good candidate as a molecular therapeutic target for CRCs. Reg IV is a potent activator of the EGF receptor/Akt/AP-1 signaling pathway in CRC (Bishnupuri et al. 2006b). Detection of Reg IV overexpression may be useful in the early diagnosis of carcinomatous transformation of adenoma (Zhang et al. 2003b). Reg IV is an important modulator of gastrointestinal cell susceptibility to irradiation; hence, it is a potential target for adjunctive treatments for human CRC and other gastrointestinal malignancies (Bishnupuri et al. 2010).

To clarify the role of Reg IV in gastric carcinogenesis and subsequent progression, Zheng et al. (2010) indicated that Reg IV expression experienced up-regulation in gastric intestinal metaplasia and adenoma and then down-regulation with malignant transformation of gastric epithelial cells. It was suggested that Reg IV expression is a good biomarker for gastric precancerous lesions and is especially related to the histogenic pathway of signet ring cell carcinoma (Zheng et al. 2010).

39.11.1.2 Marker for Hormone Refractory Metastatic Prostate Cancer

Gene, Reg IV is differentially expressed in the LAPC-9 hormone refractory xenograft. Consistent with its up-regulation in a hormone refractory xenograft, it is expressed in several prostate tumors after neoadjuvant hormone ablation therapy. As predicted by its sequence homology, it is secreted from transiently transfected cells. It is also expressed strongly in a majority of hormone refractory metastases. In comparison, it is not expressed by any normal prostate specimens and only at low levels in approximately 40% of primary tumors. Reports support Reg IV is a candidate marker for hormone refractory metastatic prostate cancer (Gu et al. 2005; Ohara et al. 2008). Serum Reg IV represents a novel biomarker for prostate cancer (Hayashi et al. 2009). Reg IV participates in 5-fluorouracil (5-FU) resistance and peritoneal metastasis, and its expression was associated with an intestinal phenotype of gastric cancer and with endocrine differentiation (Yamagishi et al. 2009; Yasui et al. 2009). Signet-ring cell carcinoma (SRCC) is a unique subtype of adenocarcinoma that is characterized by abundant intracellular mucin accumulation. Reg IV staining and claudin-18 staining can aid in diagnosis of gastrointestinal SRCC (Oue et al. 2008).

39.12 Islet Neogenesis-Associated Protein (INGAP) in Hamster

39.12.1 Characterization

Islet neogenesis-associated protein (INGAP) is expressed in islets and in exocrine pancreatic cells of normal hamsters. INGAP is a 175-amino-acid pancreatic acinar protein that

stimulates pancreatic duct cell proliferation in vitro and islet neogenesis in vivo. The INGAP gene is a novel pancreatic gene expressed during islet neogenesis whose protein product is a constituent of Ilotropin and is capable of initiating duct cell proliferation, a prerequisite for islet neogenesis. Induction of islet neogenesis by cellophane wrapping (CW) reverses streptozotocin-induced (STZ) diabetes. Administration of Ilotropin, a protein extract isolated from CW pancreata, causes recapitulation of normal islet ontogeny and reverses STZ diabetes. Although INGAP shows homology to the PAP and Reg/PSP families of genes, the increased expression of INGAP in CW is unlikely to be a result of acute pancreatitis (Rafaeloff et al. 1997). Sasahara et al. (2000) reported a INGAP-related protein (INGAPrP). While INGAP expressed in cellophane-wrapped pancreas and not in normal pancreas, the INGAPrP was abundantly expressed in normal pancreas. INGAP could be a potentially useful tool to treat conditions in which there is a decrease in β-cell mass (Del Zotto et al. 2000). A 6 kb of hamster genomic INGAP has identified a 3 kb 5-prime region with core promoter elements that is rich in transcription factor binding sites and six exons for the coding region. Analysis of promoter activity reveals stimulus-responsive DNA elements which have been identified though deletion analysis. Comparison of transcription factor binding sites in INGAP to the related gene Reg-IIIδ exposes potential sites for differential gene regulation (Taylor-Fishwick et al. 2003).

The ubiquitous localization of INGAP suggests its possible role in the physiological process of islet growth and its protective effect upon streptozotocin-induced diabetes (Flores et al. 2003). It participates in the regulation of islet neogenesis, and PDX-1/INGAP-positive cells represent a new stem cell subpopulation at an early stage of development, highly activateable in neogenesis (Flores et al. 2003; Gagliardino et al. 2003). Findings implicate PDX-1 in a possible feedback loop to block unbridled islet expansion (Taylor-Fishwick et al. 2006). INGAP expression is probably restricted to pancreas cells exerting its effect in a paracrine fashion. INGAP would be released and circulate bound to a serum protein from where it is bound and inactivated by the liver. Tissue binding could also explain INGAP's immunocytochemical presence in small intestine, where it could affect epithelial cell turnover (Borelli et al. 2007). INGAP stimulates cells in the pancreatic duct epithelium of healthy dogs (putative islet progenitor cells) to develop along a neuroendocrine pathway and form new islets in response to INGAP peptide. The INGAP might be an effective therapy for diabetes (Pittenger et al. 2007)**.** INGAP is localized to the pancreatic endocrine cells in mouse. INGAP- and insulin-immunoreactive cells are mutually exclusive, with INGAP-immunoreactive cells being preserved after streptozotocin-mediated destruction of β-cells. Findings reveal that INGAP and/or related group 3 Reg proteins have a conserved expression in the pancreatic islet (Taylor-Fishwick et al. 2008).

39.12.2 Properties of Pentadecapeptide from INGAP

A pentadecapeptide having the 104–118 amino acid sequence of INGAP (INGAP-PP) affects insulin secretion and transcript profile expression in cultured normal pancreatic neonatal rat islets. Islets cultured with INGAP-PP released significantly more insulin in response to glucose than those cultured without the peptide. INGAP-PP enhances specifically the secretion of insulin and the transcription of several islet genes, many of them directly or indirectly involved in the control of islet metabolism, β-cell mass and islet neogenesis. Studies strongly indicate an important role of INGAP-PP, and possibly of INGAP, in the regulation of islet function and development (Barbosa et al. 2006; Barbosa et al. 2008). Whilst the peptides with scrambled sequences showed no definite prevalent structure in solution, INGAP-PP maintained a notably stable tertiary fold, namely, a conformer with a central β-sheet and closed C-terminal. Such structure resembles the one corresponding to the amino acid sequence of human PAP-1, which presents 85% sequence homology with INGAP. These results explain why the two scrambled sequences tested showed no biological activity, while INGAP-PP significantly increases β-cells function and mass both in vitro and in vivo conditions. These data can help to obtain more potent INGAP-PP analogs, suitable for the prevention and treatment of diabetes (McCarthy et al. 2009)

Administration of the peptide fragment of INGAP (INGAP peptide) has been demonstrated to reverse chemically induced diabetes as well as improve glycemic control and survival in an animal model of type 1 diabetes. Cultured human pancreatic tissue has also been shown to be responsive to INGAP peptide, producing islet-like structures with function, architecture and gene expression matching that of freshly isolated islets. Studies in normoglycemic animals show evidence of islet neogenesis. These clinical studies suggest an effect of INGAP peptide to improve insulin production in type 1diabetes and glycemic control in type 2 diabetes (Lipsett et al. 2007). The INGAP peptide acts as a mitogen in the peripheral nervous system (PNS), and enhances neurite outgrowth from DRGs in vitro. The neuritogenic action of INGAP peptide correlates with an increase in ^{3}H-thymidine incorporation and mitochondrial activity and promotes Schwann cell proliferation in the DRG which releases trophic factors that promote neurite outgrowth (Tam et al. 2002).

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Emerging Groups of C-Type Lectins

40

G.S. Gupta

40.1 Layilin Group of C-Type Lectins (Group VIII of CTLDS)

40.1.1 Layilin: A Hyaluronan Receptor

Layilin, a ~55-kDa lectin, is a widely expressed integral membrane hyaluronan receptor, originally identified as a binding partner of talin located in membrane ruffles. It is recruited to membrane ruffles in cells induced to migrate in in vitro wounding experiments and in peripheral ruffles in spreading cells. Layilin is a transmembrane C-type lectin (Fig. 40.1) and binds specifically to hyaluronan (HA) but not to other tested glycosaminoglycans and belongs to group VIII of CTLDs. The other member of this group is chondrolectin. Layilin's ability to bind hyaluronan reveals an interesting parallel between layilin and CD44, because both can bind to cytoskeleton-membrane linker proteins through their cytoplasmic domains and to hyaluronan through their extracellular domains. This parallelism suggests a role for layilin in cell adhesion and motility (Banerji et al. 1999; Bono et al. 2001). There is no sequence homology with known hyaluronan receptors, including CD44, RHAMM (receptor for HA-mediated motility), or LYVE-1 (lymphatic vessel endothelial HA receptor-1). Thus, by binding to HA, layilin may facilitate cell migration by mediating early interactions between spreading cells and the extracellular matrix (ECM). Since, hyaluronan is involved in invasion of a variety of tumor cells and layilin was detected in the human lung cell line A549, layilin might play crucial roles in lymphatic metastasis of lung carcinoma. Suppression of layilin expression by iRNA significantly increased survival of tumor-bearing mice. The suppression of layilin expression might be a promising strategy for treatment of human lung carcinoma (Chen et al. 2008).

40.1.2 Interactions of Layilin

The actin cytoskeleton plays a significant role in change of cell shape and motility. Several adaptor proteins, including talin, maintain the cytoskeleton-membrane linkage by binding to integral membrane proteins and to the cytoskeleton. The talin binds to integrins, vinculin, actin and layilin. A ten-amino acid motif in layilin cytoplasmic domain is sufficient for talin binding. Layilin colocalizes with talin in ruffles and binds to talin's ~50-kDa head domain (amino acids 280–435). This region overlaps a binding site for focal adhesion kinase (Borowsky and Hynes 1998). The other known binding partners of layilin include other members of the talin/band 4.1/ERM (ezrin, radixin, and moesin) family of cytoskeletal-membrane linker molecules. The neurofibromatosis 2 tumor suppressor protein, merlin, is commonly mutated in human benign brain tumors. Merlin and radixin interact with the carboxy-terminal domain of layilin. This suggests that layilin may mediate signals from extracellular matrix to the cell cytoskeleton via interaction with different intracellular binding partners and thereby be involved in the modulation of cortical structures in the cell (Bono et al. 2005; Scoles 2008). The talin F3 domain binds to short sequences in the layilin cytoplasmic domain, integrins, and PIPK1γ (Wegener et al. 2008).

40.1.3 Functions of Layilin

The colocalization of layilin with talin and the binding interaction between them make layilin as a good candidate for a membrane-binding site for talin in ruffles. The colocalization of both epitope-tagged and endogenous layilin with talin in actin-rich membrane ruffles and significant homology to proteins with C-type lectin activity of layilin leads to suggest two general models for layilin

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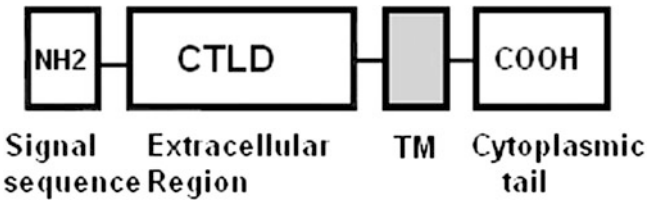

Fig. 40.1 The protein domains of layilin. Sig (NH_2-terminal signal sequence), Lectin (layilin's extracellular part which is homologous to C-type lectins) and TM (transmembrane) domain

function. In the first model, layilin acts in cell migration by anchoring to the membrane talin, which in turn binds F-actin. This chain of interactions may transmit force from actin to the membrane, resulting in its characteristic deformation into ruffles. In this scenario, layilin may serve simply as a talin docking site, or it could be an early-acting adhesion molecule that binds extracellular matrix, nucleating the formation of focal contact precursors at the cell periphery. In this case, layilin would be functioning analogously to selectins, which mediate transient adhesion between rolling leukocytes and the endothelium followed by tight integrin-mediated adhesion. However, whereas selectins mediate cell adhesion in specialized cells of immune system, it was hypothesized that layilin performs fundamental cell adhesion tasks common to most cells because layilin and talin are present in many different tissues. Layilin would thus form transient adhesion sites between ruffles and extracellular matrix that are refined into focal contacts after integrin recruitment. This could occur in both spreading and migrating cells. Talin, which can bind both layilin and integrins, may provide continuity between the two types of cell–matrix linkages as transient layilin-containing structures mature into integrin-containing focal adhesions. Integrin extracellular matrix receptors are known to be signaling as well as adhesion receptors, and if layilin encounters matrix early in the process of cell adhesion it may also signal. The three internally repeated motifs in the layilin cytoplasmic domain are potential binding sites for cytoskeletal or signaling molecules in addition to talin. Conservation of these motifs in hamster and human layilins suggests that they are of importance, and two of these repeats form a binding-site for talin (Borowsky and Hynes 1998). In the second model, layilin may function as an endo- or phagocytic receptor, similar to some other C-type lectins such as the macrophage mannose receptor. This model is suggested by the observation that talin is present in phagocytic cups in macrophages and at the sites of uptake of some bacterial pathogens (Young et al. 1992). In addition, the layilin cytoplasmic domain contains two YXXΦ motifs (YNVI, YDNM). These motifs are similar to sequences that mediate clathrin-dependent endocytosis of other membrane proteins by binding to the μ_2 chain of the AP-2 adaptor complex (Ohno et al. 1995; Marks et al. 1997; Borowsky and Hynes 1998).

40.1.4 Chondrolectin (CHODL)

The *Chondrolectin (Chodl)*, a novel human gene encoding chondrolectin is localized at chromosome 21q21 and consists of six exons and five introns. The ORF of *Chodl* encodes a type I transmembrane protein containing a single CRD of C-type lectins in its extracellular portion. CHODL expresses in testis, prostate, and spleen and the expression of CHODL is mainly limited to vascular muscle of testis, smooth muscle of prostate stroma, heart muscle, skeletal muscle, crypts of small intestine, and red pulp of spleen. CHODL is an N-glycosylated protein with a molecular weight of ~36 kDa and shows a predominantly perinuclear localization in transiently transfected COS1 cells. Although the predicted CHODL protein shares a significant homology (45% overall and 60% within the CRD) with layilin, a hyaluronan receptor, there was no specific interaction between CHODL and hyaluronan using cetylpyridinium chloride precipitation (Weng et al. 2002).

The mouse orthologue of human chondrolectin gene, *Chodl* is located at chromosome 16C3 and consists of six exons and five introns. The putative full-length mouse cDNA of *Chodl* consists of 2393 bp, with an ORF of 839 bp, 243 bp of 5′ untranslated region and 1310 bp of 3′ untranslated region. The predicted CHODL protein containing one CRD of C-type lectin in its extracellular portion shares a similarity (45%) with human layilin. In adult mice, CHODL is preferentially expressed in skeletal muscle, testis, brain, and lung. During gestation, E 7–15 its expression is up-regulated (Weng et al. 2003).

40.2 Tetranectin Group of Lectins (Group IX of CTLDs)

The tetranectin group of CTLD-containing secreted proteins has three members in both human and mouse: tetranectin (TN), cartilage-derived C-type lectin, and stem cell growth factor (SCGF). Each polypeptide has an N-terminal tail rich in charged residues, a central neck region, and a C-terminal CTLD. The neck region mediates homotrimerization through the formation of a coiled-coil of α-helices. It is not known if carbohydrate recognition plays a role in the function of TN group of proteins. The CTLDs in these proteins

resemble CTLDs of the galactose-binding subtype, but attempts to demonstrate sugar-binding activity have not been successful.

40.2.1 Tetranectin

By stimulating plasminogen activation, Tetranectin (TN) may regulate proteolysis of extracellular matrix components in development, tissue remodelling and cancer, and influence the proteolytic activation of proteases and growth factors. Tetranectin is a member of the C-type lectin family and can be detected in serum and the extracellular matrix. It is composed of three identical, non-covalently linked 20 kDa subunits, and binds to the fourth kringle domain of plasminogen, which can stimulate plasminogen activation in vitro (Høgdall 1998). Each of the mature TN monomer of 181 amino acids consists of three functional domains encoded by separate exons (Fuhlendorff et al. 1987; Wewer and Albrechtsen 1992). A short lysine-rich region at the N terminus binds to heparin (Lorentsen et al. 2000). This domain is followed by an α-helical domain responsible for multimerization by forming a triple coiled-coil α-helix (Nielsen et al. 1997). The final 132 amino acids comprise a C-type lectin-like domain, homologous to the CRD of calcium-dependent animal lectins (Drickamer 1999) and contain binding sites for calcium and plasminogen (Graversen et al. 1998). The three intrachain disulfide bonds connect Cys residues 50–60, 77–176, and 152–168. The TN is homologous (17–24% identical positions) with those parts of the asialoglycoprotein receptor family that are considered to be extracellular. The TN has no structures corresponding to those parts of the receptors considered to be intracellular and membrane anchoring. The sequence of TN is also homologous (22–23% identical positions) with the C-terminal globular domain of the core protein of the cartilage proteoglycan (Fuhlendorff et al. 1987). In addition, O-linked glycosylation of the TN molecule at threonine 4 has been described (Fuhlendorff et al. 1987; Jaquinod et al. 1999). Tetranectin has a distinct binding site in its N-terminus that mediates binding to complex sulfated carbohydrates (e.g., heparin) (Lorentsen et al. 2000). The N-terminus of TN could, therefore, mediate binding to ECM components. The crystal structure of TN is similar to that of mannose binding protein, a member of the collectin group of the C-type lectin superfamily, except that the presence of an extra disulfide bridge in TN may restrict the flexibility of the C-type lectin-like domain (Nielsen et al. 1997). The CTLD of TN undergoes structural rearrangements upon calcium binding and in its calcium-free form binds to proteins with kringle domains, such as plasminogen and to isolated kringle-4 (Clemmensen et al. 1986), apparently to its lysine-binding site. Recombinant tetranectin- as well as natural tetranectin from human plasma was shown to be a homo-trimer in solution as are other lectins in collectin family of C-type lectins.

The gene for human TN is about 12 kbp and contains two intervening sequences. The gene encodes a protein of 202 amino acid residues, with a signal peptide of 21 amino acid residues, followed by the TN sequence of 181 amino acid residues (M_r = 20,169 Da) (Wewer and Albrechtsen 1992). The 3′ noncoding region contained a single polyadenylation signal and a 26-residue poly A tail. This protein is produced locally by cells of the stromal compartment of tumors and is deposited into the extracellular matrix. Since TN binds to plasminogen, it could function as an anchor and/or reservoir for plasminogen and similar substances that regulate tumor invasion and metastasis as well as tumor angiogenesis (Wewer and Albrechtsen 1992).

By screening a human chromosome 3 somatic cell hybrid mapping panel, Durkin et al. (1997) localized the human TN gene to 3p22–p21.3, which is distinct from the loci of two human connective tissue disorders that map to the short arm of chromosome 3, MFS2 and LRS. The human TN gene consists of three exons (Durkin et al. 1997). Each separate exon encodes specific functional domains. The short exon 1 encodes the heparin binding site of TN. The second exon encodes an α helix protein, which governs the trimerisation of TN monomers by assembling them into a triple helical coiled structural element. The final exon encodes a C-type lectin-like domain, homologous to the carbohydrate recognition domains of calcium dependent animal lectins (Nielsen et al. 2000). Biochemical evidence shows that an N-terminal domain encoded within exons 1 and 2 of the tetranectin gene is necessary and sufficient to govern subunit trimerization (Holtet et al. 1997). The human, mouse, and chicken TN cDNA sequences and gene structures are very similar (Berglund and Petersen 1992; Ibaraki et al. 1995; Sørensen et al. 1995, 1997; Wewer and Albrechtsen 1992; Xu et al. 2001). Neame et al. (1992) isolated a protein from reef shark (Carcharhinus springeri) cartilage that bears a striking resemblance to TN monomer originally described by Clemmensen et al. (1986). The amino acid sequence had 166 amino acids and a calculated molecular weight of 18,430. The shark protein was 45% identical to human TN, indicating that it was in the family of mammalian C-type lectins and an analog of human TN (Neame et al. 1992).

Murine Tetranectin: The mouse TN 992-bp cDNA with an open reading frame of 606 bp is identical in length to the human TN cDNA encoding a protein of 202 aa including a signal peptide of 21 aa. An overall identity of 79–87% in amino acids between human and murine TN revealed a high evolutionary conservation of the protein. The highest

expression of mouse TN was found in lung and skeletal muscle. The sequence analysis revealed a difference in both sequence and size of the non-coding regions between mouse and human cDNAs. The cloned murine TN was mapped to region F1-F3 on mouse chromosome 9. Mapping of the transcription start point (tsp) suggests that murine TN has more than one of these. In addition, no consensus TATA-box was found uptream for the putative tsp(s) in the 5′-flanking region of the gene, indicating that the murine TN promoter belongs to the TATA-less class of genes (Ibaraki et al. 1995; Sørensen et al. 1997).

40.2.2 Cell and Tissue Distribution

Tetranectin shows a wide tissue distribution. Predominantly, it was found in secretory cells of endocrine tissue like pituitary, thyroid, parathyroid glands, and the liver, pancreas, and adrenal medulla. Endocrine cells with a known protein or glycoprotein hormonal production such as chromophils (pituitary), follicular and parafollicular cells (thyroid), chief cells (parathyroid), hepatocytes (liver), islet cells (pancreas) and ganglion cells of the adrenal medulla displayed a convincing, positive staining reaction for TN, which varied from cell to cell within the different tissues. The liver showed a distinct and universal reaction within almost all hepatocytes, thus raising suspicion of producing the bulk of TN to the blood. Suspicion indicates that this protein might have a dual function, serving both as a regulator in the secretion of certain hormones and as a participant in the regulation of the limited proteolysis, which is considered important for the activation of prohormones (Christensen et al. 1987; Jensen et al. 1987). TN was found in endothelial and epithelial tissues, particularly in cells with a high turn-over or storage function such as gastric parietal and zymogenic cells, absorptive surface epithelium of the small intestine, ducts of exocrine glands and pseudostratified respiratory epithelium. Also mesenchymal cells produced a TN positive staining reaction, which was most conspicuous in mast cells, but also present in some lymphocytes, plasma cells, macrophages, granulocytes, striated and smooth muscle cells and fibroblasts (Christensen et al. 1989). Hermann et al. (2005) described the in vivo and in vitro localisation of TN in human and murine islet cells. The amount and localization of TN is influenced by different culture conditions. Tetranectin may play an important role in the survival of islets in the liver after islet transplantation.

A distinct accumulation of TN was observed in the surrounding extracellular matrix of various carcinomas where it colocalized with plasminogen (De Vries et al. 1996). Plasma levels of TN are approximately 100 nM in healthly adults (Jensen and Clemmensen 1988). Tetranectin is also found in a mobilizable set of granules in neutrophils (Borregaard et al. 1990), in monocytes (Nielsen et al. 1993), fibroblasts (Clemmensen et al. 1991), platelets (Christensen 1992), and in various tissue locations like cartilage and the extracellular matrix of developing or regenerating muscle (Wewer et al. 1994; Clemmensen et al. 1991; Wewer et al. 1998). Furthermore, TN is a crucial component of the extracellular matrix during muscle cell development and regeneration. During muscle cell development and regeneration, TN expression marks active myogenesis in vivo and in vitro (Wewer et al. 1998a). The decrease in concentration of TN in serum after myocardial infarction (Kamper et al. 1998a) suggests consumption of circulating soluble TN during myocardial repair. A role in skeletal formation during development was evidenced since targeted deletion of the protein results in spinal deformity (Iba et al. 2001a). Iba et al. (2009) also supported that TN might play a role in the wound healing process.

Crab-eating macaque serum showed the strongest reaction for TN, followed by horse and cat. Serum from cow, goat, pig, mouse and chicken reacted weakly, while dog, trout, and the amphibian and the reptile species did not react (Thougaard et al. 2001). TN has been demonstrated in normal brain and CSF. In amniotic fluid a significant, positive correlation exists between TN concentration and gestational age. Fetal TN has been correlated to fetal maturation (Høgdall et al. 1998). Tetranectin is enriched in the cartilage of the shark. During osteogenesis, in the newborn mouse, strong TN immunoreactivity was found in the newly formed woven bone around the cartilage anlage in the future bone marrow and along the periosteum forming the cortex. TN may play an important direct and/or indirect role during osteogenesis and my have a potential role in mineralization in vivo and in vitro (Wewer et al. 1994).

40.2.3 Interactions of Tetranectin

Interactions with Complex Sulfated Polysaccharides

Tetranectin interacts with complex sulfated polysaccharides (Clemmensen 1989; Lorentsen et al. 2000), in a lysine sensitive way, specifically with apolipoprotein A (Kluft et al. 1989a), and in a calcium dependent fashion with fibrin (Kluft et al. 1989b). The distinct binding properties of TN to plasminogen and its expression in normal tissue development point to an important physiological function of TN in tissue formation and repair. Fourth kringle domain of plasminogen can stimulate plasminogen activation in vitro. In the search for new ligands for the plasminogen kringle-4 binding-protein TN, it has been found that TN specifically bound to the plasminogen-like hepatocyte growth factor and tissue-type plasminogen activator. The dissociation constants of these complexes were found to be within the same order of magnitude as the one for the plasminogen-tetranectin complex. TN did not interact with the kindred proteins: macrophage-stimulating protein, urokinase-type plasminogen activator and prothrombin (Westergaard et al. 2003).

Kluft et al. (1989a) demonstrated reversible binding of apolipoprotein-A to kringle-4-binding with an apparent K_D of 0.013 μM/l, where as Lys- and Glu-plasminogen showed an apparent K_D of 0.5 μM/l. It suggested that plasminogen and lipoprotein-A show functional analogy in their binding to TN, but TN primarily targets at lipoprotein-A (Kluft et al. 1989a). TN binds to fibrin and the amount of TN bound to fibrin varies with TN in plasma. Normally, it constitutes a constant percentage of 13–17% of plasma TN. The TN, which is reduced in serum after blood clotting can, upon coagulation, be released from platelets and become partially bound to fibrin (Kluft et al. 1989b). TN may interact with angiostatin, which is formed in cancer tissues by proteolytic degradation of plasminogen. The predominant form of angiostatin produced in cancer tissues is AST^{K1-4} (O'Reilly et al. 1994; Gately et al. 1997; Richardson et al. 2002), which inhibits cancer progression and metastasis by inhibiting cancer-related angiogenesis. Mogues et al. (2004) demonstrated that TN binds to the kringle 1–4 form of angiostatin (AST^{K1-4}) and reduces its ability to bind to ECM of endothelial cells or to inhibit endothelial cell growth.

Plasminogen and Heparin-Binding Sites in Tetranectin: TN, related to corresponding regions of the mannose-binding proteins, is known specifically to bind the plasminogen kringle-4 protein domain, an interaction sensitive to lysine. Surface plasmon resonance and isothermal calorimetry binding analyses using single-residue and deletion mutant TN derivatives showed that the kringle-4 binding site resides in the CRD and includes residues of the putative carbohydrate binding site. Furthermore, the interaction is sensitive to calcium in addition to lysine (Graversen et al. 1998). The heparin-binding site in TN resides not in the CRD but within the N-terminal region, comprising the 16 amino acid residues encoded by exon 1. In particular, the lysine residues in the decapeptide segment KPKKIVNAKK (TN residues 6–15) at the N terminus are shown to be of primary importance in heparin binding (Lorentsen et al. 2000).

Tetranectin-Binding Site on Plasminogen Kringle-4: It was reported that all amino acid residues of plasminogen kringle-4 were involved in binding to TN. Notably, one amino acid residue of plasminogen kringle-4, Arg^{32}, not involved in binding trans-aminomethyl-cyclohexanoic acid (t-AMCHA), is critical for binding TN. Moreover Asp^{57} and As ^{55} of plasminogen kringle-4, which both were found to interact with low molecular weight ligand with an almost identical geometry in the crystal of the complex, are not of equal functional importance in t-AMCHA binding. Mutating Asp^{57} to an Asn totally eliminates binding, whereas the Asp^{55} to Asn, like the Arg^{71} to Gln mutation, was found only to decrease the affinity (Graversen et al. 2000a).

A site, involving Lys^{148}, Glu^{150}, and Asp^{165} in TN is known to mediate calcium-sensitive binding to plasminogen kringle-4. Substitution of Thr^{149} in TN with a tyrosine residue considerably increases the affinity for plasminogen kringle-4, and, in addition, confers affinity for plasminogen kringle 2. This new interaction is stronger than the binding of wild-type TN to plasminogen kringle-4. The insight into molecular determinants is important for binding selectivity and affinity of C-type lectin kringle interactions (Graversen et al. 2000b).

40.2.4 Crystal Structure of Tetranectin

Tetranectin is a homotrimeric protein (TN3) which contains a C-type lectin-like domain. The TN3 domain can bind calcium. In absence of calcium, the TN3 domain binds a number of kringle-type protein ligands. The structure of calcium free-form of TN3 (apoTN3) has been studied by NMR. Compared to the structure of the calcium-bound form of TN3 (holoTN3), the core region of secondary structural elements is conserved, while large displacements occur in the loops involved in calcium or K4 binding. A conserved proline, present in *cis* conformation in holoTN3, is in apoTN3 predominantly in the *trans* conformation. Backbone dynamics indicated that, in apoTN3 especially, two of the three calcium-binding loops and two of the three K4-binding residues exhibit increased flexibility, whereas no such flexibility was observed in holoTN3. In apoTN3, the residues critical for K4 binding, are spanned over a large conformational space. Study indicated that the K4-ligand-binding site in apoTN3 is not preformed (Nielbo et al. 2004).

The recombinant human TN and the CRD of TN3 have been crystallized. TN3 crystallizes in the tetragonal space group $P4_22_12$ with cell dimensions a = b = 64.0, c = 75.7 A and with one molecule per asymmetric unit. The crystals of TN are rhombohedral, space group R3, with the hexagonal axes a = b = 89.1, c = 75.8 A, and diffract to at least 2.5 A. The asymmetric unit contains one monomer of TN. The rhombohedral space group indicates that trimers of TN are formed in accordance with the observation of trimerization in solution (Kastrup et al. 1997). The two C-terminal domains, TN23 (residues 17–181) of human recombinant TN have been crystallized in two different space groups. Crystals belonging to the monoclinic space group C2 showed unit-cell parameters: a = 160.4, b = 44.7, c = 107.5 A, β = 127.6º. TN23 crystallizes in a rhombohedral space group, with unit-cell parameters a = b = c = 107.4 A, α = β = γ = 78.3º. A full data set to 4.5 A, collected from the monoclinic crystals, shows that TN23 crystallizes as a trimer, with one trimer in the asymmetric unit (Nielsen et al. 2000).

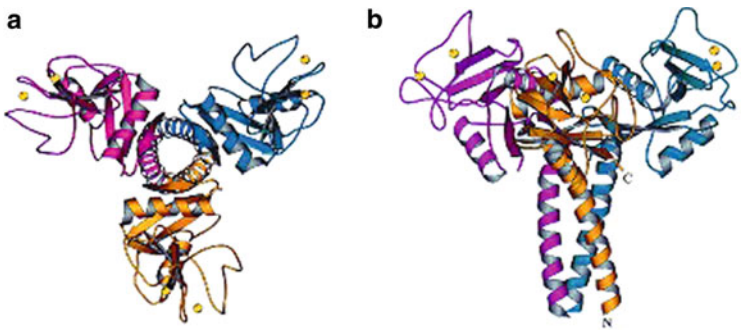

Fig. 40.2 (**a** and **b**): The overall structure of the TN trimer viewed (**a**) along and (**b**) perpendicular to the threefold axis (Reprinted by permission from Macmillan Publishers Ltd: FEBS Letters, Nielsen et al. © 1997)

Human tetranectin is a homotrimer forming a triple α-helical coiled coil. Each monomer consists of a CRD connected to a long α-helix. TN has been classified in a distinct group of the C-type lectin superfamily but has structural similarity to the proteins in the group of collectins. TN has three intramolecular disulfide bridges. Two of these are conserved in the C-type lectin superfamily, whereas the third is present only in long-form CRDs. In TN, the third disulfide bridge tethers the CRD to the long helix in the coiled-coil. The residues 26–52 of TN form a long α-helix (E2), which is connected to the CRD. The CRD of TN has characteristic topology similar to other proteins of the C-type lectin superfamily. However, structural differences occur at insertions and deletions in TN as compared to human lithostathine, human MBP, and human E-selectin (Nielsen et al. 1997). Two calcium ions have been located at same positions as calcium-binding sites 1 and 2 in structures of MBPs. In TN, the ligands are Asp^{116}, Glu^{120}, Gly^{147}, Glu^{150}, and Asn^{151} at calcium site 1 and Gln^{143}, Asp^{145}, Glu^{150}, and Asp^{165} at calcium site 2. TN, as well as lithostathine, contains a long-form CRD with three disulphide bridges in contrast to MBPs and E-selectin, which have short-form CRDs with two disulfide bridges. In TN, the disulfide bridge Cys^{50}-Cys^{60} tethers the E2 helix to a short L-strand of the CRD, whereas this disulfide bridge is part of a loop in lithostathine.

The long E2 helix provides trimerization of TN by the formation of a triple α-helical coiled coil (Fig. 40.2a, b). Only few contacts were observed among the CRDs of the trimer, in agreement with the observation that the isolated CRD of TN is a monomer in solution. The trimeric structure of TN (TN3) resembles those of rat MBP and human MBP, in which the neck region forms a long α-helix. Each monomer is built of two distinct regions, one region consisting of six β-strands and two α-helices, and the other region is composed of four loops harboring two calcium ions. The calcium ion at site 1 forms an eightfold coordinated complex and has Asp^{116}, Glu^{120}, Gly^{147}, Glu^{150}, Asn^{151}, and one water molecule as ligands. The calcium ion at site 2, which is believed to be involved in recognition and binding of oligosaccharides, is sevenfold coordinated with ligands Gln^{143}, Asp^{145}, Glu^{150}, Asp^{165}, and two water molecules. One sulfate ion was located at the surface of TN, forming contacts to Glu^{120}, Lys^{148}, Asn^{106} of a symmetry-related molecule, and to an ethanol molecule (Kastrup et al. 1998). The orientations of the CRDs are determined by the coiled-coil structure, by intramolecular interactions between the helix and its CRD, and also by intermolecular interactions between each of the helices of the coiled-coil and a neighboring CRD. The trimerization of TN as well as the fixation of the CRDs relative to the helices in the coiled-coil indicates a demand for high specificity in the recognition and binding of ligands (Nielsen et al. 1997).

40.2.5 Functions of Tetranectin

Tissue Remodeling

The function(s) of TN in postnatal life have not been elucidated although there is evidence for roles in tissue remodeling, coagulation, and cancer. Tetranectin may affect coagulation and angiogenesis through interaction with fibrin and angiostatin (a plasminogen fragment), respectively. During development, TN stimulates muscle cell differentiation and fibre formation, and promotes bone mineralization. Tetranectin-deficient mice develop a spinal deformity. TN was originally isolated as a plasminogen-binding protein that can enhance plasminogen activation in presence of tissue plasminogen activator (Clemmensen et al. 1986). Tetranectin binds to plasminogen through a calcium-sensitive interaction of its C-terminal domain with kringle-4 domain of plasminogen (Graversen et al. 1998; Graversen et al. 2000b). Because of the distinct binding properties of TN and its dynamic expression in development and disease, one may expect that TN plays a role in tissue remodeling. The precise function of TN in these processes, however, is not known, and no human genetic disorders have yet been associated with mutations in the single-copy TN gene (Durkin et al. 1997).

Osteoblastic Activity

TN is also expressed during bone development, and transfection studies suggest that TN can induce osteogenesis (Wewer et al. 1994). Studies indicated that the expression of TN in osteoblastic cells is regulated by Dex and TGF-β 1 and that the TN expression is tightly linked to the process of mineralization. Expression of TN is completely inhibited by treatment with retinoic acid, irrespective of the stage of cell differentiation. Retinoic acid downregulates the TN expression in human osteoblastic cells independent of the stage of cell differentiation, and is correlated with inhibition of mineralization (Iba et al. 2001a). Targeted loss of TN expression interferes with proper postnatal development of the vertebral bodies and results in a mild spine deformity. The TN-deficient mice exhibit kyphosis, a type of spinal deformity characterized by an increased curvature of the thoracic spine. The spines of these mice revealed an apparently asymmetric development of the growth plate and of the intervertebral disks of the vertebrae. TN-null mice had a normal peak bone mass density and were not more susceptible to ovariectomy-induced osteoporosis than were their littermates (Iba et al. 2001b).

40.2.6 Association of TN with Diseases

The concentration of TN in human serum decreases in pathological conditions such as cancer (Høgdall 1998), myocardial infarction (Kamper et al. 1998a), and rheumatoid arthritis (RA) (Jensen and Clemmensen 1988; Christensen 1992; Kamper et al. 1998b). TN decreases with the increase of RA activity from group to group. Study points to the usefulness of TN assessment as a specific fibrinolytic marker in the evaluation of disease activity in patients with RA. Reports suggest the implication of TN in the impaired regulation of fibrinolysis associated with the inflammatory process (Kamper et al. 1998b). Acute coronary syndrome (ACS) is triggered by the occlusion of a coronary artery usually due to the thrombosis caused by an atherosclerotic plaque (Bugge et al. 1995). Some of the proteins identified have been associated with ACS whereas others (such as ς-1-B-glycoprotein, Hakata antigen, Tetranectin, Tropomyosin-4) constitute novel proteins that are altered in this pathology (Darde et al. 2010). Altered abundance of TN among few proteins suggests that it may be potential biomarker for alcohol abuse (Lai et al. 2009).

In Prognosis of Cancer

Tetranectin is significantly reduced in patients with various malignancies and may be related to the pathogenesis of cancer spread and metastasis (Jensen and Clemmensen 1988). TN has been detected in tumors of the breast, colon, stomach, and ovary, whereas no TN immunoreactivity was seen in the corresponding normal epithelium of the breast, colon, or ovary (Christensen and Clemmensen 1991; Arvanitis et al. 2002; Obrist et al. 2004). Notably, TN was colocalised together with plasminogen at the invasion front of cutaneous melanomas (De Vries et al. 1996). In case of ovarian cancer, decreased plasma levels of TN were a stronger predictor of adverse prognosis than cancer stage (Høgdall 1998). Furthermore, TN is present in the stroma of various cancers (e.g., breast, ovary, colon), whereas it is not present in normal tissue from which the cancers arose (Wewer and Albrechtsen 1992; Christensen and Clemmensen 1991; Tuxen et al. 1995). Positive staining for TN in cancer stroma has also been strongly correlated with cancer progression (Høgdall 1998). While several investigators have reported that plasma and serum TN concentrations in patients with malignant tumors correlate with disease stage and survival (Blaakaer et al. 1995; Høgdall 1998; Jensen and Clemmensen 1988; Høgdall 1998; Obrist et al. 2004), others found that TN expression in tumor tissue correlated with tumor histological grading (Høgdall 1998). A significantly shortened survival was found for patients with low serum TN values compared to patients with serum TN levels above one of the cutoff levels. Thus, serum TN determination may be valuable in the selection of patients with relapse of ovarian cancer for new treatment strategies in future studies (Deng et al. 2000; Høgdall et al. 2002b). While TN was a strong prognostic variable in patients with advanced ovarian cancer, CA125 was a strong prognostic factor in patients with a localised ovarian cancer and of no prognostic value in patients with advanced cancer (Høgdall et al. 2002a). Serum TN seems to be useful prognostic factor in metastatic breast cancer (Høgdall 1998; Begum et al. 2009; 2010). A slight, but significant reduction in serum TN was found in pelvic inflammatory disease patients. The finding is important in the assessment of TN used as a potential screening marker for ovarian cancer, or as a diagnostic tool for pelvic tumors (Høgdall 1998). Low serum levels of TN are associated with high serum levels of CASA or YKL-40 with increased risk of second-line chemoresistance in patients with ovarian cancer (Gronlund et al. 2006).

In patients with multiple myeloma, either untreated or previously treated, serum levels were found to be significantly reduced (Nielsen et al. 1990). The TN, in association with the circulating intercellular adhesive molecule-1 and IL-10, may be involved in the metastatic cascade of B-chronic lymphocytic leukemia (B-CLL). The findings and good performance characteristics of TN and cICAM-1 in B-CLL suggest the potential usefulness of these adhesive molecules as prognostic markers in B-CLL (Kamper et al. 1999). Colonic neoplasia is associated with a decrease in the tissue distribution of TN, without an obvious change in the tissue level, and a low plasma TN level (Verspaget et al. 1994). Høgdall et al. (2002a) confirmed that TN is a strong prognostic factor in patients with colorectal cancer and may be valuable as a prognostic variable in future studies

evaluating new treatment strategies for colorectal cancer. TN is expressed in a subgroup of bladder cancer patients with a higher risk of recurrence who may take benefit from a closer follow-up (Brunner et al. 2007).

Neurological Diseases

TN may play a role in neurological diseases and may serve as a diagnostic aid in multiple sclerosis (MS). TN appears in CSF of MS patients (Stoevring et al. 2005; 2006; Hammack et al. 2004). Wang et al. (2010a) suggest that CSF-TN and serum-TN are potential biomarkers in epilepsy and drug-refractory epilepsy and would be useful for diagnosis. The application of biomarkers may potentially improve the efficiency of the diagnosis for Parkinson's disease (PD), since no reliable biomarker has been identified to date. Preliminary results of Wang et al. (2010b) suggest that TN and apoA-I may serve as potential biomarkers for PD, though further validation is needed.

40.2.7 Cartilage-Derived C-Type Lectin (CLECSF1)

Cartilage-derived C-type lectin is expressed in cartilage, where it may have a role in organizing the extracellular matrix. Cartilage is a tissue that is primarily extracellular matrix, the bulk of which consists of proteoglycan aggregates constrained within a collagen framework. Candidate components that organize the extracellular assembly of the matrix consist of collagens, proteoglycans and multimeric glycoproteins. Neame et al. (1999) described the human gene structure of a potential organizing factor, a cartilage-derived member of the C-type lectin superfamily (CLECSF1; C-type lectin superfamily) related to tetranectin. The CLECSF1 is restricted to cartilage and the gene is located on chromosome 16q23. The 10.9 kb of sequence upstream of the first exon was characterized. Similarly to human TN, there are three exons. The residues that are conserved between CLECSF1 and TN suggest that the cartilage-derived protein forms a trimeric structure similar to that of TN, with three N-terminal α-helical domains aggregating through hydrophobic faces. The globular, C-terminal domain that has been shown to bind carbohydrate in some members of the family and plasminogen in TN, is likely to have a similar overall structure to that of TN.

40.2.8 Stem Cell Growth Factor (SCGF): Tetranectin Homologous Protein

The SCGF is expressed in skeletal and connective tissues, where it stimulates proliferation and differentiation of haematopoietic precursor cells. The cDNA encoding stem cell growth factor (SCGF; 245 aa), a human growth factor for primitive hematopoietic progenitor cells, has been reported by Hiraoka et al. (1997). This protein consists of 323, 328 and 328 aa in the human, murine and rat forms, the latter two of which share 85.1% and 83.3% aa identity, and 90.4% and 90.4% aa similarity to the human protein, respectively. Because the newly identified human clone encodes the protein longer by 78 aa than that identified earlier, the longer clone was termed as hSCGF-α and the shorter one as hSCGF-β. SCGF is a new member of the C-type lectin superfamily, and shows the greatest homology to TN among the members of the family (27.2–33.7% aa identity and 46.0–40.6% aa similarity). SCGF transcripts are detected in spleen, thymus, appendix, bone marrow and fetal liver. The SCGF gene is located on chromosome 19 at position q13.3 for human form, and on chromosome 7 at position B3-B5 for murine form, which are close to flk-2/flt3 ligand and interleukin-11 genes of both human and murine species (Mio et al. 1998).

40.3 Attractin Group of CTLDs (Group XI)

The autosomal recessive gene of attractin has received great attention within last few decades. The attractin group of CTLD-containing type 1 transmembrane proteins has two widely-expressed members in both human and mouse: attractin (ATRN) and attractin-like protein (ATRNL1).

40.3.1 Secreted and Membrane Attractins

Attractin, initially identified as a soluble human plasma protein with dipeptidyl peptidase IV (DPPIV) activity that is expressed and released by activated T lymphocytes, has also been identified as the product of the murine mahogany gene with connections to control of pigmentation and energy metabolism. The mahogany gene product, however, is a transmembrane protein, raising the possibility of a human membrane attractin in addition to the secreted form. The genomic structure of human attractin reveals that soluble attractin arises from transcription of 25 sequential exons on human chromosome 20p13, where the 3′ terminal exon contains sequence from a long interspersed nuclear element-1 (LINE-1) retrotransposon element that includes a stop codon and a polyadenylation signal. The mRNA isoform for membrane attractin splices over the LINE-1 exon and includes five exons encoding transmembrane and cytoplasmic domains with organization and coding potential almost identical to that of the mouse gene. The relative abundance of soluble and transmembrane isoforms is differentially regulated in lymphoid tissues. Since activation of peripheral blood leukocytes with phytohemagglutinin

induces strong expression of cell surface attractin followed by release of soluble attractin, results suggest that a genomic event unique to mammals, LINE-1 insertion, has provided an evolutionary mechanism for regulating cell interactions during an inflammatory reaction (Tang and Duke-Cohan 2000).

40.3.2 Attractin has Dipeptidyl Peptidase IV Activity?

Dipeptidyl peptidase IV (DPPIV) is a serine type protease with an important modulatory activity on a number of chemokines, neuropeptides and peptide hormones. It is also known as CD26 or adenosine deaminase (ADA; EC 3.5.4.4) binding protein. DPPIV has been demonstrated on the plasma membranes of T cells and activated natural killer or B cells as well as on a number of endothelial and differentiated epithelial cells. A soluble form of CD26/DPPIV has been described in serum. Attractin, the human orthologue of mouse mahogany protein, was postulated to be responsible for the majority of DPPIV-like activity in serum. 95% of the serum dipeptidyl peptidase activity is associated with a protein with ADA-binding properties. The natural DPPIV serum enzyme was confirmed as CD26 (Durinx et al. 2000). However, the attractin acts as a receptor or adhesion protein rather than a protease (Friedrich et al. 2007). Human peripheral blood monocytes express a DPPIV-like enzyme activity, which could be inhibited completely by the synthetic DPPIV inhibitor. Drugs directed to DPPIV-like enzyme activity can affect monocyte function via attractin inhibition (Wrenger et al. 2006). The expression of DPPV and attractin in circulating blood monocytes of human subjects are influenced by metabolic abnormalities with obesity being an important factor (Laudes et al. 2010). In the CNS, attractin has been detected in neurons and in glioma cell lines at different degree of transformation. In human U373 and U87 glioma cells, membrane-bound attractin displays hydrolytic activity between 5% and 25% of total cellular DPPIV-like enzyme activity, respectively. Attractin presence in glioma, but not in normal glial cells, together with its differential enzymatic activity, suggests its role in growth properties of tumors of glial cell origin (MalÃk et al. 2001).

Attractin is a normal human serum glycoprotein of 175 kDa that was found to be rapidly expressed on activated T cells and released extracellularly after 48–72 h. There are two mRNA species with hematopoietic tissue-specific expression that code for a 134-kDa protein with a putative serine protease catalytic serine. Both have large extracellular regions consisting of a membrane-distal epidermal growth factor (EGF) domain followed by a CUB domain; two EGF domains; five Kelch domains; a cysteine-rich/plexin domain; a CTLD; a second cysteine-rich/plexin domain; and two laminin EGF domains. Except for the latter two domains, the overall structure shares high homology with the *Caenorhabditis elegans* F33C8.1 protein, suggesting that attractin has evolved new domains and functions in parallel with the development of cell-mediated immunity (Duke-Cohan et al. 1998, 2000). Attractin is found in both membrane-bound and secreted forms as a result of alternative splicing.

40.3.3 Attractin-Like Protein

The gene dosage effect of melanocortin-4 receptor (MC4R) on obesity suggests that regulation of MC4-R expression is critically important to the central control of energy homoeostasis. In order to identify putative MC4-R regulatory proteins, Haqq et al. (2003) identified a positive clone that shares 63% amino acid identity with the C-terminal part of the mouse attractin gene product, a single-transmembrane-domain protein characterized as being required for agouti signaling through MC4R. A direct interaction between this (ATRNL1) and the C-terminus of the mouse MC4-R was confirmed and the regions in this interaction involved residues 303–313 in MC4-R and residues 1280–1317 in ATRNL1. ATRNL1 is highly expressed in brain, but also in heart, lung, kidney and liver. Furthermore, co-localization analyses in mice showed co-expression of ATRNL1 in cells expressing MC4-R in a number of regions known to be important in the regulation of energy homoeostasis by melanocortins, such as the paraventricular nucleus of hypothalamus and the dorsal motor nucleus of the vagus (Haqq et al. 2003).

Attractin and Attractin-like 1 are highly similar type I transmembrane proteins. *Atrn* null mutant mice have a pleiotropic phenotype including dark fur, juvenile-onset spongiform neurodegeneration, hypomyelination, tremor, and reduced body weight and adiposity, implicating ATRN in numerous biological processes. Bioinformatic analysis indicated that Atrn and Atrnl1 arose from a common ancestral gene early in vertebrate evolution. Characterization of Atrnl1 loss- and gain function mutant mice suggested that *Atrnl1* mutant mice were grossly normal with no alterations of pigmentation, CNS pathology or body weight. *Atrn* null mutant mice carrying a β-actin promoter-driven *Atrnl1* transgene had normal, agouti-banded hairs and significantly delayed onset of spongiform neurodegeneration, indicating that overexpression of ATRNL1 compensates for loss of ATRN. Thus, two genes are redundant from the perspective of gain-of-function but not loss-of-function mutations (Walker et al. 2007). The cytoplasmic region of ATRNL1 interacts with the cytoplasmic tail of the MC4R, a G-protein-coupled receptor in the brain which regulates appetite and metabolism, but the

function of ATRNL1 is not understood. The CTLDs in attractin and ATRNL1 contain some of the residues which in other CTLDs are involved in binding mannose-type sugars. It is not known if carbohydrate recognition by the CTLDs is involved in the functions of these proteins.

A heterozygous deletion of the *Attractin-like* (*ATRNL1*) gene in a patient presented with a distinctive phenotype comprising dysmorphic facial appearance, ventricular septal defect, toe syndactyly, radioulnar synostosis, postnatal growth retardation, cognitive impairment with autistic features, and ataxia. The phenotype of mice with homozygous *Atrn* mutations overlaps considerably with the features observed in this patient. It was postulated that patient's phenotype is caused by the deletion of ATRNL1 (Stark et al. 2010).

40.3.4 Functions of Attractin

Multiple Functions: Attractin is known to play multiple roles in regulating physiological processes that are involved in monocyte-T cell interaction, agouti-related hair pigmentation, and control of energy homeostasis. Attractin affects the balance between agonist and antagonist at receptors on melanocytes, modifies behaviour and basal metabolic rate, intervenes in the development of CNS and its functions. Attractin modulates an interaction between activated T cells and macrophages. The loss of murine membrane attractin due to mahogany mutation results with severe repercussions upon skin pigmentation and control of energy metabolism. In each of these latter instances, there is a strong likelihood that attractin is moderating the interaction of cytokines with their respective receptors. Attractin is performing a similar function in the immune system through capture and proteolytic modification of the N-terminals of several cytokines and chemokines. This regulatory activity allows cells to interact and form immunoregulatory clusters and subsequently aids in downregulating chemokine/cytokine activity once a response has been initiated. These two properties are likely to be affected by the balance of membrane-expressed to soluble attractin (Duke-Cohan et al. 2000). The attractin protein is involved in the suppression of diet-induced obesity. Its expression in brain has a significant relationship with obesity and indicates the therapeutic potential of attractin in the treatment of obesity. Furthermore, the murine attractin locus is located in a region harboring several QTL for body weight and fatness.

Membrane-Bound Attractin Is a Co-Receptor for Agouti and Regulates Pigmentation in Skin: Melanocortin-1 receptor (MC1R) and its ligands, α-melanocyte stimulating hormone (αMSH) and agouti signaling protein (ASIP), regulate switching between eumelanin and pheomelanin synthesis in melanocytes. ASIP-MC1R signaling includes a cAMP-independent pathway through attractin and mahogunin, while the known cAMP-dependent component requires neither attractin nor mahogunin. Attractin may be a component of a pathway for regulated protein turnover that also involves mahogunin, a widely-expressed E3 ubiquitin ligase found at particularly high levels in brain. Membrane-bound attractin is a co-receptor for Agouti, the paracrine signaling molecule in the skin which regulates hair pigmentation by antagonizing the MC1R (Hida et al. 2009; Jackson 1999). Genetic, biochemical and pharmacological studies in humans and rodents have established that signaling through the G-protein-coupled melanocortin-4 receptor (MC4R) by pro-opiomelano- cortin (POMC)-derived ligands plays a critical role in the central suppression of appetite. As a consequence, malfunction of this signaling system leads to the development of obesity. It has been shown that melanocortin signaling can be modulated by attractin, apparently acting as a co-receptor for the inhibitory ligand agouti. Haqq et al. (2003) demonstrated that the cytosolic tail of an attractin-like protein binds directly and specifically to the C-terminal region of MC4R, raising the possibility that proteins of the attractin family influence melanocortin receptor function through multiple mechanisms (Carlson and Moore 1999; Yeo and Siddle 2003).

Agouti is expressed ubiquitously, such as lethal yellow, have pleiotropic effects that include a yellow coat, obesity, increased linear growth, and immune defects. The *mahogany* mutation suppresses the effects of lethal yellow on pigmentation and body weight. Results of genetic studies place mahogany downstream of transcription of *Agouti* but upstream of melanocortin receptors. Positional cloning of *mahogany* gene *Mgca* predicted a protein of 1,428 aa with a single-transmembrane-domain that is expressed in many tissues, including pigment cells and the hypothalamus. The extracellular domain of the Mgca protein is the orthologue of human attractin, a molecule produced by activated T cells that has been implicated in immune-cell interactions (Gunn et al. 1999).

Mahogunin Ring Finger-1 and Attractin Act Through Common Pathway: Oxidative stress, ubiquitination defects and mitochondrial dysfunction are common factors associated with neurodegeneration. Mice lacking mahogunin ring finger-1 (MGRN1) or attractin (ATRN) develop age-dependent spongiform neurodegeneration through an unknown mechanism. It has been suggested that they act through a common pathway. As MGRN1 is an E3 ubiquitin ligase, it was suggested that many mitochondrial proteins were reduced in *Mgrn1* mutants. Mitochondrial dysfunction was obvious many months before onset of vacuolation, implicating this as a causative factor. Compatible with the hypothesis that ATRN and MGRN1 act in the same pathway, mitochondrial dysfunction and increased oxidative stress were also observed in the brains of Atrn mutants. Study of *Mgrn1* and *Atrn* mutant mice can provide insight into molecular mechanism common to many neurodegenerative disorders (Sun et al. 2007; Kadowaki et al. 2007; Walker and Gunn 2010).

Cerebral Spongiform Changes: A mutation characterized by mahogany coat color, sprawling gait, tremors, and severe vacuolization of cerebrum, brainstem, granular layer of cerebellum and spinal cord was discovered in a stock of Mus castaneus mice. Histopathological analysis of brains from Mice homozygous for 2 known mahogany attractin (*Atrnmg*) mutants showed that they also have severe spongiform changes. This surprising finding raises questions about the mechanism by which mahogany controls appetite and metabolic rate, as reported (Bronson et al. 2001). A null mutation for *mahoganoid* causes a similar age-dependent neuropathology that includes many features of prion diseases but without accumulation of protease-resistant prion protein. The gene mutated in *mahoganoid* encodes a RING-containing protein with E3 ubiquitin ligase activity in vitro. Similarities in phenotype, expression, and genetic interactions suggest that *mahoganoid* and *Atrn* genes are part of a conserved pathway for regulated protein turnover whose function is essential for neuronal viability (He et al. 2003b).

Secreted Attractin Disrupts Neurite Formation in Differentiating Cortical Neural Cells In Vitro: Mutations at *Atrn* locus affects the secreted form mRNA, which is down-regulated in discrete regions of human brain while membrane attractin mRNA is well represented, resulting in the apparent absence of secreted attractin protein in CSF. In vitro, transcription of secreted form of attractin mRNA is strongly down-regulated upon differentiation of a human cortical neuron-derived cell line (HCN-1A) to a mature neuron phenotype in response to nerve growth factor. Tang and Duke-Cohan (2002) proposed that inappropriate expression of secreted attractin in the CNS blocks membrane attractin function and that its presence, either by leakage from the periphery, aberrant transcription, or release from inflammatory foci may affect neuron extracellular interactions leading to neurodegeneration in the human.

Attractin/Mahogany Protein in Rodent CNS: Both the secreted and membrane-bound forms of ATRN may be involved in the development and maintenance of the CNS. ATRN was intensely expressed in most neurons and dendrites of large neurons, such as cortical pyramidal neurons and cerebellar Purkinje neurons in CNS of rat and mice. Intense ATRN expression was also observed in the neuropil of gray matter in many regions of the CNS and indicates that ATRN is more widely expressed throughout the CNS. Expression of ATRN by various cell types suggests that ATRN serves multiple functions in the CNS (Nakadate et al. 2008). The zitter (zi/zi) rat, a loss-of-function mutant of the glycosylated transmembrane ATRN exhibits widespread age-dependent spongiform degeneration, hypomyelination, and abnormal metabolism of reactive oxygen species (ROS) in the brain. The onset of the impairment of oligodendrocyte differentiation occurs in a non-cell autonomous manner in zi/zi rats (Nakadate et al. 2008; Sakakibara et al. 2008; Ueda et al. 2008).

The Mahogany Is a Receptor Involved in Suppression of Obesity: The pathogenesis of obesity is multifactorial and involves the interaction of genetic and environmental factors. A number of important signaling molecules play important roles in obesity. One family of these molecules is the melanocortin system, which consists of several components: (1) melanocortin peptides; (2) the five seven-transmembrane G-protein coupled melanocortin receptors (MCRs); (3) the endogenous MCR antagonists, agouti and agouti-related protein; (4) the endogenous melanocortin mediators, mahogany, and syndecan. This system plays a key role in the central nervous system control of feeding behaviour and energy expenditure (Carlson and Moore 1999; Yang and Harmon 2003).

Expression of *mahogany* gene is broad; in situ hybridization analysis emphasizes the importance of its expression in the ventromedial hypothalamic nucleus, a region that is intimately involved in the regulation of body weight and feeding. Genetic studies indicate that the *mahogany* locus does not suppress the obese phenotype of the MC4-R null allele or those of the monogenic obese models (Lep^{db}, tub and Cpe^{fat}). However, mahogany can suppress diet-induced obesity, the mechanism of which is likely to have implications for therapeutic intervention in common human obesity. The amino-acid sequence of the mahogany protein suggests that it is a large, single-transmembrane-domain receptor-like molecule, with a short cytoplasmic tail containing a site that is conserved between *Caenorhabditis elegans* and mammals. It was suggested that mahogany can act as a signaling receptor (Nagle et al. 2000).

Attractin in Testis: The ATRN is expressed in Leydig cells, primitive spermatogonia, primary spermato- cytes, spermatids, Sertoli cells and peritubular myoid cells. The attractin protein was mainly located on the cell membrane and cytoplasm, and its mRNA distributed in the nucleus and cytoplasm. The rat testis has the ability of synthesizing the ATRN protein throughout sexual development. The loss of ATRN in mice showed no significantly different pathological changes from the control mice in 3-month-old ATRN (*mg-3J*) mice. But age-related *Atrn* gene progressively lost its function and caused testis vacuolization and impaired sperm function, which may be responsible for the impairment of male reproductive ability (Li et al. 2009). Human sperm-associated antigen 11 (SPAG11) iso-proteins, closely related to β-defensins in structure and function, are predominantly expressed in male reproductive tract, where their best-known roles are in innate host defense and reproduction. Human SPAG11B isoform D (SPAG11B/D) interacts with ATRN. SPAG11B/D and the ATRN interacting proteins are expressed in the proximal epididymis, and function in immunity and fertility control. Radhakrishnan et al. (2009) showed that SPAG11B/D is both a substrate and a potent inhibitor of TPSAB1 activity. Llike SPAG11B/D, ATRN is associated with spermatozoa.

40.3.5 Genetic and Phenotypic Studies of *Mahogany/Attractin* Gene

Pigment-Type Switching: The mouse *mahogany* mutation affects melanocortin signaling pathways that regulate energy homeostasis and hair color. The gene mutated in *mahogany* mice encodes attractin that is broadly expressed and conserved among multicellular animals. The mouse mutations *mahogany (mg)* and *mahoganoid (md)* are negative modifiers of the *Agouti* coat color gene, which encodes a paracrine signaling molecule that induces a switch in melanin synthesis from eumelanin to pheomelanin. Results suggest that *md* and *mg* interfere directly with Agouti signaling, possibly at the level of protein production or receptor regulation (Miller et al. 1997). The mouse *Attractin* (*Atrn*) (formerly *mahogany*) gene has been proposed as a downstream mediator of Agouti signaling because yellow hair color and obesity in lethal yellow (A^y) mice are suppressed by the *mahogany* ($Atrn^{mg}$) mutation. Atrn mRNA was found widely distributed throughout the CNS, with high levels in regions of the olfactory system, some limbic structures, regions of the brainstem, cerebellum and spinal cord. In the hypothalamus, ATRN mRNA was found in specific nuclei including the suprachiasmatic nucleus, the supraoptic nucleus, the medial preoptic nucleus, the paraventricular hypothalamic nucleus, the ventromedial hypothalamic nucleus, and the arcuate nucleus. Results suggest a broad spectrum of physiological functions for *Atrn* gene product (Lu et al. 1999).

Mutations of the mouse *Attractin* gene were originally recognized because they suppress Agouti pigment type switching. Agouti protein, a paracrine signaling molecule normally limited to skin, is ectopically expressed in lethal yellow (A^y) mice, and causes obesity by mimicking agouti-related protein (Agrp), found primarily in the hypothalamus. Mouse attractin is a widely expressed protein whose loss of function in *mahogany* ($Atrn^{mg\text{-}3J}/Atrn^{mg\text{-}3J}$) mutant mice blocks the pleiotropic effects of A^y. Results showed that attractin is a low-affinity receptor for agouti protein, but not Agrp, in vitro and in vivo. Histopathologic abnormalities in $Atrn^{mg\text{-}3J}/Atrn^{mg\text{-}3J}$ mice and cross-species genomic comparisons indicate that Atrn has multiple functions distinct from both a physiologic and an evolutionary perspective (He et al. 2001).

Characterization of two additional *Atrn* alleles, $Atrn^{mg}$ and $Atrn^{mg\text{-}L}$ by Gunn et al. (2001), who examined in parallel the phenotypes of homozygous and compound heterozygous animals showed that the three alleles have similar effects on pigmentation and neurodegeneration, with a relative severity of $Atrn^{mg\text{-}3J} > Atrn^{mg} > Atrn^{mg\text{-}L}$, which also corresponds to the effects of three alleles on levels of normal Atrn mRNA. Animals homozygous for $Atrn^{mg\text{-}3J}$ or $Atrn^{mg}$, but not $Atrn^{mg\text{-}L}$, showed reduced body weight, reduced adiposity, and increased locomotor activity, all in the presence of normal food intake. These results confirm that the mechanism responsible for the neuropathological alteration is a loss—rather than gain—of function and indicate that abnormal body weight in *Atrn* mutant mice is caused by a central process leading to increased energy expenditure, and that the pigmentation is more sensitive to levels of *Atrn* mRNA than the nonpigmentary phenotypes (Gunn et al. 2001).

Pleiotropic phenotype in *dark-like* (*dal*) mutant mouse includes dark dorsal hairs and reproductive degeneration. Their pigmentation phenotype is similar to *Atrn* mutants, which also develop vacuoles throughout brain. Testicular degenerated *dal* mutant males showed reduced serum testosterone and developed vacuoles in their testes. Genetic crosses placed *da*l upstream of the *melanocortin 1 receptor (Mc1r)* and downstream of *agouti,* although *dal* suppressed the effect of agouti on pigmentation but not body weight. $Atrn^{mg\text{-}3J}$ and dal showed additive effects on pigmentation, testicular vacuolation, and spongiform neurodegeneration, but transgenic over-expression of *Attractin-like-1 (Atrnl1)*, which compensates for loss of ATRN, did not rescue *dal* mutant phenotypes. Results suggest that *dal* and *Atrn* function in the same pathway and that identification of *dal* gene will provide insight into molecular mechanisms of vacuolation in multiple cell types (Cota et al. 2008).

The Mouse Coat Color Mutant Mahoganoid (md): *Mahoganoid* is a mouse coat-color mutation whose pigmentary phenotype and genetic interactions resemble those of *Atrn*. The mouse coat color mutant *mahoganoid* (*md*) darkens coat color and decreases the obesity of A^y mice that ectopically over-express agouti-signaling protein. The phenotypic effects of *md* are similar to those of coat color mutant mahogany ($Atrn^{mg}$). The mahoganoid encodes a 494-amino acid protein containing a C3HC4 RING (really interesting new gene) domain that may function as an E3 ubiquitin ligase. The mutations in mahoganoid allelic series (*md,* md^{2J}, md^{5J}) are all due to large retroviral insertions. In *md* and md^{2J}, the result is minimal expression of the normal size transcripts in all tissues examined. Unlike $Atrn^{mg}/Atrn^{mg}$ animals, there was no evidence of neurological deficit or neuropathology in *md*/*md* mice. Body weight and body mass index (a surrogate for adiposity) measurements of B6.C3H-*md-A* *md*/+ and *md*/*md* animals on 9% and 45% kcal fat diets indicate that *mahoganoid* does not suppress body weight in B6.C3H animals in a gene dose-dependent fashion. *Mahoganoid* effects on energy homeostasis are, therefore, most evident in the circumstances of epistasis to hypothalamic over-expression of ASP in A^y and possible other obesity-causing mutations (Phan et al. 2002).

Genes Controlling the Synthesis of Eumelanin and Pheomelanin: Mutations that affect the balance between the synthesis of eumelanin and pheomelanin provide a powerful set of tools with which to understand general aspects of cell signaling. It has been demonstrated that pheomelanin synthesis is triggered by the ability of Agouti protein to inhibit signaling through the *Melanocortin 1 receptor (Mc1r)*. The pigmentary effects of Agouti are suppressed by previously existing coat-color mutations *mg*, *md*, and *Umbrous* (*U*). Double mutant studies, with animals deficient for *Mc1r* or those which carry A^y, indicated that *mg* and *md* are genetically upstream of the Mc1r, and can suppress the effects of A^y on both pigmentation and body weight. Positional cloning has identified the gene mutated in mahogany as a single transmembrane-spanning protein whose ectodomain is orthologous to human ATRN (Barsh et al. 2000). The coat color mutations *mg* and *md* prevent hair follicle melanocytes from responding to Agouti protein. The gene mutated in *mg*, encodes ATRN that functions as an accessory receptor for Agouti protein. The gene mutated in *mahoganoid*, which is also known as *Mahogunin (Mgrn1*), encodes an E3 ubiquitin ligase. Like Attractin, Mahogunin is conserved in invertebrate genomes, and its absence causes a pleiotropic phenotype that includes spongiform neurodegeneration (He et al. 2003a).

Control of Metabolic Rate: Mouse attractin is likely to have additional roles outside melanocortin signaling (Gunn and Barsh 2000). The *mahogany (mg) or Attractin* (*Atrn*)*)* locus was originally identified as a recessive suppressor of *agouti,* a locus encoding a skin peptide that modifies coat color by antagonizing the MSH-R or MC1-R. Certain dominant alleles of *agouti* cause an obesity syndrome when ectopic expression of the peptide aberrantly antagonizes the MC4-R, a related melanocyte-stimulating hormone receptor expressed in hypothalamic circuitry and involved in the regulation of feeding behavior and metabolism. Reports indicate that *mg*, when homozygous, blocks not only the ability of *agouti* to induce a yellow coat color when expressed in the skin of the lethal *yellow mouse* (A^Y), but also the obesity resulting from ectopic expression of *agouti* in the brain. Detailed analysis of *mg*/*mg* A^Y/*a* animals, demonstrates that *mg*/*mg* blocks the obesity, hyperinsulinemia, and increased linear growth induced by ectopic expression of the agouti peptide. Remarkably, however, *mg*/*mg* did not reduce hyperphagia in the A^Y/*a* mouse. Furthermore, *mg*/*mg* induced hyperphagia and an increase in basal metabolic rate in the C57BL/6J mouse in the absence of A^Y. Consequently, although mahogany is broadly required for agouti peptide action, it also appears to be involved in the control of metabolic rate and feeding behavior independent of its suppression of *agouti* (Dinulescu et al. 1998).

Hypomyelination in Rat Zitter: In absence of attractin there is a decline in plasma membrane glycolipid-enriched rafts. The structural integrity of lipid rafts depends upon cholesterol and sphingomyelin, and can be identified by partitioning of ganglioside GM1. Despite a significant fall in cellular cholesterol with maturity, and a lesser fall in both membrane and total cellular GM1, these parameters lag behind raft loss, and are normal when hypomyelination/neurodegeneration has already begun thus supporting consequence rather than cause. These findings can be recapitulated in Atrn-deficient cell lines propagated in vitro (Azouz et al. 2007) Histopathological analysis of brains from Mice homozygous for 2 known mahogany attractin (*Atrnmg*) mutants showed that they also have severe spongiform changes. This surprising finding raises questions about the mechanism by which mahogany controls appetite and metabolic rate, as recently reported (Bronson et al. 2001).

The rat zitter (zi) mutation induces hypomyelination and vacuolation in CNS, which results in early-onset tremor and progressive flaccid paresis. Kuramoto et al. (2001) found a marked decrease in ATRN mRNA in the brain of the zi/zi rat and identified zi as an 8-bp deletion at a splice donor site of *Atrn*. Rat *Atrn* gene encoded two isoforms, a secreted and a membrane form, as a result of alternative splicing. The zi mutation at the *Atrn* locus darkened coat color when introduced into agouti rats, as also described in *mahogany* (*mg*) mice, carrying the homozygous mutation at *Atrn* locus. Transgenic rescue rats showed that the membrane-type ATRN complemented both neurological alteration and abnormal pigmentation in zi/zi rats, but that the secreted-type ATRN complemented neither mutant phenotype. Furthermore, *mg* mice exhibited hypomyelination and vacuolation in the CNS associated with body tremor (Kuramoto et al. 2001). Ultrastructurally these vacuoles mainly consisted of splitting of myelin lamella both in the periaxonal and intermyelinic spaces. Linkage analysis using intercross progeny between the myelin vacuolation (mv) rat, named after the pathologic characteristics, and normal control rat strains showed that the mv phenotypes were cosegregated with polymorphic markers adjacent to the *Atrn* (Attractin, formerly zi [zitter]) locus on rat chromosome 3. Discovery of the rat null mutation *Atrn(mv)*, different from *Atrn(zi)*, provides a new animal model for studying the functions of the attractin protein (Kuwamura et al. 2002). Studies suggest that lack of *Atrn* gene expression induced neurodegeneration by a decrease in active ERK through an intracellular signaling via oxidative stress (Muto and Sato 2003). These studies indicate that membrane ATRN has a critical role in normal myelination in CNS and provide insights into the physiology of myelination as well as the etiology of myelin diseases. Izawa et al. (2008) indicated that the attractin defect results in oligodendrocyte dysfunction, and is associated with astrogliosis and microglial

activation in mv rats. Attractin may be directly involved in the function of oligodendro-cytes in CNS myelination. Results indicate that myelinogenesis but not oligodendrogenesis is severely altered both in the white and gray matter of mv rats (Izawa et al. 2010).

The Hamster Black Tremor (bt) Mutation: The hamster black tremor (bt) mutation induces a black coat color and a defective myelination in CNS that manifests as a tremor. On the other hand, loss-of-function mutations of the *Atrn* gene, such as *Atrnmg*, *Atrnmg-L*, and *Atrnmg-3J* in mice, and *Atrnzi* in rats, induce both darkening of coat color and hypomyelination and vacuolation in CNS. The close resemblance of mutant phenotypes led to postulate that the bt/bt hamster also might harbor a mutation in *Atrn*. While the human and rat *Atrn* genes encode both membrane- and secreted-type proteins, the hamster *Atrn* gene encoded only membrane-type protein with 1,427 amino acids, as in the case of the mouse. Hamster ATRN had 93.6%, 96.8%, and 96.8% identities with human, rat, and mouse membrane-type Attractin. In the brain of the bt/bt hamster, aberrant transcripts with more than three size species were observed, and the most predominant transcript encoded the truncated Attractin without transmembrane domain. The hamster bt mutation was the approximately 10-kb retrotransposon-like insertion into the *Atrn* gene, which resulted in aberrant transcripts (Kuramoto et al. 2002).

40.3.6 Therapeutic Applications of ATRN/*Mahogany* Gene Products

The C-terminal peptide product encoded by *mahogany* gene crosses the blood–brain barrier (BBB) by a transport mechanism that is saturable. The ability of this system indicates the therapeutic potential of mahogany (1377–1428) in the treatment of obesity (Kastin and Akerstrom 2000). Avy/agouti (A^{vy}) mice have late onset obesity related to over-expression of agouti signaling protein (ASP) in the hypothalamus. As mahogany modulates the actions of ASP, Pan and Kastin (2007) tested the transport of mahogany peptide across BBB. The brain uptake of mahogany peptide was significantly higher in young A^{vy} mice, and it preceded the surge of fat mass quantified by NMR. The results suggest a role of accelerated BBB transport in the epigenetics of A^{vy} mice.

The proliferative responses of T lymphocytes of a subset of patients with CVID are abnormally low. This may be due to abnormalities in extracellular interactions or signaling defects downstream from membrane-associated receptors. Attractin is a rapidly expressed T cell activation antigen involved in forming an association between T cells and monocytes. Due to the likely role of attractin in cell guidance and amplification of immune response, results indicate that the lack of up-regulation of the molecule in patients with CVID may in turn affect any further step of productive immune response. This finding may imply a potential therapeutic role for this novel molecule (Pozzi et al. 2001). Attractin is a reliable secreted marker for high-grade gliomas. Additionally, it may be an important mediator of tumor invasiveness, and thus, a potential target in future therapies (Khwaja et al. 2006). ATRN may play a protective role against environmental toxins that implies a potential therapeutic effect of ATRN for neurodegenerative diseases (Paz et al. 2007).

40.4 Eosinophil Major Basic Protein 1 (EMBP1) (Group XII of CTLD)

40.4.1 Characterization of EMBP

Eosinophils are implicated in the combat of infections caused by helminth parasites and viruses and are associated with tissue damage in a variety of diseases (Gleich 2000). A distinctive group of proteins form the eosinophil granule. Among these, the eosinophil major basic protein 1 (EMBP1) is the most abundant eosinophil granule protein that forms the crystalline core of the granule and belongs to Group XII in CTLD classificatiion. Proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein), also known as PRG2, is the protein which in humans is encoded by the *PRG2* gene. EMBP is a 14 kDa (117 amino acid residues) intensely basic protein whose complement of basic residues consists of arginines, giving rise to a calculated pI of 11.4 and a net charge of 15.0 at pH 7 (Barker et al. 1988; McGrogan et al. 1988). However, pro-EMBP is almost neutral with a calculated pI of 6.2, because of the presence of a large number of acidic residues in the 90 residue long pro-portion (pI 3.9). It has been suggested that the pro-portion of EMBP serves predominantly to protect cells from the extreme basicity of EMBP during transport from the Golgi to the eosinophil granule (Barker et al. 1991).

EMBP is a monomer under physiological conditions that readily polymerizes in solution forming insoluble aggregates because of the presence of five free thiol groups (in addition to the four cysteines involved in disulfide bond formation) in the protein (Oxvig et al. 1994a). It has been shown to be non-glycosylated, contrasting with the pro-protein, which is heavily glycosylated with N-glycans, O-glycans, and glycosaminoglycans, raising the molecular weight to between 30 and 50 kDa (Oxvig et al. 1994b). During eosinophilopoiesis, EMBP is initially transcribed as a pro-molecule containing a markedly acidic pro-piece. The promolecule can be identified in eosinophil granules, and current evidence suggests that it is processed here into EMBP, which then condenses to form the eosinophil granule core (Popken-Harris et al. 1998). A EMBP homologue (EMBPH) similar to EMBP has also been identified in human eosinophil granule lysates (Plager et al. 1999).

40.4.2 Functions of EMBP

EMBP is expressed in eosinophils, basophils, and placental X cells. In eosinophils, the protective acidic region is removed and the CTLD covalently polymerizes to form insoluble aggregates that make up the crystalline core of eosinophil granules. EMBP is a cytotoxin deployed against helminth parasites, and possibly also against bacteria, fungi and cancerous or infected cells. EMBP triggers release of histamine from mast cells and basophils, and activates neutrophils and alveolar macrophages. Release of EMBP and its deposition onto tissues is linked to hypersensitivity reactions and causes tissue dysfunction in inflammatory and allergic conditions, including asthma. Towards the end of pregnancy, pro-EMBP circulates in a complex with pregnancy-associated plasma protein A, angiotensin and C3dg. EMBPH is expressed in eosinophils. It is less basic and less potent than EMBP, but has similar biological activities. The CTLDs in these proteins do not contain the sequence motifs associated with sugar-binding activity. However, EMBP binds heparin through a novel calcium-independent carbohydrate-recognition mechanism.

EMBP is a potent helminthotoxin and is cytotoxic toward bacteria and mammalian cells in vitro (Swaminathan et al. (2001) and may have important roles in allergic and inflammatory reactions. The EMBP causes release of histamine from mast cells and basophils, activates neutrophils and alveolar macrophages, and is directly implicated in epithelial cell damage, exfoliation and bronchospasm in asthma (Swaminathan et al. 2001). The exact mechanism of EMBP action is not known, although it has been speculated that the highly basic nature of the protein contributes to its toxicity.

The concentration of EMBP is elevated in biological fluids (e.g. sputa and bronchoalveolar lavage fluids) from patients with asthma and other eosinophil-associated diseases. Deposition of EMBP onto damaged tissues in various human diseases is strongly associated with dysfunction of those tissues, and instillation of EMBP into the lung of monkeys causes a tenfold increase in bronchial hyperreactivity, as well as marked bronchospasm (Gundel et al. 1991). EMBP appears to damage cells by disrupting the bilayer lipid membrane (Gleich et al. 1993) or altering the activity of enzymes within tissues (Hastie et al. 1987). Evidence also implicates pro-EMBP as an inhibitor of human pregnancy-associated plasma protein A (Overgaard et al. 2000).

40.4.3 Crystal Structure of EMBP

EMBP does not exhibit high sequence similarity to any known proteins, but has weak sequence identity (23–28%) with C-type lectin domains of mannose-binding protein, human lithostathine (LIT), and the low affinity IgE receptor FceRII (Patthy 1989). Swaminathan et al. (2001) reported the crystal structure of EMBP at 1.8 Å resolution. This protein is structurally related to members of the CTLD family, but is significantly different from known CTLDs, lacking the potential to bind carbohydrates in a calcium dependent manner. EMBP are likely to be proteoglycans such as heparin, leading to new interpretations for EMBP function (Swaminathan et al. 2005). Evidence of binding of EMBP to heparin and heparin disaccharide has been presented. The crystal structure of EMBP in complex with a heparin disaccharide and in the absence of Ca^{2+} is shown in Fig. 40.3, a report of any C-type lectin with this sugar. Structural analysis shows that the potential carbohydrate ligands recognized by EMBP are likely to be different from those in other CTLDs.

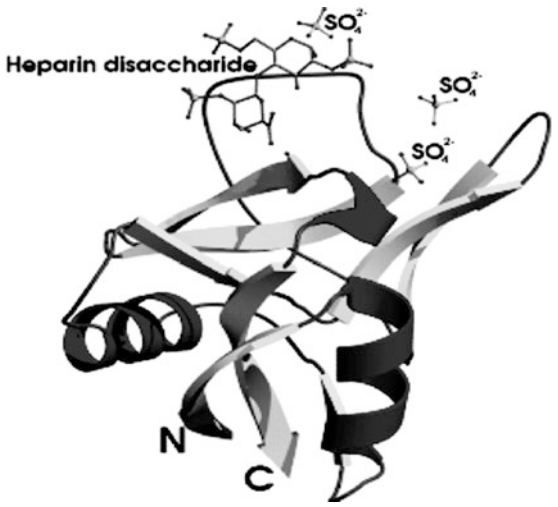

Fig. 40.3 Crystal structure of EMBP in complex with heparin disaccharide (PDB: 2BRS). The sugars recognized by EMBP are likely to be proteoglycans such as heparin, leading to new interpretations for EMBP function (Printed with permission from Swaminathan et al. 2005 © American Chemical Society)

40.4.4 DEC-205-Associated C-Type Lectin (DCL)-1

DEC-205-associated C-type lectin (DCL)-1 was also formerly classified as a member of group XII. However, DCL-1 is a type 1 transmembrane protein with an extracellular CTLD and neck region and may be classified as belonging to a new group of CTLD-containing proteins. DCL-1 was discovered in cells associated with the development of Hodgkin's lymphoma. Intergenic splicing of DCL-1 and DEC-205 (a CTLD-containing type 1 transmembrane protein of the mannose receptor family (Chaps 15 and 35) results in production of a fusion protein containing both DCL-1 and DEC-205 extracellular domains, plus the transmembrane and cytoplasmic regions of DCL-1. Non-intergenically spliced DCL-1 transcripts are present in myeloid and B lymphocyte cell lines.

Fig. 40.4 Domaim organization of Integral membrane protein (IDD/DGCR2) (Group XIII of CTLD)

40.5 Integral Membrane Protein, Deleted in Digeorge Syndrome (IDD) (Group XIII of CTLDs)

Integral membrane protein, deleted in DiGeorge syndrome (IDD) is a unique CTLD-containing type 1 transmembrane protein expressed in a range of cell types and notably in developing nervous system. The extracellular region consists of a CTLD sandwiched between a membrane-distal low density lipoprotein (LDL)-receptor class A domain and a membrane-proximal von Willebrand Factor C domain (Fig. 40.4). IDD also has a sizeable intracellular region.

40.5.1 DiGeorge Syndrome Critical Region 2 (DGCR2)

IDD may function as an adhesion receptor in cell-cell or cell-matrix interactions during cell differentiation and migration, particularly in the nervous system, or may be involved in signaling. The IDD gene lies within the DiGeorge syndrome critical region – the minimal region of chromosome 22. Deletions of the 22q11.2 have been associated with a wide range of developmental defects (notably DiGeorge syndrome, velocardiofacial syndrome, conotruncal anomaly face syndrome and isolated conotruncal cardiac defects) classified under the acronym CATCH 22. The DGCR2 gene encodes a novel putative adhesion receptor protein, which could play a role in neural crest cells migration, a process which has been proposed to be altered in DiGeorge syndrome (Taylor et al. 1997). The CTLD in IDD is not known to have sugar-binding activity. Alternative splicing of gene results into multiple transcript variants. The DGCR2 gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, and zebrafish.

SEZ-12 is one of the seizure-related cDNAs which was isolated from primary cultured neurons from mouse cerebral cortex with or without pentylene tetrazol (PTZ). SEZ-12 expression is transiently down-regulated in mouse brain by injection of PTZ. The deduced amino acid sequence of SEZ-12 revealed that it encodes membrane-bound C-type lectin and has a significant homology to that of human cDNA of DGCR2 and IDD, associated with the DiGeorge syndrome. The message was expressed ubiquitously in various organs with low-abundance. The cloned transmembrane protein was probably involved in cell-cell interaction. Findings suggest that transmembrane signaling in neuronal cells may have an important role in PTZ-induced seizure (Kajiwara et al. 1996). Several lines of evidence have established the presence of an association between a 3-Mb deletion in chromosome 22q11 and schizophrenia with a reduced expression of DGCR2 (Iida et al. 2001; Shifman et al. 2006). However, Georgi et al. (2009) did not support a significant role of DGCR2 in the aetiology of schizophrenia in the German population.

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Family of CD93 and Recently Discovered Groups of CTLDs

41

G.S. Gupta

41.1 Family of CD93

CD93 is a ~120 kDa *O*-sialoglycoprotein that within the hematopoietic system is selectively expressed on cells of the myeloid lineage. CD93 in humans is encoded by the *CD93* gene (Nepomuceno et al. 1997; Webster et al. 2000). CD93 is a C-type lectin transmembrane receptor which plays a role not only in cell–cell adhesion processes but also in host defence. CD93 belongs to the Group XIV C-Type lectin family, a group containing two other members, endosialin (CD248) and thrombomodulin (TM, CD141), a well characterized anticoagulant. All of them contain a C-type lectin domain, a series of epidermal growth factor like domains (EGF), a highly glycosylated mucin-like domain, a unique transmembrane domain and a short cytoplasmic tail. Due to their strong homology and their close proximity on chromosome 20, CD93 has been suggested to have arisen from thrombomodulin gene through a duplication event. Sequence analysis revealed that CD93 is identical to a protein on human phagocytes termed C1q receptor (C1qRp). The mouse homologue of human C1qRp is known as AA4. The AA4 and TM are derived from a common ancestor since both genes are co-localized to the same region of the chromosome 2 and also because they share similar domain composition and organization.

Endosialin (tumor endothelial marker 1) is expressed preferentially by tumor endothelial cells but not by normal endothelium. Its protein domain architecture is homologous to that of CD93 and TM (CD141), suggesting a similar function in mediating cell-cell interactions. Endosialin colocalization with thrombomodulin suggests that these proteins may have complementary functions in tumor progression (Brady et al. 2004). The complement C1q receptor (C1qRp) and TM are involved in cell-cell interactions and innate immune host defense. However, not much is known on the lectin 14A, a member of CD93 family of C-type lectins.

41.2 CD93 or C1q Receptor (C1qRp): A Receptor for Complement C1q

41.2.1 CD93 Is Identical to C1qRp

The first component of complement, C1, is a multi-molecular complex comprising C1q and the Ca^{2+}-dependent tetramer $C1r_2$-$C1s_2$. The C1q is an essential component of innate complement (C) system and is crucial to ward off infection and clear toxic cell debris (e.g. amyloid fibrils, apoptotic cells) in natural way. Another important function of C1q is its ability to bind to a wide range of cell types resulting in the induction of cell-specific biological responses. These cells include polymorphonuclear leukocytes, monocytes, lymphocytes, dendritic cells, endothelial cells and platelets. Interaction of C1q with endothelial cells and platelets, for example, leads to cellular activation followed by release of biological mediators and/or expression of adhesion molecules, all of which contribute, directly or indirectly to the inflammatory process. These specific responses are mediated by the interaction of C1q with C1q binding proteins or receptors on the cell surfaces. Several candidate C1q receptors [C1q receptor for phagocytosis enhancement (C1qRp/cC1qR/CD93, a 120 kDa, O-sialoglycoprotein), complement receptor (CR) 1, calreticulin (CRT: Chap. 2), and binding protein for globular head of C1q (gC1qR/p33, a 33 kDa homotrimeric protein)] have been studied and reviewed for their structure-function relationships (McGreal and Gasque 2002). Cell-surface molecule, C1qRp, has emerged as a defence collagen receptor for C1q, as well as MBL and surfactant protein A (Chaps. 23–25). C1qRp (known as AA4 antigen in rodents) is the antigen recognized by a pro-adhesive monoclonal antibody (mNI-11) and antibodies against CD93. Although the specific role of each of these receptors in a given C1q-mediated cellular response is yet to be known, all of them may participate in the inflammatory processes associated with vascular or atherosclerotic

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lesions, autoimmune diseases, or infections. Study of structure of cC1qR/CD93 and gC1qR/p33, both of which have been characterized on the basis of their ability to bind C1q, and their role in infection and inflammation is the subject of recent and future investigations (Ghebrehiwet and Peerschke 2004). Within the hematopoietic system CD93 is selectively expressed on cells of the myeloid lineage. CD93 is identical C1qRp, which was shown to mediate enhancement of phagocytosis in monocytes and suggested to be a receptor of C1q and two other structurally related molecules. Cells expressing CD93 have enhanced capacity to bind C1q. Furthermore, immature DCs express CD93/C1qRp, and mature DCs, known to have reduced capacity for antigen uptake and to have lost the ability to phagocytose, show weak-to-negative CD93/C1qRp expression (Steinberger et al. 2002). Narayanan et al. (1997) investigated the distribution of receptors for C1q-collagen domain (cC1qR) and globular domain (gC1qR) in adult human lung fibroblasts. The cC1qR was expressed predominantly by a population of human lung fibroblasts, while the 38 kD gC1qR was produced by all cells. Therefore, two lung fibroblast subtypes may be distinguished based on production of cC1qR and another protein which binds to C1q-globular domain.

41.2.2 Characterization of CD93/C1qR$_P$

The CD93/C1qR$_P$ is a type I membrane protein of 631 amino acid containing a region homologous to C-type lectin carbohydrate recognition domain. The C1qR$_P$ is a 100 kDa M_r protein (126,000 M_r under reducing conditions). Monoclonal antibodies that immunoprecipitate 126 kDa M_r polypeptide inhibit the enhancement of phagocytosis triggered not only by C1q but also by MBL and pulmonary SP-A providing evidence that this polypeptide is a functional receptor that mediates the enhancement of phagocytosis. Specifically, the *Cd93* gene is located at 84 cM on murine chromosome 2 (Kim et al. 2000) and encodes a type I O-glycosylated transmembrane protein whose domain structure includes an amino-terminal C-type lectin domain, a tandem array of five EGF-like repeats, a single hydrophobic transmembrane region, and a short cytoplasmic domain that contains a PDZ binding domain and a moesin interaction site (Bohlson et al. 2005; Kim et al. 2000; Zhang et al. 2005). This domain structure bears a unique resemblance to the selectin family of adhesion molecules (Dean et al. 2001; Kim et al. 2000; Rosen 2004). Both post-translational glycosylation and the nature of the amino acid sequence of the protein explain to the difference between its predicted molecular weight and its migration on SDS-PAGE. The mature C1qRp contains a relatively high degree of O-linked glycoslyation, and engaged in cell surface ligation of SP-A, as well as C1q and MBL, and enhances phagocytosis. The O-glycosylation is important in the stable cell surface expression of C1qR$_P$/CD93 (Park and Tenner 2003). While the cDNA for C1qRp encodes a 631 amino acid membrane protein, CHO cells transfected with the cDNA of the C1qR$_P$ coding region express a surface glycoprotein with the identical 126,000 M_r as the native C1qR$_P$ (Nepomuceno et al. 1999). CD93, similar to C1qR$_P$, is expressed by endothelial cells, cells of myeloid lineage, platelets, and early hematopoietic stem cells and is an important lineage-specific marker of early B-cell developmental stages (Cancro 2004; Danet et al. 2002; Fonseca et al. 2001; Nepomuceno and Tenner 1998). Normally, CD93 is expressed at high levels on pro-, pre- and immature bone marrow (BM) B-cell progenitors as well as transitional (TR) B cells in the periphery (Cancro 2004). Additionally, CD93 is subject to metalloprotease-mediated ecto-domain cleavage or shedding, which is characteristic of several inflammatory mediators and adhesion molecules including TNF-α, TGF-α, TGF-β, EGF, CD44, and L-selectin (Bohlson et al. 2005a). In phagocytic cells, it has been characterized as contributing to the enhancement of FcR- and CR1-induced phagocytosis triggered by innate immune system defense collagens such as C1q and MBL (Park and Tenner 2003). There seems a novel mechanism of regulated expression of CD93 that may have implications in stem cell development and inflammation. CD93 is susceptible to ectodomain shedding. Bohlson et al. (2005a) identified multiple stimuli that trigger shedding, and identified both a soluble form of CD93 in human plasma and intracellular domain containing cleavage products within cells that may contribute to the physiologic role of CD93 (Bohlson et al. 2005a). The C1qR from U937 cells and human tonsil lymphocytes interacts with C1q, MBP, conglutinin and SP-A (Malhotra et al. 1990). The interaction of SP-A with U937 cells triggers the expression of an intracellular pool of C1qR (Malhotra et al. 1992).

41.2.3 Two Types of Cell Surface C1q-Binding Proteins (C1qR)

Synonyms of C1qR are: C1q/MBL/SPA receptor, C1qR(p), C1qr1, C1qrp, C1qRp, cell surface antigen AA4 and CD93. Selective expression of C1qR$_P$ in specific cell types supports the hypothesis that there is more than one C1q receptor mediating the diverse responses triggered by C1q (Nepomuceno and Tenner 1998; Guan et al. 1994; Tenner 1998). A 60-kDa calreticulin-homologue which binds to the collagen-like "stalk" of C1q (called cC1qR) and a 33-kDa protein with affinity for the globular "heads" of the molecule (called gC1qR), have been described. The two molecules are also secreted by Raji cells and peripheral blood lymphocytes and can be isolated in soluble form from serum-free culture supernatant. The two purified soluble proteins had

immunochemical and physical characteristics similar to their membrane counterparts in the sense that both bound to intact C1q and to their respective C1q ligands, cC1q and gC1q. In addition, N-terminal amino acid sequence analyses of the soluble cC1qR and gC1qR were found to be identical to the reported sequences of the respective membrane-isolated proteins. Ligand blot analyses using biotinylated membrane or soluble cC1qR and gC1qR showed that both bind to the denatured and nondenatured A-chain and moderately to the C-chain of C1q. Moreover, like their membrane counterparts, the soluble proteins were found to inhibit serum C1q hemolytic activity. Although cC1qR was released when both peripheral blood lymphocytes and Raji cells were incubated in phosphate-buffered saline for 1 h under tissue culture conditions, gC1qR was releasable only from Raji cells, suggesting that perhaps activation or transformation leading to immortalization is required for gC1qR release (Peterson et al. 1997).

gC1q-binding Proteins (gC1qR): Differences from cC1qR

Human neutrophils have multiple C1q-binding proteins. Ligand-binding studies with the globular domain of C1q revealed two gC1q-binding proteins (gC1qR): a 33 kDa protein (pI 4.5) mainly in the neutrophil plasma membrane and an 80–90 kDa protein (pI 4.1–4.2) located mainly in the granules. Direct binding studies showed that C1q bound to this higher molecular weight protein under physiological conditions. In contrast, anti-cC1qR antibody, which recognizes a protein binding to collagenous tails of C1q, detected only a 68,000 M_r protein in the plasma membrane. Both the 33,000 and 68,000 M_r receptors appear early on the surface of differentiating HL-60 cells. Phorbol myristate acetate treatment of neutrophils down-regulated both the receptors from cell surface, and significant amounts of soluble gC1qR were in cell media supernatants, suggesting receptor shedding or secretion**.** gC1qR, unlike cC1qR, did not bind to other C1q-like ligands, namely mannose binding protein, surfactant protein-A, surfactant protein-D, or conglutinin under normal ionic conditions, suggesting a greater specificity for C1q than the "collectin" type receptor (cC1qR). Rather, gC1qR only bound purified C1q, and the binding was enhanced under low ionic conditions and in the absence of calcium. C1qR/CD93 is also proteolytically cleaved from the surface of activated human monocytes and neutrophils in response to inflammatory signals in vitro and a soluble form of CD93 (sCD93) exists in human plasma. Inflammation triggers release of sCD93 in vivo and identifies the inflammatory macrophage as a source of sCD93 (Greenlee et al. 2008, 2009).

Ghebrehiwet et al. (1997) showed that biotinylated cC1qR binds to recombinant as well as native gC1qR and the binding sites for cC1qR are located within N-terminal residues 76 through 93 of the mature form of gC1qR and within residues 204 through 218. The evidence suggests that cC1qR is able to form a complex with gC1qR and may associate with gC1qR on the cell surface (Ghebrehiwet et al. 1997).

C1q receptor (C1qR/collectin receptor/cC1qR) with an almost complete amino acid sequence identity with calreticulin (CRT) is located on the surface of many cell types. C1qR also interacts with the collectins SP-A, MBL, CL43 and conglutinin via a cluster of charged residues on the collagen tails of the ligands. The C1q and collection binding site on C1qR/CRT is localised to the residues 160–283 (Stuart et al. 1997).

Collectins and C1q Enhance Mononuclear Phagocytosis Through a Common Receptor: Enhanced monocyte FcR- and CR1-mediated phagocytosis by MBP, C1q, and SP-A suggests acommon structural feature of the collagen-like domains that may provide a basis for this immunologically important function. MBP also enhances FcR-mediated phagocytosis by both monocytes and macrophages, and stimulated CR1-mediated phagocytosis in human culture-derived macrophages and in phorbol ester-activated monocytes. The mAb that recognizes a 126 kDa cell surface protein and inhibits C1q-enhanced phagocytosis, also inhibited the MBP-mediated enhancement of phagocytosis**.** This common feature among defense collagen receptors suggests that the enhancement of phagocytosis by MBP and C1q share at least one critical functional component, the $ClqR_P$ (Nepomuceno et al. 1997; Tenner et al. 1995). mAbs that recognize a cell surface $C1qR_P$ inhibit C1q-, MBL- and SP-A-mediated enhancement of phagocytosis. Similar inhibition of enhancement of phagocytosis by these mAbs suggests that $C1qR_P$ is a common component of a receptor for these macromolecules. The $C1qR_P$ is cross-linked directly by monoclonal anti-$C1qR_P$ or engaged as a result of cell surface ligation of SP-A, C1q and MBL resulting in enhanced phagocytosis (Nepomuceno et al. 1999).

41.2.4 Tissue Expression and Regulation of CD93/C1qRp

$CD93/ClqR_P$ was originally identified in mice as an early B cell marker through the use of AA4.1 monoclonal antibody. Then this molecule was shown to be expressed on an early population of hematopoietic stem cells, which give rise to the entire spectrum of mature cells in the blood. Now CD93 is known to be expressed by a wide variety of cells such as platelets, monocytes, microglia and endothelial cells. In the immune system CD93 is also expressed on neutrophils, activated macrophages, B cell precursors until the T2 stage in the spleen, a subset of DCs and of natural killer cells.

Though C1qRp is identical to CD93 protein, a study failed to demonstrate a direct interaction between CD93 and C1q under physiological conditions. Radioiodinated C1qR and CRT bind to C1q with identical characteristics (McGreal et al. 2002).

Studies show that human monocyte-derived DCs express gC1qR and cC1qR; their expression on the cell surface is maturation dependent, and immature DCs secrete C1q (Vegh et al. 2003). Immature DCs express C1qR, gC1qR and cC1qR/CR and, accordingly, display a vigorous migratory response to soluble C1q with maximal cell movement. In contrast, mature DCs neither express C1qR nor do move to a gradient of soluble C1q. C1q functions as a chemotactic factor for immature DC, and migration is mediated through ligation of both gC1qR and cC1qR/CR (Vegh et al. 2006).

The TGF-β and TNF-α upregulate the mRNA levels of cC1qR and collagen but do not affect gC1qR mRNA levels significantly. It was also indicated that different subsets of human lung fibroblasts respond differently to inflammatory mediators (Lurton et al. 1999). Protein kinases regulate the expression of CD93 on the cell surface. The CD93 expression on human monocyte-like cell line (U937), a human NK-like cell line (KHYG-1), and a human umbilical vein endothelial cell line (HUV-EC-C) involves the PKC isoenzymes (Ikewaki et al. 2006). PKC activator phorbol myristate acetate (PMA) effectively up-regulated CD93 expression on cultured cell lines and its regulation was mainly controlled by a PKCδ-isoenzyme (Ikewaki et al. 2007).

41.2.5 Functions

41.2.5.1 Adhesion, Migration and Phagocytosis

The CD93 was initially thought to be a receptor for C1q, but now is thought to instead be involved in intercellular adhesion and in the clearance of apoptotic cells. The intracellular cytoplasmic tail of this protein contains two highly conserved domains which may be involved in CD93 function. Indeed, the highly charged juxtamembrane (JX) domain has been found to interact with moesin, a protein known to play a role in linking transmembrane proteins to the cytoskeleton and in the remodelling of the cytoskeleton (Fig. 41.1). The interaction of moesin with CD93 cytoplasmic domain is modulated by binding of other intracellular molecules to the C11 region and implies that a PIP_2 signaling pathway is involved in CD93 function (Norsworthy et al. 2004; Zhang and Bohlson 2005). This process appears crucial for adhesion, migration and phagocytosis, three functions in which CD93 may be involved. CD93 is required for efficient engulfment of apoptotic cells via an unknown mechanism. Neonatal rat microglia are known to express C1qRp. Interaction of these cells with substrate-bound C1q was shown to enhance both FcR-and CR1-mediated phagocytosis. Results suggest that C1q in areas of active degeneration may promote the phagocytic capacity of microglia via interaction with microglial C1qRp (Webster et al. 2000). Reports also indicate that besides such-well-known complement regulatory molecules as CD55 (DAF), CD46 (MCP), CD35 (CR1) and CD59(HRF), C1qR too is able to regulate complement activity (van den Berg et al. 1995).

41.2.5.2 CD93 in Maintenance of Plasma Cells in Bone Marrow

In the context of late B cell differentiation, CD93 has been shown to be important for the maintenance of high antibody titres after immunization and in the survival of long-lived plasma cells in the bone marrow. Indeed, CD93 deficient mice failed to maintain high antibody level upon immunization and present a lower amount of antigen specific plasma cells in the bone marrow. CD93, expressed during early B-cell development, appeared to be restimulated during plasma-cell differentiation. High CD93/CD138 expression was restricted to antibody-secreting cells both in T-dependent and T-independent responses as naive, memory, and germinal-center B cells remained CD93-negative. CD93 was expressed on (pre)plasmablasts/plasma cells, including long-lived plasma cells. Strikingly, while humoral immune responses initially proceeded normally, CD93-deficient mice were unable to maintain antibody secretion and bone-marrow plasma-cell numbers, demonstrating that CD93 is important for the maintenance of plasma cells in bone marrow niches (Chevrier et al. 2009).

41.2.5.3 Role in Fertilization

Complement components might play a role in fertilization. C1q has the ability to promote sperm agglutination in a capacitation-dependent manner as well as an effect on sperm-oolemma binding and fusion. The gC1qR is present on the surface of capacitated sperm. Confocal and immunofluorescence microscopy revealed an increase in receptor expression over the rostral portion of the sperm head after capacitation. It appears that gC1qR may play a role in human fertilization (Grace et al. 2002).

41.2.6 CD93 in Pathogenesis of SLE

The association between C1q and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) is well established. The SLE is characterized by the presence of multiple autoantibodies and high levels of circulating immune complexes. In a cross-sectional study it was found that higher titres of antibodies against cC1qR/CRT are present in sera of SLE patients compared with normal donors. Deficiency in C1q is considered to be a

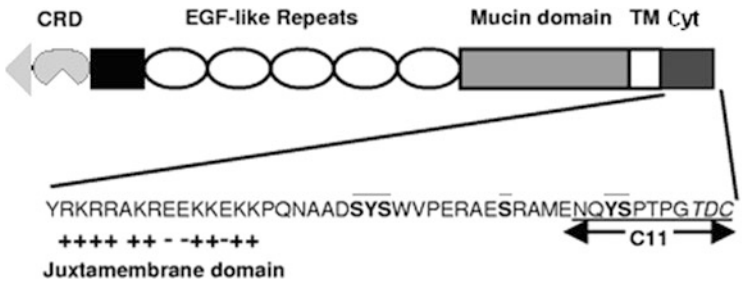

Fig. 41.1 Schematic representation of C1QRp/CD93. CD93 has been shown to influence the phagocytic activity in response to defense collagens such as C1q. CD93 has a 47 amino acid cytoplasmic tail and modulates phagocytosis. The cytoplasmic tail contains a highly charged juxtamembrane (Jx) region, and charge (+ or −) is indicated below the single-letter amino acid residue. Potential sites of serine or tyrosine phosphorylation are marked by horizental line. Cytoplasmic (*underlined*) 11 amino acids region has been implicated in signal transduction; this region contains a class I PDZ-binding domain (*italics*). Abbreviations: CRD, Carbohydrate recognition domain; EGF, epidermal growth factor; TM, transmembrane domain; Cyt, cytosolic region (Bohlson et al. 2005b)

strong susceptibility factor and is corroborated by the fact that ~92% of the known cases of hereditary deficiency in C1q develop rheumatic disease. C1q-deficient mice have shown the presence of glomerulonephritis with immune deposits and a large number of apoptotic bodies in the diseased glomeruli suggesting a defect in the clearance of apoptotic cell by macrophages and DCs. Although these data are consistent with the hypothesis that C1q deficiency may induce a generalized failure to clear immune complexes and apoptotic cells, this concept alone cannot wholly explain why individuals with C1q deficiency are prone to develop SLE. Therefore, C1q in conjunction with other surface molecules must play a more fundamental role in immunoregulation, especially those processes that regulate T cell function and tolerance. In support of this hypothesis is the finding that C1q causes inhibition of mitrogen-induced T cell-proliferative response by interaction with C1q receptors. Furthermore, macrophages and possibly DCs not only synthesize but also display C1q as a type II cell surface molecule, especially at sites of inflammation. Although it is not yet known what role the surface-expressed C1q plays, it is tempting to assume that it plays a role in the priming of naive T cells by DCs (Ghebrehiwet and Peerschke 2004; van den Berg et al. 1998). However, in a contradiction, C1qRp expression and regulation was not altered on peripheral blood monocytes of SLE patients. Possible relations with disease activity and medication need further investigations (Moosig et al. 2006)

41.3 Murine Homologue of Human C1qRp (AA4)

A murine genomic clone is 73% identical in sequence with the coding region for human $C1qR_P$ cDNA. Chromosomal localization of the human and murine genes demonstrated that these genes are syntenic. Murine cell lines of diverse myeloid origins are shown to respond to interaction of C1q with the enhancement of phagocytosis similar to that seen in human peripheral blood monocytes. $mC1qR_P$ is expressed in murine myeloid cell lines, but not in a mouse epithelial cell line, similar to the cell type expression of the human gene product (Kim et al. 2000). A 110–130-kDa membrane glycoprotein, expressed by rat NK cells, is a structural orthologue of the phagocytosis-stimulating receptor for complement factor C1q and mannose-binding lectin on human macrophages, C1qRp (receptor for C1q, regulating phagocytosis). Rat C1qRp is a monomeric type I integral membrane protein consisting of 643 amino acids with an N-terminal lectin-like domain, five epidermal growth factor-like domains, a transmembrane domain and a 45-residue cytoplasmic domain. It is encoded by a single gene on rat chromosome 3q41–q42 and is 67% and 87.5% identical at the amino acid level to human and mouse C1qRp, respectively. Rat C1qRp is expressed by resting and by activated NK cells, on subpopulations of NKR-$P1^+$ T cells (NK/T cells), dendritic cells, macrophages, neutrophils and granulocytes, but not by B cells or NKR-$P1^-$ T cells. Expression of this innate immune receptor is therefore not restricted to hematopoietic cells of the myeloid lineage, but is also expressed on subsets of cells of lymphoid origin (Løvik et al. 2000) The C1qRp, present on macrophages and neutrophils, is presumed to stimulate phagocytosis in these cells. In rat NK cells, an intracellular Ca^{2+}-response is induced upon stimulation of C1qRp with mAbs, LOV3 and LOV8. The response induced by the antibodies was owing to the Ca^{2+} mobilization from intracellular calcium stores (Løvik et al. 2001). Murine mast cells express specific receptors for C1q. Both cC1qR and gC1qR are present on these cells. C1qRs may play a significant role in mast cell function and regulation by providing an important signal through which mast cells can be recruited to inflammatory sites of increased C1q concentration (Ghebrehiwet et al. 1995).

The major site of AA4 expression in all tissue tested is on endothelial cells and that despite the apparent involvement of AA4 in the phagocytic response, it is not expressed by tissue macrophages. Although AA4 has been described on all hematopoietic progenitors, only circulating immature B cells, monocytes and NK cells but not T cells and

neutrophils expressed AA4 (Dean et al. 2001). The CD93-mRNA is highly induced after transient focal cerebral ischemia. CD93 protein is upregulated in endothelial cells, but also in selected macrophages and microglia. Harhausen et al. (2010) speculated that CD93-neuroprotection is mediated via suppression of the neuroinflammatory response through downregulation of CCL21 (Harhausen et al. 2010).

The murine fetal stem cell marker AA4 is a known homologue of the human phagocytic C1q receptor involved in host defense. The rat AA4 displays similar domain composition and organization to thrombomodulin. The rat AA4 was encoded by a single transcript of 7 kb expressed constitutively in all tissues and was expressed predominantly by pneumocytes and vascular endothelial cells. AA4 was identified as a glycosylated protein of 100 kDa expressed by endothelial cells > platelets > NK cells and monocytes (ED1+ cells). The staining was associated to the cell surface and intracytoplasmic vesicles. Interestingly, alveolar epithelial cells (lung) but not other epithelia (e.g. uterus) were strongly stained for AA4. Although AA4 has been described on all hematopoietic progenitors, only circulating immature B cells, monocytes and NK cells but not T cells and neutrophils expressed AA4. These results support the idea that AA4/C1qRp is involved in some cell-cell interactions (Dean et al. 2000, 2001).

The hematopoietic system of mice is established during the early to midgestational stage of development. Although AA4.1$^+$ and AA4.1$^-$ cells had equivalent potency to generate myeloid cell lineages, the lymphoid potential in ES-cell-derived cells was largely restricted to the cells expressing AA4.1. The same cell type was present abundantly in the early yolk sac and in fewer numbers (~5% of that in the yolk sac) in the caudal half of the developing embryos. These results suggest that AA4.1 is a cell surface marker that can identify the earliest lymphohematopoietic progenitors in mouse development (Yamane et al. 2009).

Zekavat et al. (2010) identified a point mutation in the NOD *Cd93* gene, which maps to the *Idd13* locus, a region encoding a high degree of penetrance for diabetes susceptibility in non-obese diabetic (NOD) mice (Kim et al. 2000; Serreze et al. 1998). In addition, they also identified this *Cd93* polymorphism in NZB/W F1 mice, to which the lupus susceptibility loci, *Wbw1* and *Nkt2*, are tightly linked (Rahman et al. 2002; Esteban et al. 2003). This point mutation is associated with aberrant expression of CD93 on NOD and NZB/W F1 B cells in the pro-/pre-, immature, and TR subsets as compared with non-autoimmune B6 mice. The *Idd13* and *Wbw1* and *Nkt2* loci are known to play a role in the regulation of the invariant natural killer T (iNKT) cell compartment (Chen et al. 2007; Esteban et al. 2003; Jordan et al. 2004; Rahman et al. 2002).

This allele carries a coding polymorphism in the first EGF-like domain of CD93, which results in an amino acid substitution from Asn→His at position 264. This polymorphism does not appear to influence protein translation or ecto-domain cleavage, as CD93 is detectable in bone-marrow-derived macrophage and B-cell precursor lysates and in soluble form in the serum. The NOD CD93 isoform causes a phenotypic aberrancy in the early B-cell developmental stages (i.e., pro-, pre-, immature, and transitional), likely related to a conformational variation. Consistent with genetic linkage, B6 CD93$^{-/-}$ and B6.NOD^{Idd13} mice were found susceptible to a profound CD4$^+$ NKT cell deficient state. Data suggest that *Cd93* may be an autoimmune susceptibility gene residing within the *Idd13* locus, which plays a role in regulating absolute numbers of CD4$^+$ NKT cells (Zekavat et al. 2010).

41.4 The gC1qR (p33, p32, C1qBP, TAP)

41.4.1 A Multi-Multifunctional Protein - Binding with C1q

The gC1qR (also named as gC1qBP) is a 33 kDa single chain, highly acidic glycoprotein that binds to the globular 'heads' of C1q. Comparison of the cDNA-derived amino acid sequences of gC1qBP reveals that either of the rodents, rat and mouse, sequences is 89.9% identical to human sequence. Recombinant rat gC1qBP binds avidly to human C1q. gC1qBP mRNA is abundantly expressed in every rat and mouse tissue analysed. Rat mesangial cells synthesise gC1qBP, but do not express gC1qBP on the cell surface. In rat serum, gC1qBP is present at low levels (Lynch et al. 1997).

Tha gC1qR was first isolated from membrane preparation of Raji cells and now appears to be ubiquitously distributed. Although, gC1qR was originally identified as a protein which binds to the globular "heads" of C1q, evidence suggests that the molecule is in fact a multi-ligand binding, multifunctional protein with affinity for diverse ligands which at best are functionally related. These molecules include: thrombin, vitronectin, and high molecular weight kininogen. The gC1qR molecule, which is identical to the transcription factors SF2 and the Tat-associated protein, or TAP, is the product of a single gene localized on chromosome 17p13.3 in human, and chromosome 11 in mouse, and is encoded by an approximately 1.5–1.6 kb mRNA. The full length cDNA encodes a primary translation protein of 282 residues and the 'mature' or membrane form of the protein isolated from Raji cells corresponds to residues 74–282 and is presumed to be generated by a site-specific cleavage and removal of the highly basic, 73-residues long, N-terminal segment during post-translational processing. The translated amino acid sequence does not predict for the presence of a conventional sequence motif compatible with a

transmembrane segment and does not have a consensus site for a GPI anchor. However, there is strong evidence which indicates that gC1qR is expressed both inside the cell and on the membrane. In addition, the membrane expression of gC1qR can be upregulated with inflammatory cytokines such as INF-gamma, TNF-α, or LPS (Ghebrehiwet and Peerschke 1998).

Although expressed on the surface of cells, an intriguing feature of the membrane-associated form of gC1qR is that its translated amino acid sequence does not predict the presence of either a sequence motif compatible with a transmembrane segment or a consensus site for a glycosylphosphatidylinositol anchor. Moreover, the N-terminal sequence of the pre-pro-protein gC1qR contains a motif that targets the molecule to the mitochondria and as such was deemed unlikely to be expressed on the surface. However, several lines of experimental evidence clearly show that gC1qR is present in all compartments of the cell, including the extracellular cell surface. First, surface labeling of B lymphocytes with the membrane-impermeable reagent sulfosuccinimidyl 6-(biotinamido) hexanoate shows specific biotin incorporation into the surface-expressed but not the intracellular form of gC1qR. Second, FACS and confocal laser scanning microscopic analyses demonstrated specific staining of Raji cells surface. Third, endothelial gC1qR, which is associated with the urokinase plasminogen activator receptor, and cytokeratin 1 bind 125I-high molecular weight kininogen in a specific manner. Fourth, native gC1qR purified from Raji cell membranes but not intracellular gC1qR is glycosylated, as evidenced by a positive periodic acid Schiff stain as well as sensitivity to digestion with endoglycosidase H and F. Finally, cross-linking experiments using C1q as a ligand indicate that both cC1qR and gC1qR are co immunoprecipitated with anti-C1q. Taken together, the evidence accumulated to date supports the concept that in addition to its intracellular localization, gC1qR is expressed on the cell surface and can serve as a binding site for plasma and microbial proteins, but also challenges the existing paradigm that mitochondrial proteins never leave their designated compartment. It is therefore proposed that gC1qR belongs to a growing list of a class of proteins initially targeted to the mitochondria but then exported to different compartments of the cell through specific mechanisms which have yet to be identified. The designation 'multifunctional and multicompartmental cellular proteins' is proposed for this class of proteins (Ghebrehiwet et al. 2001).

Platelets are involved in the development of many types of vascular lesions. In addition to their role in primary hemostasis, they participate in inflammatory processes that may contribute to the development of thrombosis, atherosclerosis and vasculitis. C1q has been shown to modulate platelet interactions with collagen and immune complexes, and has been identified at the sites of vascular injury and inflammation, as well as in atherosclerotic lesions. Platelets express a variety of C1q binding sites, including gC1qR/p33 (gC1qR) (Peerschke and Ghebrehiwet 2001).

The gC1qR (or P32) protein is a binding protein for nuclear pre-mRNA splicing factor SF2/ASF and numerous other nuclear and cell surface proteins, yet is targeted to the mitochondrial matrix compartment where these proteins are not present (Soltys et al. 2000). The endothelial protein p33/gC1qR is thought to mediate the assembly of components of the kinin-forming and complement-activating pathways on the surface of cardiovascular cells. Fractionation studies demonstrated that the vesicular but not the membrane fraction of EA.hy926 cells is rich in p33. It seems that externalization of p33 must precede its complex formation with target proteins on the endothelial cell surface (Dedio and Muller-Esterl 1996).

The binding of the ligand C1q by recombinant gC1qR was indistinguishable from binding shown by gC1qR isolated from Raji cells. gC1qR as a novel vitronectin-binding protein that may participate in the clearance of vitronectin-containing complexes or opsonized particles or cooperate with vitronectin in the inhibition of complement-mediated cytolysis (Ghebrehiwet et al. 1994; Lim et al. 1996).

41.4.2 Human gC1qR/p32 (C1qBP) Gene

The 7.8-kbp human gC1qR/p32 (C1qBP) gene was cloned and found to consist of 6 exons and 5 introns. Analysis of a 1.3-kb DNA fragment at the 5′-flanking region of this gene revealed the presence of multiple TATA, CCAAT, and Sp1 binding sites. Subsequent 5′ and 3′ deletion of this fragment confined promoter elements to within 400 bp upstream of the translational start site. Because the removal of the 8-bp consensus TATATATA at -399 to -406 and CCAAT at -410 to -414 did not significantly affect the transcription efficiency of the promoter, GC-rich sequences between this TATA box and the translation start site may be very important for the promoter activity of the C1qBP gene. One of seven GC-rich sequences in this region binds specifically to PANC-1 nuclear extracts and the transcription factor Sp1. Primer extension analysis mapped three major transcription start regions. The farthest transcription start site is 49 bp upstream of the ATG translation initiation codon and is in close proximity of the specific SP1 binding site (Tye et al. 2001).

41.4.3 Ligand Interactions

41.4.3.1 A Receptor for C1q

Although originally isolated as a receptor for C1q by virtue of its specificity for the globular heads of that molecule, a large body of evidence has now been accumulated which shows that in addition to C1q, gC1qR can serve as a receptor for diverse ligands including proteins of the intrinsic coagulation/bradykinin forming cascade, as well as antigens of cellular, bacterial, and viral origin. Furthermore, since gC1qR has been shown to regulate the functions of protein kinase C (PKC), it is postulated that gC1qR-induced signaling cascade may involve activation of PKC. Collective reports suggest that gC1qR plays an important role in blood coagulation, inflammation, and infection. It is still unclear as to how the molecule is anchored on the membrane since its sequence is devoid of a classical transmembrane domain or a glycosylphosphatidylinositol (GPI) anchor. Furthermore, while recombinant gC1qR can bind to cell surfaces suggesting that it may bind directly to the phospholipid bilayer, experiments showed that, at least in vitro, gC1qR does not bind to unilamellar vesicle preparations of either phosphatidylcholine (PC) or phosphatidylserine: phosphatidylcholine. The three-dimensional structure of gC1qR.

41.4.3.2 Interaction of High Molecular Weight Kininogen

Kininogens, the precursor proteins of the vasoactive kinins, bind specifically, reversibly, and saturably to platelets, neutrophils, and endothelial cells. Two domains of the kininogens expose major cell binding sites: domain D3 that is shared by H- and L-kininogen and domain D5H that is exclusively present in H-kininogen. The kininogen cell binding sites were mapped to 27 residues of D3 ("LDC27") and 20 residues of D5H ("HKH20″", respectively) (Herwald et al. 1995; Hasan et al. 1995). An endothelial binding protein of 33 kDa similar to gC1qR has been indicated to mediates the assembly of critical components of the kinin-generating pathway on the surface of endothelial cells, thereby linking the early events of kinin formation and complement activation (Herwald et al. 1996).

To further probe cellular trafficking routes of gC1qR, Dedio et al. (1999) over-expressed human gC1qR in a mammalian cell and monitored cell surface exposure of recombinant gC1qR by virtue of its capacity to bind labeled H-kininogen. Transient transfection of COS1 cells with the full-length cDNA of human gC1qR resulted in a high level of recombinant protein that matched the pool of endogenous gC1qR present in these cells. Overexpression of gC1qR did not significantly increase the number of H-kininogen binding sites exposed by the transfected cells thus denying the possibility that alternative routing of gC1qR to the surface of COS1 cells occurs at significant levels. Hence gC1qR has the capacity to tightly bind H-kininogen, but because gC1qR is routed to mitochondria it cannot fulfill the postulated functions as a cell docking site for kininogens and complement factors (Dedio et al. 1999).

The gC1qR has been shown to bind a number of plasma proteins involved in the coagulation and kinin systems. Biotinylated gC1qR was found to bind to fibrinogen in a manner which was specific and inhibited by excess soluble fibrinogen or polyclonal antibodies directed against either gC1qR or fibrinogen. Observations may suggest a potential role for gC1qR in modulating fibrin formation particularly at local sites of immune injury or inflammation (Lu et al. 1999).

High molecular weight kininogen (HK) and factor XII bind to human umbilical vein endothelial cells (HUVEC) in a zinc-dependent and saturable manner indicating that HUVEC express specific binding site(s) for those proteins. The binding of HK and factor XII to HUVECs occurs via a 33-kDa cell surface glycoprotein that appears to be identical to gC1qR but with a site on gC1qR distinct from that which binds C1q (Joseph et al. 1996; 2001). However, unlike C1q, whose interaction with gC1qR does not require divalent ions, the binding of HK to gC1qR is absolutely dependent on the presence of zinc. Zinc can induce the exposure of hydrophobic sites in the C-terminal domain of gC1qR involved in binding to HK/FXII (Kumar et al. 2002).

Cell surface proteins reported to participate in the binding and activation of the plasma kinin-forming cascade includes gC1qR, cytokeratin 1 and u-PAR. Each of these proteins binds high molecular weight kininogen (HK) as well as Factor XII. The studies suggest that formation of HK (and Factor XII) binding sites along endothelial cell membranes consists of bimolecular complexes of gC1qR-cytokeratin 1 and u-PAR-cytokeratin 1, with gC1qR binding being favored (Joseph et al. 2004).

Human p32 (also known as SF2-associated p32, p32/TAP, and gC1qR) localizes predominantly in the mitochondrial matrix. It is thought to be involved in mitochondrial oxidative phosphorylation and in nucleus-mitochondrion interactions. The three-dimensional structure of gC1qR identified unique structural features that may serve not only to anchor the protein but also to explain its affinity for such a diversity of plasma as well as microbial and viral ligands (Ghebrehiwet et al. 2002; Jiang et al. 1999). The crystal structure reveals that p32 adopts a novel fold with seven consecutive antiparallel β-strands flanked by one N-terminal and two C-terminal α-helices. Three monomers form a doughnut-shaped quaternary structure with an unusually asymmetric charge distribution on the surface. The implications of the structure on previously proposed functions of p32 are discussed and new specific functional properties are suggested (Jiang et al. 1999).

41.4.3.3 Adrenergic Receptor

gC1qR was found to bind with the carboxyl-terminal cytoplasmic domain of the α1B-adrenergic receptor (amino acids 344–516) in a yeast two-hybrid screen of a cDNA library prepared from the rat liver. The gC1qR interaction with an arginine-rich motif in the C-tail of hamster α1B-adrenergic receptors seems to control their expression and subcellular localization. Studies suggest that gC1qR interacts specifically with α1B- and α1D-, but not α1A-adrenergic, and this interaction depends on the presence of an intact C-tail. This suggests a role for gC1qR in regulating the cellular localization and expression of adrenergic receptors (Pupo and Minneman 2003; Xu et al. 1999).

41.4.3.4 Interaction with Bacteria and Viruses

On the surface of activated platelets, gC1qR has been shown to serve as a binding site for *Staphylococcus* aureus and this binding is mediated by protein A. Since the binding of *S. aureus* to platelets is postulated to play a major role in the pathogenesis of endocarditis, gC1qR may provide a suitable surface for the initial adhesion of the bacterium. Recent data also demonstrate that the exosporium of Bacillus cereus, a member of a genus of aerobic, Gram-positive, spore-forming rod-like bacilli, which includes the deadly Bacillus anthracis, contains a binding site for gC1qR. Therefore, by virtue of its ability to recognize plasma proteins such as C1q and HK, as well as bacterial and viral antigens, cell-surface gC1qR not only is able to generate proinflammatory byproducts from the complement and kinin/kallikrein systems, but also can be an efficient vehicle and platform for a plethora of pathogenic microorganisms (Peerschke and Ghebrehiwet 2007).

InlB is a *Listeria monocytogenes* protein that promotes entry of the bacterium into mammalian cells by stimulating tyrosine phosphorylation of the adaptor proteins Gab1, Cbl and Shc, and activation of phosphatidyl-inositol (PI) 3-kinase. However, Braun et al. (2000) demonstrated a direct interaction between InlB and the gC1qR. The platelet gC1qR is a cellular binding site for *Staphylococcal* protein A which suggests an additional mechanism for bacterial cell adhesion to sites of vascular injury and thrombosis (Nguyen et al. 2000).

Circulating, nonenveloped Hepatitis C virus (HCV) core protein interacts with immunocytes through gC1qR. The HCV core protein is the protein expressed during the early phase of HCV infection and suppresses host immune responses, including anti-viral cytotoxic T-lymphocyte responses in a murine model. Like C1q, HCV core protein can inhibit T-cell proliferative responses in vitro. Biochemical analysis of the interaction between core and gC1qR indicates that HCV core binds the region spanning amino acids 188–259, a site distinct from the binding region of C1q (Kittlesen et al. 2000). Binding of HCV core to gC1qR on T cells leads to impaired Lck/Akt activation and T-cell function (Yao et al. 2004). Up-regulation of gC1qR expression is a distinctive feature of MC, and dysregulated shedding of C1qR molecules contributes to vascular cryoglobulin-induced damage via the classic complement-mediated pathway (Sansonno et al. 2009). The gC1qR is the main cellular partner of the hepatitis B virus P22 which was colocalized with the endogenous gC1qR in both the cytoplasm and the nucleus but never in the mitochondria (Lainé et al. 2003). The gC1qR is an effective inhibitor of HIV-1 infection, which prevents viral entry by blocking the interaction between CD4 and gp120. Since gC1qR is a human protein, it is most probably not antigenic in humans. It would seem logical, therefore, to consider gC1qR or its fragments involved in the CD4 binding as potential therapeutic agents (Szabo et al. 2011).

41.4.4 Role in Pathology

The localization of gC1qR and its ligands C1q and HK in atherosclerotic lesions, and the ability of gC1qR to modulate complement, kinin, and coagulation cascades, suggests that gC1qR may play an important role in promoting inflammation and thrombosis in atherosclerotic lesions (Peerschke et al. 2004).

Sequencing of the gene encoding tumor-associated gC1qR did not reveal any consistent tumor-specific mutations. However, histochemical staining demonstrated marked differential expression of gC1qR in thyroid, colon, pancreatic, gastric, esophageal and lung adenocarcinomas compared to their nonmalignant histologic counterparts. In contrast, differential expression was not seen in endometrial, renal and prostate carcinomas. Despite high expression in breast carcinoma, gC1qR was also expressed in nonmalignant breast tissue. Although the precise relation of gC1qR to carcinogenesis remains unclear, the finding of tumor overexpression and the known multivalent binding of gC1qR to a variety of circulating plasma proteins as well as its involvement in cell-to-cell interactions suggest that gC1qR may have a role in tumor metastases (Rubinstein et al. 2004).

Membrane type 1 matrixmetallo-proteinase (MT1-MMP), a key enzyme in cell locomotion, is known to be primarily recruited to the leading edge of migrating cells. MT1-MMP via its cytoplasmic tail directly associates with a compartment-specific regulator gC1qR. Although a direct functional link between these two proteins remains uncertain, observations suggest that the transient associations of gC1qR with the cytoplasmic tail of MT1-MMP are likely to be involved in the mechanisms regulating presentation of the protease at the tumor cell surface (Rozanov et al. 2002).

41.5 Thrombomodulin

The thrombomodulin group of CTLD-containing cell surface proteins has three members in both human and mouse: thrombomodulin, CD93, endosialin, and C-type lectin 14A. The extracellular region of each of these type 1 transmembrane proteins consists of a CTLD projected from the cell surface by a Sushi domain, a number of epidermal growth factor (EGF) domains, and a region rich in proline, serine and threonine that carries multiple O-linked glycans. The short cytoplasmic tail often contains tyrosine-based motifs.

41.5.1 Localization

Thrombomodulin (TM) (CD141) or BDCA-3 is an integral membrane glycoprotein, initially described on an endothelial cell surface. Most of the vascular TM is contained in capillaries which comprise greater than 99% of endothelial surface area. Immunohistochemical analysis of human tissues demonstrated TM on endothelia of blood vessels and lymphatics except for in the CNS (Maruyama et al. 1985; Ishii et al. 1986; DeBault et al. 1986). The syncytiotrophoblast of the placenta contains TM, while hepatic sinusoids and the postcapillary venules of lymph nodes are devoid of it. The TM has been identified on the squamous epithelium of the epidermis (Yonezawa et al. 1987) and in human platelets where their number per endothelial cell is low (Suzuki et al. 1988). TM has been found in a variety of cultured cells, including NIH 3T3 cells, A549 small cell lung cancer cells and CHO cells. Some endothelial neoplasms express TM. Choriocarcinoma but not choriocarcinoma cell lines have been found to express TM (Dittman and Majerus 1990). The antigen described as BDCA-3 has turned out to be identical to TM (Dzionek et al. 2000, 2002). Thus it was revealed that this molecule also occurs on a very rare (0.02%) subset of human dendritic cells called MDC2. Its function on these cells is unknown at present, but apparently, TM has at least one other ligand apart from thrombin, because anticoaglulation is a common place function, in contrast to the rarity of MDC2 cells. In humans, TM is encoded by the THBD gene (Wen et al. 1987).

41.5.2 Characteristics

The human TM is a 75 kDa protein when analyzed on SDS-PAGE. After reduction of disulfide bonds, the Mr was 105 kDa, indicating secondary structure involving multiple cystine bridges. This secondary structure renders TM stable under extremes of pH and under denaturing conditions. The cDNAs for human (Wen et al. 1987; Suzuki et al. 1987) and murine TM (Dittman and Majerus 1989) have been sequenced. The structure resembles the low density lipoprotein (LDL) receptor (Goldstein et al. 1985) (Fig. 41.2). The initiating methionine at amino terminal is followed by an 18 amino acid hydrophobic leader sequence. The mature protein starts with sequences distantly homologous to CTLDs such as the asialoglycoprotein receptor (residues 19–179) (Patthy 1988). The large area of identity between the mouse and human cDNAs in the lectin domain implies that the conserved sequence has some unknown function. Human TM consists of ten structural elements: an N-terminal domain homologous to the family of C-type lectins (residues 1–226), six tandemly repeated epidermal growth factor (EGF)-like1 domains (residues 227–462), a Ser/Thr-rich region (residues 463–497), a transmembrane domain (residues 498–521), and a short cytoplasmic tail (residues 522–557) (Jackman et al. 1986; Suzuki et al. 1987; Wen et al. 1987) (Fig. 41.2). The short cytoplasmic tail contains several potential sites for phosphorylation, and phosphorylation in cells has been associated with increased endocytosis and degradation of TM. Thrombomodulin is glycosylated on asparagine residues (Stearns et al. 1989).

The TM gene (*THBD*) maps to chromosome 20p11.2, contains a single exon and no introns, and spans 4 kb (Ireland et al. 1996; Wen et al. 1987). Both the human (Jackman et al. 1987; Shira et al. 1988) and mouse genes are intronless, for which no special function is known. The TM transcribes a single mRNA of approximately 3,700 bases, consistent with the size of cDNAs for human (3,693 bases) and mouse (3,658 bases) TM. In humans the 5′ untranslated region is 150 bases, the coding region is 1,725 bases encoding 575 amino acids, and the 3′ untranslated region is 1,779 bases. The 3′ untranslated region is highly conserved between species and contains the sequence TTATTTAT, which has been associated with short mRNA half-lives (Shaw and Kamen 1986). Several potential polyadenylation signals, AATAAA are also present in the cDNAs of three species.

41.5.3 Regulation of TM Activity

TM is subject to regulation by internalization of the cell-surface molecule, with loss of protein C activating activity. Thrombin binding to TM can induce internalization of thrombin-TM complexes, with transport to lysosomes, release and degradation of thrombin, and subsequent return of TM to the cell surface (Dittman and Majerus 1990). Cytokines also affect the expression of TM. Tumor necrosis factor (TNF) rapidly alters the anticoagulant properties of the endothelial cell surface by inducing expression of the procoagulant, tissue factor, and reducing TM activity. The reduction in activity is associated with endocytosis and

lysosomal degradation of TM. TNF also inhibits transcription of TM mRNA; the subsequent decrease in TM synthesis may also contribute to the decline in TM activity. Other cytokines, interleukin-1 (Naworth et al. 1986) and endotoxin (Moore et al. 1987) also cause decreased TM activity on endothelial cell surfaces. In fact, thrombin increases TM transcription rates, mRNA levels, and total TM protein in cultured mouse hemangioma cells (Dittman et al. 1989). Infusion of TNF into humans increases the production of fibrin, indicating activation of coagulation. Protein C activation was also increased in contrast to what might have been predicted by the in vitro effect of TNF on TM activity and protein C activation. This observation is consistent with additional regulation of TM in vivo by components of the hemostatic system, perhaps by thrombin as discussed above (Dittman and Majerus 1990).

41.5.4 Structure-Function Relations - Binding to Thrombin

Structural requirements for thrombin binding to TM and cofactor activity have been studied by mutagenesis of recombinant human TM. Deletion of the fourth EGF-like domain abolished cofactor activity but did not affect thrombin binding. Deletion of either the fifth or the sixth EGF-like domain markedly reduced both thrombin binding affinity and cofactor activity. Studies suggest that the anticoagulant function of TM is mediated by EGF-like domains 4, 5, and 6 (EGF456) (Zushi et al. 1989). While TM binds to EGF-like domains 5 and 6 (Kurosawa et al. 1988), EGF-like domain 4 is not required for thrombin binding but is essential for accelerating protein C activation (Tsiang et al. 1992; Parkinson et al. 1992). Membrane association is not necessary for cofactor activity (Kurosawa et al. 1988; Suzuki et al. 1989), and the smallest fully active soluble TM fragment consists of EGF456 (Zushi et al. 1989; Hayashi et al. 1990). The EGF456 contains the region required for thrombin binding and protein C activation. Neither mouse nor bovine TM contains the serine-glycine-X-glycine signal sequence required for heparin-like glycosoaminoglycan attachment 58; human TM has an unfavored site for such modification.

Thrombin binding sequences were also localized by assaying the ability of synthetic peptides derived from TM. The two most active peptides corresponded to (a) the entire third loop of the fifth EGF-like domain ($Kp = 85 \pm 6$ μM) and (b) parts of the second and third loops of the sixth EGF-like domain ($Kp = 117 \pm 9$ μM). Studies suggest that thrombin interacts with two discrete elements in TM. Deletion of the Ser/Thr-rich domain dramatically decreased both thrombin binding affinity and cofactor activity and also prevented the formation of a high molecular weight TM species containing chondroitin sulfate. Substitutions of this domain with polypeptide segments of decreasing length and devoid of glycosylation sites progressively decreased both cofactor activity and thrombin binding affinity. This correlation suggests that increased proximity of the membrane surface to the thrombin binding site may hinder efficient thrombin binding and the subsequent activation of protein C. Membrane-bound TM therefore requires the Ser/Thr-rich domain as an important spacer, in addition to EGF-like domains 4–6 (EGF456), for efficient protein C activation. The EGF456 appears to have anticoagulant properties which make it more suitable anticoagulant in extracorporeal circulation in monkeys (Suzuki et al. 1998).

41.5.4.1 The Disulfide Bonding Pattern

The disulfide bonding pattern of the fourth and fifth EGF-like domains within the smallest active fragment of TM has been determined. The disulfide bonding pattern of the fourth EGF-like domain was (1–3, 2–4, 5–6). Surprisingly, the disulfide bonding pattern of the fifth domain was (1–2, 3–4, 5–6), which is different from EGF or any other EGF-like domain analyzed so far. The observation that not all EGF-like domains have an EGF-like disulfide bonding pattern reveals an additional element of diversity in the structure of EGF-like domains (White et al. 1996).

41.5.4.2 Critical Amino Acids in TM

Studies suggest that the region 333–350 in EGF3-4 is critical for protein C activation by the thrombin-TM complex and the region 447–462 in EGF6 is critical for thrombin binding (Parkinson et al. 1992). The 80–90% of TM activity is lost by oxidation of Met^{388}, located within the short interdomain loop between EGF-like domains 4 and 5. The interdomain loop is critical in the biological anticoagulant properties of TM (Clarke et al. 1993). Site-directed mutagenesis suggested that amino acid Asp^{349} of EGF456, in a recombinant protein is essential for retaining full protein C-activating cofactor activity. Thus, Asp^{349} in the fourth EGF-like structure of TM plays a role in its Ca^{2+}-mediated binding to protein C (Zushi et al. 1991).

Although, the fourth EGF-like domain is unnecessary for high affinity thrombin binding, yet it is required for cofactor activity. Two regions of the fourth EGF-like domain were identified as essential for cofactor activity: (1) the sequence consisting of amino acids Glu^{357}, Tyr^{358}, and Gln^{359} shared by the overlapping first and second disulfide loops, and (2) the amino-terminal region of the third disulfide loop containing amino acids Glu^{374}, Gly^{375}, and Phe^{376} (Lentz et al. 1993). In another study, mutants between Cys^{333} and Cys^{462} of TM were expressed in E. coli. In EGF4, which is essential for protein C activation by the thrombin-TM complex, critical residues were: Asp^{349}, Glu^{357}, Tyr^{358}, and Phe^{376}. In EGF5-EGF6, critical residues within a proposed

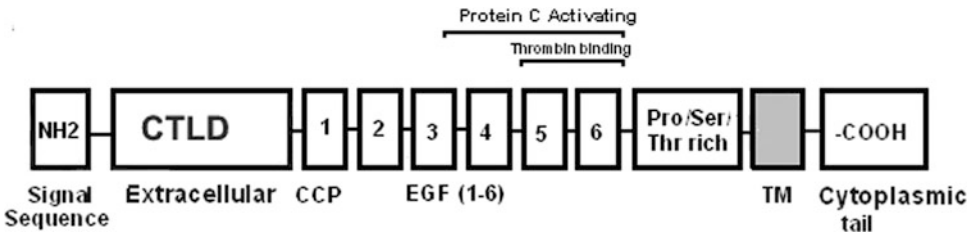

Fig. 41.2 Domain organization of Thrombomodulin: TM is composed of five structural domains. Extending from a short cytoplasmic tail and transmembrane domain is a serine/threonine–rich region to which a chondroitin sulfate moiety that optimizes anticoagulant function is attached. Next is a domain that consists of six epidermal growth factor (EGF)-like repeats, four of which are responsible for the protein's anticoagulant and antifibrinolytic functions. The NH_2-terminal domain has two modules. The first, adjacent to the EGF-like domain, is a ~70–amino acid residue hydrophobic region. The second, which is ~155–amino acid residues long, has homology to C-type lectins (Conway et al. 2002)

acidic thrombin-binding region were: Glu^{408}, Tyr^{413}, Ile^{414}, Leu^{415}, Asp^{416}, Asp^{417}, Asp^{423}, Ile^{424}, Asp^{425}, and Glu^{426}. A potential Ca^{2+}-binding site, which is comprised of residues Asp^{423}, Asp^{425}, Glu^{426}, Asn^{439}, Leu^{440}, and Phe^{444}, overlaps the thrombin-binding region. Asp^{461}, in the C-loop of EGF6 shown to be critical for thrombin and residues Asp^{398}, Asp^{400}, Asn^{402}, and Asn^{429} in EGF5 were critical for binding. Thus, 22 residues are critical in the region comprising EGF4-6, which is essential for thrombin binding and protein C activation by the thrombin-TM complex (Nagashima et al. 1993). These results suggest that amino acids critical for TM cofactor activity are located near the junction between the two subdomains of the fourth EGF-like domain.

41.5.4.3 Methionine Oxidation Is Deleterious for TM

Although TM is a large protein, only EGF4,5-like domains are required for anticoagulant function. These two domains must work together, and the linker between the two domains contains a single methionine residue, Met^{388}. Oxidation of Met^{388} is deleterious for TM activity. Structural studies, both X-ray and NMR, of wild type and variants at position 388 showed that Met^{388} provides a key linkage between the two domains. Oxidation of the methionine has consequences for the structure of the fifth domain, which binds to thrombin. The functional consequences of oxidation of Met^{388} include decreased anticoagulant activity. Oxidative stress from several causes is reflected in lower serum levels of activated protein C and a higher thrombotic tendency, and this is thought to be linked to the oxidation of Met^{388} in TM. Thus, TM structure and function are altered in a subtle but functionally critical way upon oxidation of Met^{388} (Wood et al. 2005).

41.5.4.4 Role of Exosites 1 and 2 in Thrombin Reaction

Thrombin has three main binding sites: the catalytic or active site, where its primary plasma inhibitor, antithrombin, binds and inhibits its coagulation activity; and two anion-binding sites, exosite 1 and exosite 2. Exosite 1 binds to fibrinogen, TM, hirudin, and the amino terminus of heparin cofactor II (HCII) (Becker et al. 1999). Exosite 2 mediates binding of thrombin to heparin and to platelet surface glycoprotein Ib-α (GPIba). Ligand binding to either exosite 1 or exosite 2 may also influence reactivity of the active site of thrombin (Fredenburgh et al. 1997). The cofactors heparin, vitronectin (VN), and TM modulate the reactivity of α-thrombin with plasminogen activator inhibitor (PAI-1). While heparin and VN accelerate the reaction by two orders of magnitude, TM protects α-thrombin from rapid inactivation by PAI-1 in presence of VN. Kinetic studies suggest that (1) heparin binds to exosite 2 of α-thrombin to accelerate the reaction by a template mechanism, (2) VN accelerates PAI-1 inactivation of α-thrombin by lowering the K_D for initial complex formation by an unknown mechanism that does not require binding to either exosite 1 or exosite 2 of α-thrombin, (3) α-thrombin may have a binding site for PAI-1 within or near exosite 1, and (4) TM occupancy of exosite 1 partially accounts for the protection of thrombin from rapid inactivation by PAI-1 in presence of vitronectin (Rezaie 1999).

Exosite 1 of thrombin consists of a cluster of basic residues (Arg^{35}, Lys^{36}, Arg^{67}, Lys^{70}, Arg^{73}, Arg^{75}, and Arg^{77} in chymotrypsinogen numbering) that play key roles in the function of thrombin. Structure suggests that the side chain of Arg^{35} projects toward the active site pocket of thrombin, but all other residues are poised to interact with TM. While studying the role of these residues in TM-mediated protein C activation by thrombin, results suggest that Arg^{35} is responsible for the Ca^{2+} dependence of protein C activation by the thrombin–TM complex and that a function for TM in the activation complex is the allosteric alleviation of the inhibitory interaction of Arg^{35} with the substrate. The results suggest that TM modulates the conformation of Arg^{35}, and a cofactor function of TM is to alleviate the inhibitory interaction of Arg^{35} with protein C in the activation complex (Rezaie and Yang 2003).

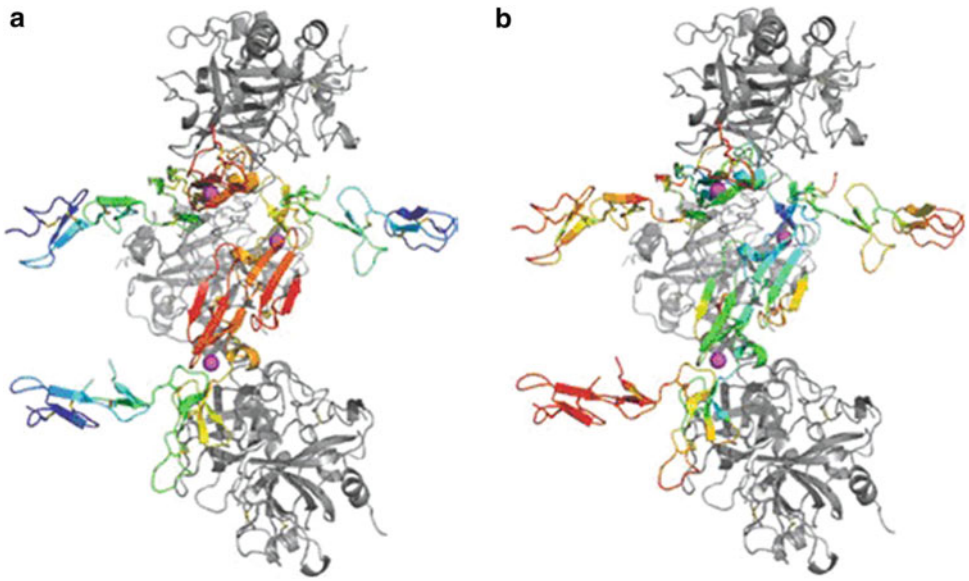

Fig. 41.3 Crystal structure of Na-free thrombin in complex with thrombomodulin: Structure of the thrombin-TM domains 4, 5, 6 (TM456) complex. (**a**) Ribbon diagram of the three complexes in the asymmetric unit, with thrombin coloured *grey* and TM456 coloured from N-to-C terminus from *blue* (EGF4) to *red* (EGF6), with *magenta* balls indicating Ca2 + ions. (**b**) The same image as in (**a**), but coloured according to B-factor to illustrate the inherent flexibility of the 4th EGF domains (Adapted with permission from Adams et al. 2009 © John Wiley and Sons)

Structural and mutagenesis data indicated that the interaction of basic residues of the heparin-binding exosite of protein C with the acidic residues of EGF4 is partially responsible for the efficient activation of the substrate by the thrombin-EGF456 complex. Similar to protein C, protein C inhibitor has a basic exosite (H-helix) that constitutes the heparin-binding site of the serpin. It was suggested that TM enhances the reactivity of protein C inhibitor with thrombin by providing both a binding site for the serpin and a conformational modulation of the extended binding pocket of thrombin (Yang et al. 2003).

41.5.5 The Crystal Structure

The crystal structure of the complex between human thrombin and the minimal cofactor fragment of TM, EGF456 revealed the features of TM-thrombin interaction and corroborated with earlier biochemical information (Fuentes-Prior et al. 2000) (Fig. 41.3). A reasonably small contact interface of ~900 Å is seen between exosite I of thrombin and EGF domains 5 and 6 of TM. Although the surfaces at the interface displayed charge complementarity, which may aid in steering the two fragments together (Myles et al. 2001) only a single salt bridge exists between lysine 110 of thrombin and aspartate^{461} of TM. The TM-thrombin complex crystal structure revealed no obvious conformational changes within the active site of thrombin, but the presence of a bound active-site inhibitor clouds this issue. However, allostery may play a role in switching the substrate preference of thrombin, as several biochemical studies suggest a tightening of the active site in response to TM binding (Koeppe et al. 2005). The cofactor effect of TM may also be partially attributable to an improved accessibility of the activation peptide of TM-bound protein C (Vindigni et al. 1997; Yang et al. 2006; Adams and Huntington 2006). The structure of EGF-like domain 4 has been determined by NMR spectroscopy. These structures are small steps toward an understanding of how TM regulates thrombin (Sadler 1997). The interaction of thrombin with a 28-residue peptide, hTM422-449, corresponding to the N-terminal subdomain of the sixth EGF-like repeat of human TM plus the junction between the fifth and the sixth EGF-like domains, characterized by NMR spectroscopy, is conformationally flexible in absence of thrombin. Upon addition of thrombin, differential resonance perturbations and transferred nuclear Overhauser effects (NOEs) are observed for TM peptide, suggesting specific and rapidly reversible binding and structuring of hTM422-449 in complex with thrombin (Tolkatchev et al. 2000).

Molecular modeling of the protein C activation based on the crystal structure of thrombin in complex with the EGF456 predicts that the binding of EGF56 to exosite 1 of thrombin positions EGF4 so that a negatively charged region on this domain juxtaposes a positively charged region of protein C. It has been hypothesized that electrostatic interactions between these oppositely charged residues of EGF4 and protein C facilitate a proper docking of the substrate into the catalytic pocket of thrombin. To test this hypothesis, Yang and Rezaie

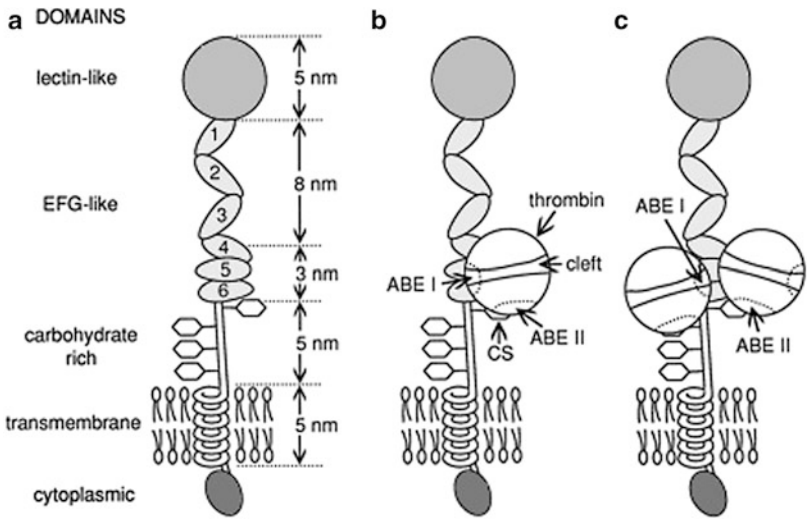

Fig. 41.4 Schematic diagram of Thrombomodulin and TM bound with thrombin. (**a**) schematic diagram of TM based on the electron microscope images of soluble TM. Domains are identified on the *left*, while their sizes are given on the *right*. EGF modules are numbered and carbohydrate moieties are represented as hexagons. The membrane is represented as a lipid bilayer, and the transmembrane region is shown as a typical single helix. (**b**) TM with one thrombin molecule bound. The locations of the catalytic cleft, anion-binding exosite I (ABE I), and anion-binding exosite II (ABE II) of thrombin are shown. Anion-binding exosite I is shown interacting with EGF-like modules 5 and 6 of TM, and anion-binding exosite II is shown interacting with the chondroitin sulfate (CS) residue on TM. (**c**) TM with two thrombin molecules bound, one via interactions of its anion-binding exosite I with EGF modules 5 and 6 and the other via interactions of its anion-binding exosite II with the chondroitin sulfate (Adapted with permission from Weisel et al. 1996 © The American Society for Biochemistry and Molecular Biology)

(2003) constructed several mutants of EGF456 and protein C in which charges of the putative interacting residues on both EGF4 (Asp/Glu) and protein C (Lys/Arg) were reversed. Results of TM-dependent protein C activation studies by such a compensatory mutagenesis approach support the molecular model that EGF4 interacts with the basic exosite of protein C (Yang and Rezaie 2003).

41.5.5.1 TM-Thrombin Complex Under EM

Weisel et al. (1996) determined the shape of SolulinTM, a soluble recombinant form of human TM missing the transmembrane and cytoplasmic domains, by electron microscopy. Solulin appears to be an elongated molecule about 20 nm long that has a large nodule at one end and a smaller nodule near the other end from which extends a thin strand. About half of the molecules form bipolar dimers apparently via interactions between these thin strands. Electron microscopy of complexes formed between Solulin and human α-thrombin revealed that a single thrombin molecule appears to bind to the smaller nodule of Solulin, suggesting that this region contains the EGF41. Epidermal growth factor-like domains 1–4 comprise the connector between the small and large nodule, which is the lectin-like domain; the thin strand at the other end of the molecule is the carbohydrate-rich region. With chondroitin sulfate-containing soluble TM produced from either human melanoma cells or CHO cells, a higher percentage of molecules bound thrombin and, in some cases, two thrombin molecules were attached to one soluble TM in approximately the same region (Weisel et al. 1996) (Fig. 41.4).

41.5.6 Functions

TM is considered a protein involved in coagulation, cancer and embryogenesis. The altered coagulation process leads to a precocious placental failure, but some studies suggest that TM has an anti-inflammatory ability through both protein C dependent and independent pathways.

41.5.6.1 TM Initiates Anticoagulant Process

Thrombomodulin is an endocytic receptor expressed primarily on endothelial cells. It exerts an anticoagulant effect through the sequestration of thrombin and activation of protein C, which degrades coagulation factors. Thrombin sequestration inhibits activation of the G-protein-coupled protease-activated receptor (PAR)-1. TM improves the catalytic efficiency of thrombin toward protein C in the presence of Ca^{2+} by ~3 orders of magnitude by improving both the Km and the kcat of the protein C activation reaction. TM forms a complex with thrombin which is responsible of converting protein C to activated protein C (Maruyama et al. 1985; Rezaie and Yang 2003; Wouwer et al. 2004; Dahlbäck and Villoutreix 2005). The TM-thrombin complex balances the substrate specificity of thrombin, erasing its procoagulant properties, and improving the conversion of protein C into its activated form (Esmon 2000; Fuentes-Prior

et al. 2000). Protein C acts on a variety of targets where as the TM-protein C system is essential for maintaining the placenta during pregnancy. TM influences inflammation and tumor progression and TM polymorphisms have been linked to thrombotic conditions such as heart attack. The activity of TM initiates an essential physiological anticoagulant process (Hofsteenge et al. 1986) which by binding to thrombin inhibits the procoagulant functions of thrombin and acts as a cofactor for thrombin catalyzed activation of protein C. This raises the speed of protein C activation 1,000-fold. Activated protein C degrades clotting factors Va and VIIIa and thereby inhibits blood clotting. TM-bound thrombin has no procoagulant effect. When bound to TM, thrombin also activates the carboxypeptidase B-like enzyme thrombin activatable fibrinolysis inhibitor (TAFI) (Bajzar et al. 1996). The binding site for thrombin has been localized by alanine-scanning mutagenesis and peptide-binding studies to the final 2 EGF domains, EGF56 (Jakubowski and Owen 1989; Kurosawa et al. 1988; Nagashima et al. 1993; Adams and Huntington 2006).

Primarily, TM modifies the substrate specificity of thrombin, apparently by an allosteric mechanism. The thrombin-TM-complex also inhibits fibrinolysis by cleaving thrombin-activatable fibrinolysis inhibitor (TAFI) into its active form, initiating an essential anticoagulant pathway. The cofactor function of membrane-associated TM requires EGF456, as well as a Ser/Thr-rich spacer between EGF-like domain 6 and the transmembrane domain. The Ser/Thr-rich domain is variably modified with a chondroitin sulfate chain that influences the affinity of thrombin binding and the calcium ion dependence of cofactor function. Deletion of the N-terminal lectin-like domain and EGF-like domains 1 and 2 had no effect on TAFI or protein C activation, whereas deletions including EGF-like domain 3 selectively abolished TM cofactor activity for TAFI activation. Thus, the anticoagulant and antifibrinolytic cofactor activities of TM have distinct structural requirements: protein C binding to the thrombin-TM complex requires EGF-like domain 4, whereas TAFI binding requires EGF-like domain 3 (Kokame et al. 1998) (Fig. 41.5).

Deletion and point mutants of soluble TM were used to compare and contrast elements of primary structure required for the activation of TAFI and protein C. The smallest mutant capable of efficiently promoting TAFI activation contained residues including the c-loop of EGF3 through EGF6. This mutant is 13 residues longer than the smallest mutant that functioned well with protein C; the latter consisted of residues from the interdomain loop connecting EGF3 and EGF4 through EGF6. Wang et al. (2000) suggest that structural elements in the thrombin-binding domain are needed for the activation of both protein C and TAFI, but more of the primary structure is needed for TAFI activation. In addition, some residues are needed for one of the reactions but not the other (Wang et al. 2000).

Single-chain urokinase-type plasminogen activator (scu-PA) can be cleaved by thrombin into a virtually inactive form called thrombin-cleaved two-chain urokinase-type plasminogen activator (tcu-PA/T), a process accelerated by TM. TM-EGF56 bound to thrombin but did not accelerate the activation of protein C. In contrast, the inactivation of scu-PA by thrombin was accelerated to the same extent as that induced by TM-LEO and TM-Ei4-6. This study demonstrated that, in addition to the chondroitin sulfate moiety, only EGF-like domains 5 and 6 are essential for the acceleration of the inactivation of scu-PA by thrombin. This differs from the domains that are critical for activation of protein C (EGF-like domains i4–6) and thrombin activatable fibrinolysis inhibitor (EGF-like domains 3–6) (Schenk-Braat et al. 2001).

Molecular Basis of TM Activation of Slow Thrombin

Thrombin is critical enzyme for producing and stabilizing a clot, but when complexed with TM it is converted to a powerful anticoagulant. Thrombin function is also controlled by Na^+. Its apparent affinity suggests that half of the thrombin generated is in a Na^+-free "slow" state and half is in a Na^+-coordinated "fast" state. While slow thrombin is a poor procoagulant enzyme, when complexed to TM it is an effective anticoagulant. A structure of thrombin complexed with EGF456 of TM in absence of Na^+ and other cofactors or inhibitors suggests that TM binds thrombin (Adams et al. 2009). As a result the thrombin component resembles structures of fast form. The Na^+ binding loop in thrombin in thrombin-TM complex is in a conformation identical to the Na^+-bound form, with conserved water molecules compensating for the missing ion. It was shown that activation of slow thrombin by TM principally involves the opening of the primary specificity pocket. Therefore, TM binding alters the conformation of thrombin in a similar manner as Na^+ coordination, resulting in an ordering of the Na^+ binding loop and an opening of the adjacent S1 pocket (Adams et al. 2009) (Fig. 41.5).

41.5.6.2 Regulator of Inflammation

Although, the TM is best characterized as a natural anticoagulant, reports have illuminated the importance of TM in the regulation of inflammation. The antigen described as BDCA-3 is identical to TM (Chap. 35) (Dzionek et al. 2000, 2002). This molecule also occurs on a very rare (0.02%) subset of human dendritic cells called MDC2. Its function on these cells is unknown at present, but apparently, TM has at least one other ligand apart from thrombin, because anticoaglulation is a common place function,

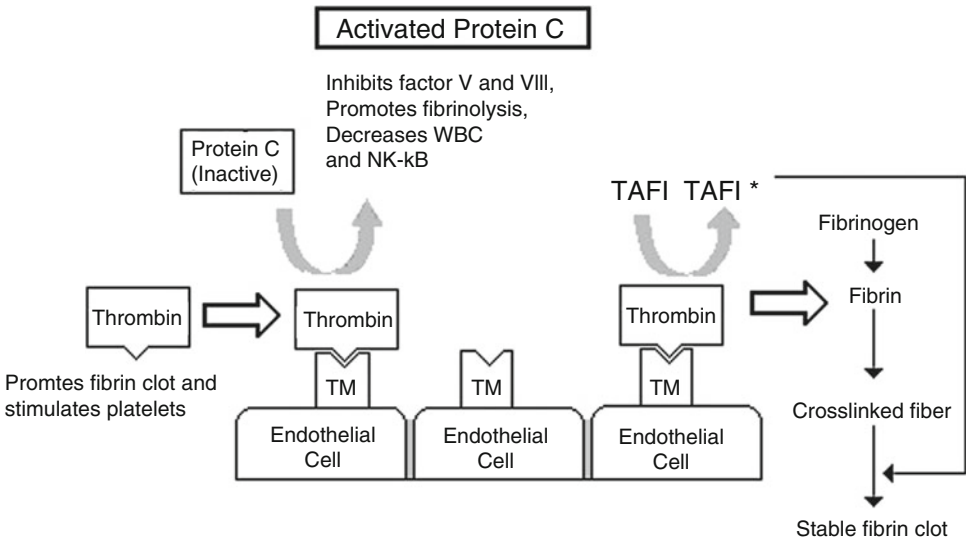

Fig. 41.5 Functions of membrane bound thrombomodulin (TM): (1) TM exerts an anticoagulant effect through the sequestration of thrombin and activation of protein C, which degrades coagulation factors. (2) TM also accelerates the proteolytic activation of a plasma procarboxypeptidase referred to as thrombin-activable fibrinolysis inhibitor (TAFI*). Activated TAFI* can be generated from the binary thrombin-TAFI complex (T-TAFI) or the ternary thrombin-thrombomodulin-TAFI complex (T-TM-TAFI) (Nesheim 2003). The anticoagulant and antifibrinolytic cofactor activities of TM have distinct structural requirements: protein C binding to the thrombin-TM complex requires EGF-like domain 4, whereas TAFI binding also requires EGF-like domain 3 (Kokame et al. 1998)

in contrast to the rarity of MDC2 cells. Thrombomodulin expression on DCs may be involved in the pathogenesis of atopy and asthma (Yerkovich et al. 2009). Thrombomodulin inhibits allergic bronchial asthma by inducing tolerogenic DCs. Treatment of bronchial asthma with TM improved lung function and reduced IgE and cells in alveolar lavage fluid by inducing tolerogenic dendritic dells. These were characterized by high expression of surface CD141/TM$^+$ and low expression of maturation markers and possessed reduced T-cell co-stimulatory activity. The TM effect was independent of its role in coagulation. Rather, it was mediated via the TM lectin domain directly interacting with DCs. Results showed that TM is a modulator of DC immunostimulatory properties and a novel candidate drug for the prevention of bronchial asthma in atopic patients (Takagi et al. 2011; Yerkovich et al. 2009). CD141$^+$ DC subset is an important functionally distinct human DC subtype with characteristics similar to those of the mouse CD8α^+ DC subset. CD141$^+$ DCs play a role in the induction of cytotoxic T lymphocyte and may be the most relevant targets for vaccination against cancers, viruses, and other pathogens (Jongbloed et al. 2010). Thrombomodulin was present in all keratinizing squamous carcinomas and the great majority (87%) of nonkeratinizing tumors in a membrane-staining pattern. It was moderately common in small cell (27%) and large cell carcinomas (25%) but relatively rare in adenocarcinomas (13%) (Miettinen and Sarlomo-Rikala 2003).

41.5.6.3 Regulator of Placental Functions

Thrombomodulin is expressed mainly on the endothelial surface of blood vessels and in the placental syncytiotrophoblast (Maruyama et al. 1985; Dittman and Majerus 1990). The functions of TM are exerted in two distinct tissues: in non-endothelial extra-embryonic tissues, required for proper function of the early placenta, and in embryonic blood vessel endothelium whose absence causes lethal consumptive coagulopathy (Bicheng et al. 2005). Disruption of the mouse gene encoding the TM leads to embryonic lethality caused by an unknown defect in the placenta. Isermann et al. (2003) showed that the abortion of TM-deficient embryos is caused by tissue factor–initiated activation of the blood coagulation cascade at the feto-maternal interface. These findings show a new function for the TM–protein C system in controlling the growth and survival of trophoblast cells in the placenta. This function is essential for the maintenance of pregnancy (Isermann et al. 2003).

41.5.7 Abnormalities Associated with Thrombomodulin Deficiency

41.5.7.1 Polymorphism and the Risk of Heart Disease

The genes encoding proteins in the TM-protein C pathway are promising candidate genes for stroke susceptibility because of their importance in thrombosis regulation and inflammatory response. Thrombosis is a dynamic balance

between factors that promote clot formation, antithrombotic mechanisms, and fibrinolysis. Central to this balance is the TM-protein C antithrombotic mechanism. TM forms a 1:1 complex with thrombin on the vascular endothelium, thereby inhibiting the procoagulant actions of thrombin and converting protein C to activated protein C. Activated protein C promotes fibrinolysis, inhibits thrombosis by inactivating clotting factors Va and VIIIa, and reduces inflammation by decreasing white blood cell and NF-kB activation (Esmon 1987, 1995, 2001; Barnes and Karin 1997). These relationships are demonstrated in Fig. 41.5**.** Because of the central role that the TM-protein C pathway plays in thrombosis regulation and inflammatory response, the genes encoding these pathway proteins are promising candidate genes regarding stroke susceptibility.

Eosinophil (EO) specific major basic protein accumulates on endocardial surfaces in the course of hypereosinophilic heart disease and promotes thrombosis by binding to the anionic TM and impairing its anticoagulant activities. Major basic protein potently inhibits the capacity of cell surface TM to generate the natural anticoagulant activated protein C. Thus, EO cationic proteins potently inhibit anticoagulant activities of the glycosylated form of TM, thereby suggesting a potential mechanism for thromboembolism in hypereosinophilic heart disease (Slungaard et al. 1993).

The TM gene (*THBD*) maps to chromosome 20p11.2, and spans 4 kb (Ireland et al. 1996). A single nucleotide polymorphism (C→T) at position + 1418 (C1418T) encodes for an amino acid change from alanine to valine at protein position 455 ($Ala^{455}Val$) (van der Velden et al. 1991). The location of this amino acid variation corresponds to the sixth EGF region of the TM protein (Fig. 41.2). This location has been shown to be responsible for the high-affinity binding of thrombin and for the suspension of thrombin at a specific position above the endothelial surface in relation to other cofactors, thereby producing optimal protein C activation by thrombin (Esmon 1995; Sadler 1997).

Studies have shown that the *THBD* $Ala^{455}Val$ polymorphism is associated with ischemic heart disease (Norlund et al. 1997; Wu et al. 2001). Based on results of Kittner et al. (1998), Cole et al. (2004) determined the association between the *THBD* $Ala^{455}Val$ polymorphism and the occurrence of ischemic stroke in young women. Schulz et al. (2004) indicated that among women aged 15–44 years, the AA genotype is more prevalent among blacks than whites and is associated with increased risk of early-onset ischemic stroke. Removing strokes potentially related to cardioembolic phenomena increased this association. Whether this association is due to population stratification, needs to be confirmed.

41.5.7.2 Spontaneous Recurrent Abortion

Reduced expression of TM in placental tissue of women were associated with spontaneous recurrent abortion and the loss of TM caused early post-implantation embryonic lethality (Isermann et al. 2001; Kaare et al. 2007; Stortoni et al. 2010). This suggested that TM expression in non-endothelial placental cells is required for a normal function of the early placenta. Further studies are needed to elucidate that the TM expression in non-endothelial placental cells is required for a normal function of the early placenta.

41.6 Endosialin (Tumor Endothelial Marker-1 or CD248)

41.6.1 A Marker of Tumor Endothelium

Endosialin (Tumor Endothelial Marker-1 or CD248) was originally described as a marker of tumor endothelium, and later as a marker of fibroblasts (Rettig et al. 1992; St Croix et al. 2000). Endosialin is a 165-kDa cell surface glycoprotein expressed by tumor blood vessel endothelium in a broad range of human cancers but not detected in blood vessels or other cell types in many normal tissues. A full-length endosialin cDNA with an open reading frame of 2274 bp encodes a type I membrane protein of 757 amino acids with a predicted molecular mass of 80.9 kDa. The sequence matches with an expressed sequence tag of unknown function in public data bases, named Tumor Endothelial Marker-1(TEM-1). Endosialin is classified as a C-type lectin-like protein, composed of a signal leader peptide, five globular extracellular domains (including a C-type lectin domain, one domain with similarity to the Sushi/ccp/scr pattern, and three EGF repeats), followed by a mucin-like region, a transmembrane segment, and a short cytoplasmic tail (Fig. 41.6). The endosialin core protein carries abundantly sialylated, O-linked oligosaccharides and is sensitive to O-sialoglycoprotein endopeptidase, placing it in the group of sialomucin-like molecules. The N-terminal 360 amino acids of endosialin show homology to thrombomodulin, a receptor involved in regulation of blood coagulation (Section 41.5), and to complement receptor C1qRp (Section 41.4) (Christian et al. 2001; Teicher 2007). Endosialin is cell surface receptor of unknown function, with a distinctive pattern of endothelial expression in newly formed blood vessels in human cancers. It is not known if carbohydrate recognition is involved in the function of proteins in the endosialin group. The CTLDs in these proteins do not contain the sequence motifs connected with sugar-binding activity.

The single copy endosialin gene was mapped to chromosome 19. Endosialin gene is intronless and encodes a 92-kDa protein that has 77.5% overall homology to the human protein. This gene is ubiquitously expressed in normal human and mouse somatic tissues and during development.

Fig. 41.6 Domain architecture of endosialin. The diagram shows the arrangement of the N-terminal signal leader peptide (*triangle*), the C-type lectin domain, the domain similar to a Sushi/SCR/CCP domain (*S*), three EGF-like repeats (*EGF*), and the transmembrane region (*TM*), flanked by two segments with low complexity regions, namely a sialomucin-like sequence (*MUCIN*) and a short, putative cytoplasmic tail (*CYT*) (Adapted from Christian et al. 2001© American Society for Biochemistry and Molecular Biology)

The murine orthologue of endosialin has been identified, opening up the analysis of developmental regulation in the embryo and in aberrant tissue remodeling. Its expression at the mRNA level is density-dependent and up-regulated in serum-starved cells. In vitro, its expression is limited to cells of embryonic, endothelial, and preadipocyte origin, suggesting that the wide distribution of its expression in vivo is due to the presence of vascular endothelial cells in all the tissues. The ubiquitous expression in vivo is in contrast to previously reported expression limited to corpus luteum and highly angiogenic tissues such as tumors and wound tissue (Opavsky et al. 2001). Further analyses confirmed selective *TEM-1*/*endosialin* expression in tumor endothelium, pericytes and a subset of fibroblast-like cells of tumor stroma in breast carcinoma, anaplastic astrocytoma and GBM (Simonavicius et al. 2008; Davies et al. 2004; Brady et al. 2004; MacFadyen et al. 2005; Carson-Walter et al. 2001; Christian et al. 2008).

Findings on mouse endosialin suggest that: (1) endosialin protein (also termed as TEM-1) is restricted to vascular endothelium and fibroblast-like cells in developing organs, and largely disappears during adulthood, (2) endothelial expression varies markedly between organs regarding spatial and temporal patterns, (3), circumscribed mesenchymal expression in fibroblast-like cells was evident throughout development, most pronounced adjacent to certain budding epithelia, as in the lung and kidney glomeruli, but unrelated to the endothelial expression. The endosialin protein persists in the stromal fibroblasts of the adult uterus (Rupp et al. 2006). Brain pericytes in culture had higher levels of endosialin/TEM 1 than TEMs-2, -3, -4, -5, -7, and -8. Endosialin was present in the cytoplasmic body and in the elongated extensions essential to pericyte function. It seems that endosialin is strongly expressed by pericytes during periods of active angiogenesis during embryonic and tumor development. With the appropriate agent, targeting endosialin may interfere with blood vessel growth during tumor development (Bagley et al. 2008a).

41.6.1.1 Upregulation of Endosialin Gene by Hypoxia

Angiogenesis is well known to be principally driven by hypoxia in growing solid tumors as well as in developing embryos (Liao and Johnson 2007). The endosialin gene transcription is induced by hypoxia predominantly through a mechanism involving hypoxia-inducible factor-2 (HIF-2) cooperating with the Ets-1 transcription factor. It was shown that HIF-2 activates the endosialin promoter both directly, through binding to a hypoxia-response element adjacent to an Ets-binding site in the distal part of the upstream regulatory region, and indirectly, through Ets-1 and its two cognate elements in the proximal promoter. Results also suggested that the SP1 transcription factor mediates responsiveness of endosialin promoter to high cell density (Ohradanova et al. 2008).

41.6.2 Interactions with Ligands

It is not clear if carbohydrate recognition is involved in the function of proteins in the endosialin group. The CTLDs in these proteins do not contain the sequence motifs connected with sugar-binding activity. Recent evidence suggests that endosialin may interact with extracellular matrix components including fibronectin (FN) and collagen types I and IV as well as Mac-2 BP/90 K in promoting vascular migration and invasion (Tomkowicz et al. 2007; Becker et al. 2008). More importantly, cells expressing endosialin exhibit enhanced adhesion to FN as well as enhanced migration through matrigel and increased adhesive properties (Tomkowicz et al. 2007). Subsequently, endosialin/collagen IV co-localization was confirmed by confocal microscopy. Other studies have demonstrated the importance of endosialin in regulating proliferation tube formation on matrigel (Bagley et al. 2008b). However, the exact mechanism(s) as to how endosialin exerts its pleiotropic cellular effects has not been addressed. Becker et al. (2008) provided evidence that PDGF receptor and the MAP kinase ERK-1/2 are involved with the endosialin-regulated signaling pathways that control proliferation of pericytes. Mac-2 BP/90 K has been identified as a specific interacting partner of endosialin. C-terminal fragment of Mac-2 BP/90 K, a binding partner for galectin-3, and collagens were responsible for endosialin binding. Intriguingly, the expression patterns of Mac-2 BP/90 K and endosialin were mutually exclusive in all human tissues. These studies demonstrate a novel repulsive interaction between endosialin on stromal fibroblasts and Mac-2 BP/90 K on tumor cells (Becker et al. 2008). Tomkowicz et al. (2010) showed that normal pericytes expressing high levels of endosialin were able to proliferate, respond to PDGF-BB stimulation by phosphorylating both the PDGF receptor and the MAP-kinase ERK-1/2, and induce the expression of immediate early transcription factor *c-Fos*. However, when endosialin expression was knocked-down, PDGF-BB-induced proliferation, ERK-1/2-phosphorylation, and *c-Fos* expression were significantly impaired. These results provide evidence for a endosialin-

dependent signal pathway that controls proliferation of human pericytes and suggest targeting this pathway for future strategies aimed at mitigating tumor angiogenesis (Tomkowicz et al. 2010).

41.6.3 Functions

Abundant endosialin expression was found in the vasculature and fibroblast-like cells of the developing mouse and human embryos (Rupp et al. 2006; MacFadyen et al. 2007; Virgintino et al. 2007). Nanda et al. (2006) have shown that endosialin is dispensable for normal development and subcutaneous tumor growth, but can modulate invasiveness and metastatic progression in an orthotopic Xenograft model of colorectal cancer. All these data support the functional involvement of endosialin in angiogenesis, a complex process of vascular branching and sprouting that plays a key role in tumor expansion and progression, and therefore represents an opportunity for therapeutic intervention against cancer. MacFadyen et al. demonstrated that endosialin was expressed by fibroblasts and pericytes associated with tumor vessels, but not on HUVECs or endothelial cells of normal or diseased tissue (MacFadyen et al. 2005, 2007). Moreover, endosialin expression was upregulated on tumor-derived pericytes and confirmed by Virgintino et al. (2007).

The functional significance of endosialin expression on pericytes and stromal cells, its upregulation during cancer, and its role in angiogenesis has largely remained unknown. The knockout (KO) mouse model showed the absence of endosialin expression, reduced growth, invasion, and metastasis of human tumor Xenografts. In addition, lack of endosialin led to an increase in small immature blood vessels and decreased numbers of medium and large tumor vessels. This abnormal angiogenic response could be responsible for the reduced tumor growth and invasion observed in endosialin KO mice, suggesting a role for endosialin in controlling the interaction among tumor cells, endothelia, and stromal matrix (Nanda et al. 2006). Data indicated that the stroma can control tumor aggressiveness and that this control varies with anatomic site. Endosialin is required for complete popliteal LN (pLN) expansion but not for coordination of B and T cell compartmentalisation or antibody production following 4-hydroxy-3-nitrophenyl)acetyl chicken γ-globulin immunisation. In vitro, Endosialin expression in human MG63 stromal cells and mouse embryonic fibroblasts leads to a pro-proliferative and pro-migratory phenotype. This correlates with a proliferating $CD248^{+}$ population observed in vivo during pLN expansion (Lax et al. 2010).

41.6.3.1 Endosialin in Cancer

Several studies confirmed that endosialin is upregulated in blood vessels and activated stromal fibroblasts in human colorectal, brain and breast tumors as well as in mouse Xenograft models (Carson-Walter et al. 2001; Brady et al. 2004; Davies et al. 2004; Madden et al. 2004; Dolznig et al. 2005; MacFadyen et al. 2005, 2007; Huber et al. 2006; Rupp et al. 2006). Endosialin expression is restricted to capillaries and shows a heterogeneous pattern typical for molecules involved in vascular reorganization. Human colon and ovarian Xenografts showed that endosialin expression was largely confined to NG2-expressing perivascular cells and not CD31-positive endothelial cells (Bagley et al. 2008b).

Endosialin is a stromal marker that is differentially expressed on fibroblasts and pericytes in the thymus, lymph node and spleen. Expression is high during LT development but largely disappears in the adult. Endosialin is re-expressed in a Salmonella-induced model of splenic enlargement; peak expression corresponding to the peak of splenic enlargement. These results suggest that endosialin expression helps define a subset of LT stromal cells which play a role in remodelling during tissue development, infection and repair (Dolznig et al. 2005; Lax et al. 2007; MacFadyen et al. 2005; Teicher 2007). Cancer-associated fibroblasts (CAF) have been implicated in promoting tumor development and have been associated with mesenchymal stem cells (MSC). MSC can form networks in a tube formation assay that is inhibited by an anti-endosialin antibody. MSC are a potential target for anticancer therapeutic intervention and endosialin expression offers a tool for the identification of MSC. Endosialin expression by both CAF and MSC further implies the potential contribution of MSC to tumor stroma via differentiation into tumor stromal fibroblasts (Bagley et al. 2009; Demoulin 2010).

In an effort to better characterize the desmoplastic response to human skin tumors, Huber et al. (2006) evaluated the expression pattern of three stromal cell markers, fibroblast-activation protein (FAP), endoglyx-1, and endosialin, in a series of melanocytic and epithelial skin tumors. FAP-positive fibroblasts were detected in all tumor tissues tested, including cases of melanocytic nevi, melanoma metastases, basal cell carcinomas, and squamous cell carcinomas. Endoglyx-1 expression was confined to normal and tumor blood vessel endothelium including 'hot spots' of neoangiogenesis within the cutaneous melanoma metastases. Endosialin was selectively induced in subsets of small- and medium-sized tumor blood vessels in melanoma metastases and squamous cell carcinomas. These data describe novel aspects of targets for novel therapeutic strategies aimed at the tumor stroma (Huber et al. 2006). Christian et al. (2008) claim that endosialin is expressed by tumor-associated myofibroblasts and mural cells and not by endothelial cells. Endosialin expression is barely detectable in normal human tissues with moderate expression only detectable in the stroma of the colon and the prostate. Corresponding cellular experiments confirmed endosialin expression by mesenchymal cells and indicated that it may

in fact be a marker of mesenchymal stem cells. Collectively, experiments validate endosialin as a marker of tumor-associated myofibroblasts and tumor vessel-associated mural cells (Christian et al. 2008).

Brain Tumors

Human brain tumors express endosialin in a heterogeneous manner. Gliomas are the most frequent primary tumors of the central nervous system in adults. The most prevalent and aggressive subclass of these is glioblastoma multiforme, which is characterized by massive neovascularization. The largest proportion of endosialin-expressing tumors was found in highly invasive glioblastoma multiforme, anaplastic astrocytomas, and metastatic carcinomas. Endosialin is not expressed in normal human adult brain but is strongly upregulated in the angiogenic vasculature of all high-grade glioma specimens. Endosialin was localized to the endothelium of small and large CD31 stained vessels and also expressed by Thy-1-positive fibroblast-like cells close to the meninges and α-smooth muscle actin-positive cells in some vessels. Endosialin colocalized with thrombomodulin, suggesting these proteins may have complementary functions in tumor progression (Brady et al. 2004). However, Simonavicius et al. (2008) demonstrated unambiguously that endosialin is not expressed by the glioma endothelial cells but on closely associated perivascular cells. Carson-Walter et al. (2009) characterized the expression pattern of endosialin in astrocytic and metastatic brain tumors and investigated its role as a therapeutic target in human endothelial cells and mouse xenograft models. Eendosialin was induced in the vasculature of high-grade brain tumors where its expression was inversely correlated with patient age. Although lack of endosialin did not suppress growth of intracranial GBM xenografts, it did increase tumor vascularity (Carson-Walter et al. 2009). The recognition of endosialin as an antigen that is selectively over-expressed in human tumor tissues offers new strategies for treating cancer. Not only do the tumor vasculature and stromal compartments upregulate endosialin but, the malignant cells of sarcomas also strongly express endosialin. Thus, endosialin holds potential value both as a therapeutic target and as a biomarker for certain human cancers (Bagley 2009; Rouleau et al. 2008; Simonavicius et al. 2008). The function of C-type lectin 14A is not known.

41.7 Bimlec (DEC 205 Associated C-type lectin-1 or DCL-1) or (CD302) (Group XV of CTLD)

BIMLEC is synonymous with C-type lectin domain family 13 member A (CLEC13A), KIAA0022 and DEC-205-associated C-type lectin-1 (DCL-1). BIMLEC is a Type I transmembrane protein with neck region and CTLD in extracellular region. This new group of C-type lectins was predicted by Zelensky and Gready (2004) in Fugu whole genome and supported by a database cDNA sequences. Group was named Bimlec, linked to DEC-205. Function of Bimlec in humans is unknown, but it is expressed as a fusion protein with DEC-205 in Hodgkin's lymphoma cells (Kato et al. 2003). The cDNA encoding DCL-1 has been identified in Reed-Sternberg cells associated with the development of classic Hodgkin's lymphoma as part of fusion protein between DEC-205 (CD205) and a C-type lectin DCL-1 (Kato et al. 2003). Although the 7.5-kb DEC-205 and 4.2-kb DCL-1 mRNA were expressed independently in myeloid and B lymphoid cell lines, the DEC-205/DCL-1 fusion mRNA (9.5 kb) predominated in the HRS cell lines. The DEC-205 and DCL-1 genes comprising 35 and 6 exons, respectively, are juxtaposed on chromosome band 2q24 and separated by only 5.4 kb. The fusion mRNA predominates in these cells, but both proteins are expressed independently also in myeloid cells and B-lymphoid cell lines. DCL-1 protein is highly conserved among the human, mouse, and rat orthologs. DCL-1 expression in leukocytes is restricted to monocytes, macrophages, granulocytes, and dendritic cells, although DCL-1 mRNA is present in many tissues. These results imply an unusual transcriptional control mechanism in HRS cells, which cotranscribe an mRNA containing DEC-205 and DCL-1 prior to generating the intergenically spliced mRNA to produce a DEC-205/DCL-1 fusion protein. Kato et al. (2007) have suggested that DCL-1 is an unconventional lectin receptor that plays roles in endocytosis and phagocytosis but also in cell adhesion and migration. In nomenclature of CD antigens this protein has been given the designation CD302.

41.8 Proteins Containing SCP, EGF, EGF and CTLD (SEEC) (Group XVI of CTLD)

Souble protein containing SCP, EGF, EGF and CTLD (SEEC) domains is a group of CTLD which has been predicted during Fugu whole-genome analysis and numbered as group XVI of CTLD. Group XVI of CTLD is well conserved between human and fish and supported by available cDNAs. The sperm-coating glycoprotein (SCP) domain, which is present in organisms from yeast and plants to mammals, but whose function is unknown (Szyperski et al. 1998), is rarely observed in combination with other domains in proteins. This SCP/CTLD combination is observed in one known protein – Nowa from hydra (Engel et al. 2002). CTLD has potential Ca-carbohydrate-binding motif (QPD) characteristic of galactose specificity

41.8.1 Nematocyst Outer Wall Antigen (NOWA)

The nematocyst is a unique extrusive organelle involved in the defense and capture of prey in cnidarians. The nematocyst outer wall antigen (NOWA) was identified in nematocysts, explosive organelles of *Hydra*, jellyfish, corals and other *Cnidaria*. Minicollagens along with glycoprotein NOWA form the major components of nematocyst capsule outer wall, which resists osmotic pressure of 15 MPa. NOWA spontaneously assembles to globular macromolecular particles that are sensitive to reduction as the native wall structure. NOWA, the major component of the outer wall, is formed very early in morphogenesis. NOWA is a 90-kDa glycoprotein which comprises a C-terminal eightfold repeat of the minicollagen cysteine-rich domain, suggesting a possible disulfide-dependent hetero-assembly of minicollagens and NOWA protein (Ozbek et al. 2004). NOWA has a modular structure with an N-terminal sperm coating glycoprotein domain, a central C-type lectin-like domain, and an eightfold repeated cysteine-rich domain at the C-terminus. Interestingly, the cysteine-rich domains are homologous to the cysteine-rich domains of minicollagens. The cysteines of these minicollagen cysteine-rich domains undergo an isomerization process from intra- to intermolecular disulfide bonds, which mediates the crosslinking of minicollagens to networks in the inner wall of the capsule. The minicollagen cysteine-rich domains present in both proteins provide a potential link between NOWA in the outer wall and minicollagens in the inner wall. Engel et al. (2002) proposed a model that integrates the role of microtubule cytoskeleton and the interaction of NOWA and minicollagen in forming the nematocyst wall. Data suggest a continuous suprastructure of the nematocyst wall, assembled from wall proteins that share a common oligomerization motif (Meier et al. 2007).

41.8.2 The Cysteine-Rich Secretory Proteins (CRISP) Super-Family

The cysteine-rich secretory proteins (CRISP), antigen 5, and pathogenesis-related 1 proteins (CAP) superfamily members are found in a remarkable range of organisms spanning each of the animal kingdoms. Within humans and mice, there are 31 and 33 individual family members, respectively, and although many are poorly characterized, a majority of them shows a notable expression bias to the reproductive tract (Gupta 2005) and immune tissues or are deregulated in cancers. CAP superfamily proteins are most often secreted and have an extracellular endocrine or paracrine function and are involved in processes including the regulation of extracellular matrix and branching morphogenesis, potentially as either proteases or protease inhibitors; in ion channel regulation in fertility; as tumor suppressor or prooncogenic genes in tissues including prostate; and in cell-cell adhesion during fertilization. Several reviews describing mammalian CAP superfamily gene expression profiles, phylogenetic relationships, protein structural properties, and biological functions draw into focus their potential role in health and disease. The nine subfamilies of the mammalian CAP superfamily include: the human glioma pathogenesis-related 1 (GLIPR1), Golgi associated pathogenesis related-1 (GAPR1) proteins, peptidase inhibitor 15 (PI15), peptidase inhibitor 16 (PI16), cysteine-rich secretory proteins (CRISPs), CRISP LCCL domain containing 1 (CRISPLD1), CRISP LCCL domain containing 2 (CRISPLD2), mannose receptor like and the R3H domain containing like proteins. The overall protein structural conservation within the CAP super-family results in fundamentally similar functions for the CAP domain in all members, yet the diversity outside this core region dramatically alters target specificity and, therefore, the biological consequences (Gibbs et al. 2008).

41.8.2.1 Human Glioma Pathogenesis-Related Protein

Specifically, the human glioma pathogenesis-related protein (GliPR) is highly expressed in the brain tumor glioblastoma multiforme and exhibits 35% amino acid sequence identity with the tomato pathogenesis-related (PR) protein P14a, which has an important role for the plant defense system. GliPR is homologous to group 1 plant pathogenesis-related proteins (PR-1) that are implicated in plant defense responses to viral, bacterial, and fungal infection (Klessig et al. 2000; van Loon et al. 2006). Since GliPR shows structural similarities with its homologous plant PR-1 proteins, mammalian testis proteins (TPX1) and the insect venom Ag-5 protein, which are secretory proteins (van Loon et al. 2006; Foster and Gerton 1996), it has been suspected that GliPR is also secreted. Comparison of the GliPR model with the P14a structure resulted into identification of a common partially solvent-exposed spatial cluster of four amino acid residues, His-69, Glu-88, Glu-110, and His-127 in the GliPR numeration. This cluster is conserved in all known plant PR proteins of class 1, indicating a common putative active site for GliPR and PR-1 proteins and thus a functional link between the human immune system and a plant defense system (Szyperski et al. 1998).

There isTGliPR is related to testes-specific, vespid and pathogenesis protein 1 (RTVP-1) (Rich et al. 1996). Increased expression of GliPR was associated with myelomonocytic differentiation in macrophages (Gingras and Margolin 2000). Whereas GliPR has been reported to act as a tumor suppressor gene inducing apoptosis in prostate cancer (Naruishi et al. 2006; Ren et al. 2004), it appears to be an oncogene in glioblastomas (Rosenzweig et al. 2006) and

Wilms tumors (Chilukamarri et al. 2007). RTVP-1 protein is reported to contain a N-terminal signal peptide sequence and a transmembrane domain (Szyperski et al. 1998). Furthermore, homology studies revealed a putative active enzymatic center in GliPR (Szyperski et al. 1998). GliPR's homology with plant PR-1 proteins raises the question whether GliPR has an evolutionarily conserved role in innate immune response and human host defense of viral infection including HIV-1. There is an early up-regulation of GliPR expression (fivefold) in CEM T cells infected with HIV-1 (Scheuring et al. 1998). Alternatively or additionally, HIV-1 may induce and exploit GliPR for viral replication. The up-regulation of GliPR by HIV-1 and the early significant inhibition of HIV-1 replication mediated by knockdown of GliPR reveal GliPR as an important HIV-1 dependency factor (HDF), which may be exploited for HIV-1 inhibition (Capalbo et al. 2010).

Data from a number of systems suggests that sequences within the C-terminal CAP domain of CAP proteins have the ability to promote cell-cell adhesion. Cloned mouse *Glipr1l1* has a testis-enriched expression profile where GliPR1L1 is post-translationally modified by N-linked glycosylation during spermatogenesis and ultimately becomes localized to the connecting piece of elongated spermatids and sperm. After sperm capacitation, however, GliPR1L1 is also localized to the anterior regions of the sperm head. Zona pellucida binding assays indicate that GliPR1L1 has a role in the binding of sperm to the zona pellucida surrounding the oocyte. Studies suggest that, along with other members of the CAP superfamily and several other proteins, GliPR1L1 is involved in the binding of sperm to the oocyte complex. Results strengthen the role of CAP domain-containing proteins in cellular adhesion and propose a mechanism whereby CAP proteins show overlapping functional significance during fertilization (Gibbs et al. 2010).

41.8.2.2 C9orf19

A human transcript, C9orf19, mapped to the genomic region involved in hereditary inclusion body myopathy (IBM2) at chromosome 9p12-p13, has been characterized. Genomic characterization of the C9orf19 gene identified five exons extending over 27.2 kb of genomic DNA, located 12 kb centromeric to the tumor suppressor RECK gene. C9orf19 mRNA is expressed in a wide range of adult tissues as a single transcript, most abundantly in lung and peripheral blood leukocytes. The predicted protein contains SCP-like extracellular protein signature classified to IPR001283, a family of evolutionary related proteins with extracellular domains, which includes the human GliPR, the human testis specific glycoprotein (TPX-1), and several other extracellular proteins from rodents (SCP), insects venom allergens (Ag5, Ag3), plants pathogenesis proteins (PR-1) and yeast hypothetical proteins (Eisenberg et al. 2002).

41.8.2.3 A 28 kDa Glycoprotein from Human Neutrophils

A 28 kDa glycoprotein has been purified from exocytosed material from human neutrophils. The deduced 245 amino acid sequence of the 2124 bp full-length cDNA showed high degree of similarity to the deduced sequences of human gene TPX-1 and of sperm-coating glycoprotein from rat and mouse. Subcellular fractionation of human neutrophils indicated that the protein is localized in specific granules, hence named SGP28 (specific granule protein of 28 kDa) (Kjeldsen et al. 1996).

41.9 Calx-*b* and CTLD Containing Protein (CBCP) (Group XVII of CTLD)

41.9.1 CBCP/Frem1/QBRICK

CBCP/Frem1/QBRICK large proteoglycan (~2,100 residues) contains a set of chondroitin sulphate proteoglycan (CSPG) repeats (homologous to the NG2 ectodomain) (Nishiyama et al. 1991), a calcium-binding Calx-β domain and a CTLD. The emerging family of extracellular matrix proteins characterized by 12 consecutive CSPG repeats and the presence of Calx-β motif(s) includes Fras1, QBRICK/Frem1, Frem2 and Frem3. Frem1 belongs to a family of structurally related extracellular matrix proteins of which Fras1 is the founding member. Fras1/Frem proteins have been shown to be strictly co-localized in the sublamina densa of mouse epithelial basement membranes during development. Frem3 was present in a broad range of epithelial basement membranes where Fras1, Frem1 and Frem2 were missing. Fras1 and Frem2 were colocalized with Frem3 in the basement membrane of certain skin parts, underlying the thin-layer, of rapidly proliferating keratinocytes, whereas Frem1 was detected only in the basement membrane of the tail (Pavlakis et al. 2008).

41.9.1.1 Fras1 and Frem1 in Sublamina Densa of Epithelial Basement Membranes

Fras1 co-localizes with the markers of epithelial basement membranes and is ultrastructurally detected underneath the lamina densa of embryonic mouse epithelia. The loss of Fras1 mainly affects the cohesiveness of the embryonic skin basement membrane with its underlying mesenchyme. Fras1 could serve as a direct link between the sublamina densa and mesenchyme. The localization of Fras1 is consistent with previous results indicating that Fras1 exerts its function below the lamina densa and that Fras1 displays the same localization pattern in all epithelial basement membranes (Dalezios et al. 2007). It was shown that basement membrane levels of collagen VII rise at late

embryonic life, concomitant with descending Fras1/Frem immunolabeling (Chiotaki et al. 2007).

Mutations in Fras1 and Frem1 have been identified in mouse models for Fraser syndrome, which display a strikingly similar embryonic skin blistering phenotype due to impaired dermal-epidermal adhesion. Frem1 originates from both epithelial and mesenchymal cells, in contrast to Fras1 that is exclusively derived from epithelia. However, both proteins are localized in an absolutely overlapping fashion in diverse epithelial basement membranes. At the ultrastructural level, Frem1 exhibits a clustered arrangement in the sublamina densa coinciding with fibrillar structures reminiscent of anchoring fibrils. Furthermore, in addition to its extracellular deposition, around E16, Frem1 displays an intracellular distribution in distinct epidermal cell types such as the periderm layer and basal keratinocytes. Since periderm cells are known to participate in temporary epithelial fusions like embryonic eyelid closure, defective function of Frem1 in these cells could provide a molecular explanation for the "eyes open at birth" phenotype, a feature unique for Frem1 deficient mouse mutants. Finally, Frem1 localization in the basement membrane is lost but not in periderm cells in the skin of Fras1$^{-/-}$ embryos. Reports indicate that besides a cooperative function with Fras1 in embryonic basement membranes, Frem1 can also act independently in processes related to epidermal differentiation (Petrou et al. 2007a).

FREM1 Mutations Cause Bifid Nose, Renal Agenesis, and Anorectal Malformations Syndrome

An autosomal-recessive syndrome of bifid nose and anorectal and renal anomalies (BNAR) was reported in a consanguineous Egyptian sibship. Alazami et al. (2009) identified a shared region of homozygosity on chromosome 9p22.2-p23 that revealed homozygous frameshift and missense mutations in FREM1, which encodes an extracellular matrix component of basement membranes. The phenotypic variability reported for different Frem1 mouse mutants suggests that the apparently distinct phenotype of BNAR in humans may represent an unrecognized variant of Fraser syndrome (Alazami et al. 2009).

41.9.1.2 Association of Fras1/Frem Family with Fraser Syndrome

Fras1 and the structurally related proteins are involved in the structural adhesion of the skin epithelium to its underlying mesenchyme. Deficiency in the individual murine Fras1/Frem genes gives rise to the bleb phenotype, which is equivalent to the human hereditary disorder Fraser syndrome, characterized by cryptophthalmos (hidden eyes), embryonic skin blistering, renal agenesis, and syndactyly. Recent studies revealed a functional cooperation between the Fras1/Frem gene products, in which Fras1, Frem1 and Frem2 are simultaneously stabilized at the lower most region of the basement membrane by forming a macromolecular ternary complex. Loss of any of these proteins results in the collapse of the protein assembly, thus providing a molecular explanation for the highly similar phenotypic defects displayed by the respective mutant mice. Petrou et al. (2008) reviewed the current knowledge regarding the structure, function, and interplay between the proteins of the Fras1/Frem family and proposed a possible scenario for the evolution of the corresponding genes.

QBRICK (Frem 1) in Hair Morphogenesis

During mouse hair morphogenesis, gene predominantly expressed at the tip of developing hair follicles encodes a protein characterized by the presence of 12 tandem repeats of ~120 amino acids and a novel N-terminal domain containing an Arg-Gly-Asp cell-adhesive motif. The protein encoded by this gene, named QBRICK, was localized at the basement membrane zone of embryonic epidermis and hair follicles, in which it was more enriched at the tip rather than the stalk region. QBRICK was active in mediating cell-substratum adhesion through integrins containing α_v or α_8 chain, but not integrin $\alpha_5\beta_1$. QBRICK colocalized with α_v-containing integrins in the interfollicular region, but with the α_8-containing integrin at the tip region of developing hair follicles. These results indicate that QBRICK is an adhesive ligand of basement membrane distinctively recognized by cells in the embryonic skin and hair follicles through different types of integrins directed to the Arg-Gly-Asp motif (Kiyozumi et al. 2005).

41.9.1.3 Genetics of Frem1 in Fraser Syndrome and Blebs Mouse Mutants

Fraser syndrome is a recessive multi-system disorder characterized by embryonic epidermal blistering, cryptophthalmos, syndactyly, renal defects and a range of other developmental abnormalities. The family of four mapped mouse blebs mutants has been proposed as models of this disorder, given their striking phenotypic overlaps. These loci have been cloned, uncovering a family of three large extracellular matrix proteins and an intracellular adapter protein which are required for normal epidermal adhesion early in development. These proteins have been shown to play a crucial role in the development and homeostasis of the kidney. The cloning and characterization of these genes and the consequences of their loss have been explored (Smyth et al. 2004). Smyth et al. (2004) reported the mutations in Frem1 in both the classic head blebs mutant and in an N-ethyl-N-nitrosourea-induced allele and showed that inactivation of the gene results in the formation of in utero epidermal blisters beneath the lamina densa of the basement membrane and also in renal agenesis. Frem1 is expressed widely in the developing embryo in regions of epithelial/mesenchymal interaction and epidermal

remodeling. Furthermore, Frem1 appears to act as a dermal mediator of basement membrane adhesion, apparently independently of the other known "blebs" proteins Fras1 and GRIP1. Since the collagen VI and Fras1 deposition in the basement membrane was normal, it indicated that the protein plays an independent role in epidermal differentiation and is required for epidermal adhesion during embryonic development (Smyth et al. 2004).

Jadeja et al. (2005) mapped myelencephalic blebs to Frem2, a gene related to Fras1 and Frem1, and showed that a Frem2 gene-trap mutation was allelic to myelencephalic blebs. Expression of Frem2 in adult kidneys correlated with cyst formation in myelencephalic blebs homozygotes, indicating that the gene is required for maintaining the differentiated state of renal epithelia. Two individuals with Fraser syndrome were homozygous with respect to the same missense mutation of FREM2, confirming genetic heterogeneity. This is the only missense mutation reported in any blebbing mutant or individual with Fraser syndrome, suggesting that calcium binding in the CALXβ-cadherin motif is important for normal functioning of FREM2.

In Frem2 mutant mice, not only Frem2 but Fras1 and QBRICK/Frem1 were depleted from the basement membrane zone. This coordinated reduction in basement membrane deposition was also observed in another Fraser syndrome model mouse, in which GRIP1, a Fras1- and Frem2-interacting adaptor protein, is primarily affected. Targeted disruption of Qbrick/Frem1 also resulted in diminished expression of Fras1 and Frem2 at the epidermal basement membrane, confirming the reciprocal stabilization of QBRICK/Frem1, Fras1, and Frem2 in this location. These proteins formed a ternary complex, raising the possibility that their reciprocal stabilization at the basement membrane is due to complex formation. The coordinated assembly of three Fraser syndrome-associated proteins at the basement membrane appears to be instrumental in epidermal-dermal interactions during morphogenetic processes (Kiyozumi et al. 2006, 2007).

41.9.2 Frem3

The Fraser syndrome protein Fras1 and the structurally related proteins Frem1, Frem2 and Frem3 comprise a novel family of extracellular matrix proteins implicated in the structural adhesion of the embryonic epidermis to the underlying mesenchyme. Fras1, Frem1 and Frem2 have been shown to be simultaneously and interdependently stabilized in the basement membrane by forming a ternary complex located underneath the lamina densa. Although Fras1, QBRICK/Frem1 and Frem2 have been shown to localize at the basement membrane through reciprocal stabilization, Frem3 localized at the basement membrane with tissue distribution patterns clearly distinct from those of other 12 CSPG repeats-containing proteins (12-CSPG proteins). In adult mice, Frem3 was present at the basement membrane underlying ductal cells of the salivary gland, retinal ganglion cells, basal cells of epidermis and hair follicles, where other 12-CSPG proteins were barely expressed. Frem3 is distinct from other 12-CSPG proteins in its tissue distribution and competence to assemble into the basement membrane (Kiyozumi et al. 2007). In absence of Fras1 the basement membrane localization of Frem3 remains unaffected in contrast to Frem1 and Frem2 which are completely abolished from the basement membrane (Petrou et al. 2007b).

41.9.3 Zebrafish Orthologues of FRAS1, FREM1, or FREM2

Using forward genetics, Carney et al. (2010) identified the genes mutated in two classes of zebrafish fin mutants. The mutants of these genes display characteristic blistering underneath the basement membrane of the fin epidermis. Three of them are due to mutations in zebrafish orthologues of FRAS1, FREM1, or FREM2, large basement membrane protein encoding genes that are mutated in mouse bleb mutants and in human patients suffering from Fraser Syndrome. In addition to mutations in Hemicentin1 (Hmcn1) Carney et al. (2010) identified the extracellular matrix protein Fibrillin2 as an indispensable interaction partner of Hmcn1 (Carney et al. 2010).

References

CD93/gC1qR/Thrombomodulin/Endosialin

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Part XIV

Clinical Significance of Animal Lectins

MBL Deficiency as Risk of Infection and Autoimmunity

42

Anita Gupta

42.1 Pathogen Recognition

In pathogen recognition by C-type lectins, several levels of complexity can be distinguished; these might modulate the immune response in different ways. Firstly, the pathogen-associated molecular pattern repertoire expressed at the microbial surface determines the interactions with specific receptors (Fig. 42.1). Secondly, each immune cell type possesses a specific set of pathogen-recognition receptors. Thirdly, changes in the cell-surface distribution of C-type lectins regulate carbohydrate binding by modulating receptor affinity for different ligands. Crosstalk between these receptors results in a network of multimolecular complexes, adding a further level of complexity in pathogen recognition (Cambi and Figdor 2005; Jack et al. 2001; Thiel et al. 2006) (see Chap. 23). MBL deficiency is genetically determined and predisposes to recurrent infections and chronic inflammatory diseases. MBL deficiency has been implicated in susceptibility and course of viral, bacterial, fungal, and protozoan infection. More than 10% of the general population may, depending on definition, be classified as MBL deficient, underlining the redundancy of the immune system. MBL-disease association studies have been a fruitful area of research, which implicates a role for MBL in infective, inflammatory and autoimmune disease processes. MBL deficiency predisposes both to infection by extra-cellular pathogens and to autoimmune disease.

42.1.1 MBL Characteristics

Mannose-binding lectin is a C-type serum lectin and is primarily produced by the liver (Bouwman et al. 2005) in response to infection, and is part of many other factors termed "http://en.wikipedia.org/wiki/Acute_phase_protein" \o "Acute phase protein" acute phase proteins. MBL is made up of 96-kDa structural units, which in turn are composed of three identical 32-kDa primary subunits. The subunits consist of an N-terminal cross-linking region, a collagenlike domain, and a C-terminal carbohydrate-recognition domain (CRD) (Chap. 23; Turner and Hamvas 2000). Circulating MBL is composed of higher-order oligomeric structures, which include dimers, trimers, tetramers, pentamers, and hexamers of the structural homotrimeric unit. The oligomeric configuration of the structural units allows the MBL molecule to have multiple CRDs, facilitating multivalent ligand binding. Each CRD of MBL is structurally identical and is able to bind a range of oligosaccharides including N-acetylglucosamine D-mannose, Nacetylmannosamine, and L-fucose (Turner 1996). Although the various sugars are bound with different affinities, the cluster-like array of multiple binding sites allows activation of complement to be most effective. MBL is considered to play a major role in innate defense against pathogens, involving recognition of arrays of MBLbinding carbohydrates on microbial surfaces. However, recent studies have shown that MBL is also involved in the recognition of self-targets, such as apoptotic and necrotic cells (Nauta et al. 2003). In plasma, MBL is associated with MBL-associated serine proteases (MASPs). Currently, three MASPs have been identified, MASP-1, MASP-2, and MASP-3 (Chap. 23).

42.1.2 Pathogen Recognition and Role in Innate Immunity

MBL belongs to the class of collectins in the C-type lectin superfamily, whose function appears to be pattern recognition in the first line of defense in the pre-immune host. MBL recognizes carbohydrate patterns, found on the surface of a large number of pathogenic micro-organisms, including bacteria, viruses, protozoa and fungi. Mannose-binding lectin binds to the repeating sugar arrays on surfaces of pathogens through multiple lectin domains and, following binding, is able to activate the complement system via an associated serum protease, MASP-2. Importantly, MBL activates the complement system through a distinctive third pathway, independent of

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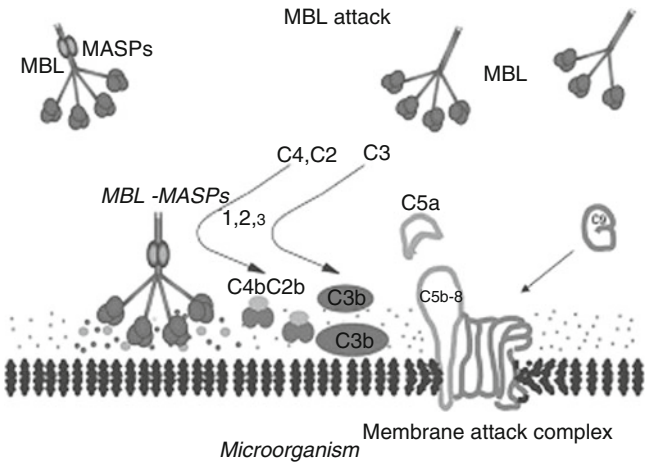

Fig. 42.1 *MBL-mediated complement attack*. Mannose-binding lectin (MBL) complexed with the MASPs binds to sugar arrays on a microorganism and mediates a complement attack through MASP2. MASPs denote MBL-associated serine proteases (Reprinted by permission from Macmillan Publishers Ltd: Genes Immun. Garred et al. © 2006). Mannan-binding lectin binds to patterns of carbohydrate groups in the correct spatial orientation. MBL is composed of two to six clusters of carbohydrate-binding lectin domains that interact with each other via a collagen-like domain (Chap. 23). Within each cluster are three separate binding sites that have a fixed orientation relative to each other; all three sites can therefore only bind when their ligands – mannose and fucose residues in bacterial cell-wall polysaccharides – have the appropriate spacing

Table 42.1 Some clinically relevant microorganisms recognized by MBL

Bacteria	Viruses
Staphylococcus aureus	HIV-1 and HIV-2
Staphylococcus pneumoniae	Herpes simplex-2
Staphylococcus pyrogenes	Influenza A
Enterococcus spp.	Hepatitis B virus
Listeria monocytogenes	Hepatitis C virus
Haemophilus influenzae	**Fungi**
Neisseria meningitidis	*Aspergillus fumigatus*
Neisseria gonorrhoeae	*Candida albicans*
Escherichia coli	*Cryptococcus neoformans*
Klebsiella spp.	*Saccharomyces cerevisiae*
Pseudomonas aeroginosae	**Protozoa**
Salmonella montevideo	*Plasmodium falciparum*
Salmonella typhimurium	*Cryptosporidium parvum*
H. pylori	Trypanosoma
Chlamydia trachomatis	
Chlamydia pneumonia	
Proprionibacterium acnes	
Mycobacterium avium	
Mycobacterium tuberculosis	
Mycobacterium leprae	
Leishmania chagasi	

both antibody and the C1 complex (Table 42.1). The MBL binds to neutral carbohydrates on microbial surfaces and recognises carbohydrates such as mannose, glucose, L-fucose, N-acetyl-mannosamine (ManNAc), and N-acetyl-glucosamine (GlcNAc). Oligomerisation of MBL enables high avidity binding to repetitive carbohydrate ligands, such as those present on microbial surfaces, including *E. coli*, *Klebisella aerogenes*, *Neisseria meningitides*, *Staphylococcus aureus*, *S. pneumonia*, *A. fumigatus and C. albicans* (c/r Kerrigan and Brown 2009). However, there is also a great variation in the binding of MBL to various organisms; *Candida albicans*, β-haemolytic group A *Streptococci* and *Staphylococcus aureus* bind with high affinity, while *Clostridium* sp. *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, b-haemolytic streptococci and *Streptococcus pneumoniae* exhibit low or no binding (Santos et al. 2001). It is also observed that some organisms (e.g. *Klebsiella* sp. and *Escherichia coli*) show a variable pattern of binding. Later, it was shown that the absence of sialic acid from the lipo-oligosaccharide (LOS) of *Neisseria meningitidis* serogroup B, serogroup C and *Neisseria gonorrhoeae* permits MBL binding and presence of sialic acid on LOS results in poor or no MBL binding. In a similar study on *Salmonella* sp, it was found that MBL binds to rough chemotype but exhibits low or no binding with smooth chemotype (Ambrosio and De Messias-Reason 2005). In order to activate the complement system after MBL binds to its target (for example, mannose on the surface of a bacterium), the MASP protein functions to cleave the blood protein C4 into C4a and C4b. The C4b fragments can then bind to the surface of the bacterium, and initiate the formation of a C3 convertase. The subsequent complement cascade, catalyzed by C3 convertase, results in creating a membrane attack complex, which causes the lysis of the pathogen to which MBL is bound (Worthley et al. 2005, 2006) (Fig. 42.1). Being an important component of innate immunity, acting as an ante-antibody and/or as a disease modifier, MBL is thought to influence disorders as diverse as meningococcal disease, rheumatoid arthritis, cystic fibrosis and recurrent miscarriage. Vulvovaginal candidiasis is a yeast infection of vulva and vagina; millions of women suffer from vulvovaginal candidiasis 5 worldwide. Women bearing MBL variant allele are at a higher risk for vulvovaginal candidiasis syndrome 343. The cervicovaginal lavage (CVL) MBL levels and gene mutation frequency were both higher in women suffering from vulvovaginal candidiasis than in controls (Liu et al. 2006b). On the other hand, MBL levels were low (0.30 ng/mL) in women with recurrent vulvovaginal candidiasis and were associated with a high gene mutation frequency compared to controls (1.28 ng/mL) (Ip and Lau 2004). Parenteral administration of MBL increased resistance

of mice to hematogenously disseminated candidiasis. Thus, MBL plays an important role in innate resistance to candidiasis, suggesting a protective role of lectin in female genital tract infection (Pellis et al. 2005).

The autologous function for MBL, perhaps, is to perform a regulatory role within the immune system. The MBL interacts with human peripheral blood cells such as B lymphocytes and natural killer cells. The MBL is capable of binding to differently glycosylated ligands on several autologous cell types via its carbohydrate-recognition domain. It was speculated that this could have functional significance at extravascular sites, but perhaps only in individuals possessing MBL genotypes conferring MBL sufficiency (Downing et al. 2003, 2005).

Nevertheless, MBL genotyping of various populations has led to the suggestion that there may be some biological advantage associated with absence of the protein. In addition, the protein also modulates disease severity, at least in part through a complex, dose-dependent influence on cytokine production. Moreover, there appears to be a genetic balance in which individuals generally benefit from high levels of the protein. These findings suggest that the concept of MBL as a protein involved solely in first line defense is an over-simplification and the protein should rather be viewed as having a range of activities including disease modulation (Turner and Hamvas 2000; Dommett et al. 2006; Worthley et al. 2006). The mechanisms and signaling pathways involved in such processes remain to be elucidated. Though the deficiency of MBL is associated with increased susceptibility to infections, Roos et al. (2004) indicated that antibody-mediated classical pathway activation can compensate for impaired target opsonization via MBL pathway in MBL-deficient individuals. Lack of MBL may be most relevant in the context of a co-existing secondary immune deficiency. Replacement therapy appears promising. The development of a recombinant product should permit the extension of MBL therapy to randomized clinical trials of sufficient size to provide clear evidence about the physiological significance of this intriguing glycoprotein. The MBL has attracted great interest as a potential candidate for passive immunotherapy to prevent infection (Gupta et al. 2008).

42.2 MBL Deficiency as Risk of Infection and Autoimmunity

42.2.1 MBL Deficiency and Genotyping

The single point mutations in exon 1 of human *MBL-2* gene appear to impair the generation of functional oligomers leading to the secondary structural abnormalities of the collagenous triple helix and a failure to form biologically functional higher order oligomers. Such deficiencies of the functional protein are common in certain populations, e.g. in sub-Saharan Africa, but virtually absent in others, e.g. indigenous Australians. There is an increased incidence of infections in individuals with such mutations and an association with the autoimmune disorders such as SLE and rheumatoid arthritis. Thus, the MBL is a potential candidate for passive immunotherapy to prevent infection.

42.2.1.1 Polymorphism in MBL Gene Is Associated in Exon 1 at Codon 52, 54, and 57

The concentration of MBL in plasma is determined genetically, primarily by the genetic polymorphism of the first exon of the structural gene and promoter region. The gene encoding MBL, *MBL2* (*MBL1* is a pseudogene), is located on long arm chromosome 10q11.2–q21 and contains four exons. A number of single nucleotide polymorphisms (SNPs) have been characterized in the gene. Exon 1 harbours three missense SNPs, giving rise to amino acid exchanges in the first part of the collagenous region. Two of these SNPs: $Gly^{54}Asp$, named 'B' and $Gly^{57}Glu$, named 'C' exchange glycine with an acetic amino acid. The third ($Arg^{52}Cys$, 'D') introduces a cysteine in the collagen region (the residue numbers includes the leader sequence of 20 residues) (Fig. 42.2). The wild type is denoted 'A'. Heterozygous individuals for D, B, and C mutations have a substantial decrease in MBL serum concentration, whereas MBL is undetectable in the serum of homozygous individuals. Three structural mutations within exon 1 at codons 52, 54, and 57 have been invariably referred to as the D, B and C variants. In addition to these three variant structural alleles, the promoter region also shows a number of SNPs as well, some of which influences the expression of MBL. The three SNPs in the coding region of the MBL2 gene those are associated with abnormal polymerization of the MBL molecule, decreased serum concentrations of MBL and strongly impaired function. These MBL SNPs are associated with increased susceptibility to infections, especially in immune-compromised persons, as well as with accelerated progression of chronic diseases. Normal serum levels of MBL range from 800 to 1,000 ng/mL in healthy Caucasians, however, wide variations can occur due to point mutations in codons 52, 54 and 57 of exon 1 and in the promotor region of the MBL gene (Turner 2003). Separate point mutations in the collagenous domain of human MBL are associated with immune-deficiency, caused by reduced complement activation by the variant MBLs as well as by lower serum MBL concentrations.

MBL deficiency with B mutation is associated with 26% of Caucasian populations (Steffensen et al. 2000). In a cohort of 236 Australian blood donors, 5 MBL promoter and coding SNPs were genotyped. Significant associations were found between both coding and promoter polymorphisms and MBL antigenic and functional levels (Minchinton et al. 2002). Point mutations in exon 1 at codons 52, 54 and 57

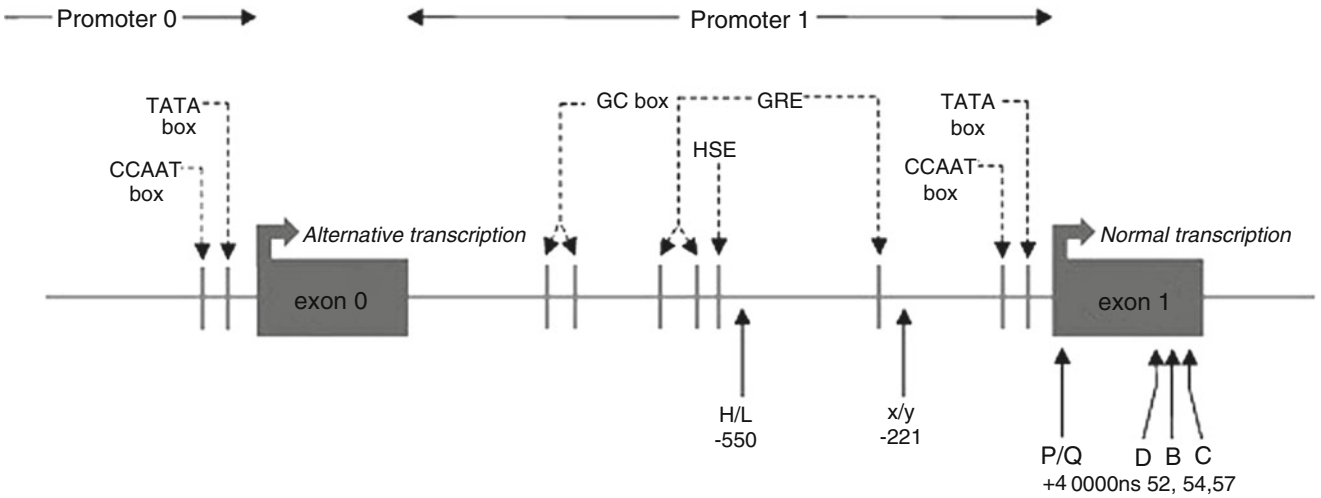

Fig. 42.2 *MBL2 polymorphisms*. Two promoters, promoter 1 and promoter 0 regulate the transcription of the human MBL2 gene. Similar to promoter 1 promoter 0 also includes a TATA box for transcription initiation, and transcription factor binding DNA sequences. Six DNA polymorphisms in the MBL2 gene are known to be associated with variation in quantity and/or function of MBL in serum. Three variants affect the expression of the MBL2 gene. They are localized in the promoter 1 (position −550, H/L variant and −221, X/Y variant) and in the 5′-untranslated region (position þ4, P/Q variant) of the MBL2 gene. Three base substitutions in exon 1 in codons 52 (D), 54 (B) and 57 (C) result in amino-acid changes (arginine to cysteine, glycine to aspartic acid and glycine to glutamic acid, respectively) and decreased level and function of MBL. The normal allele is named A proteases (Reprinted by permission from Macmillan Publishers Ltd: Genes Immun. Garred et al. © 2006)

and a promoter polymorphism at −221 bp of MBL gene were associated with increased susceptibility to various infectious diseases (Steffensen et al. 2003; Roos et al. 2006). The codon 54 mutation was frequent in both a British Caucasian and a Hong Kong Chinese population. The replacement of glycine-54 with an aspartic acid residue disrupts the fifth Gly-Xaa-Yaa repeat in the collagen-like domain of each 32 kDa MBP peptide chain and prevents the formation of the normal triple helix (Sumiya et al. 1991). Super et al. (1992) suggested that this genotype occurs in 5% of the population and encodes a functional protein. Super et al. (1992) also suggested that the $Gly^{54}Asp$ allele does not account for a deficiency state, but instead suggested that MBP may have two predominant allelic forms that have overlapping function and differ only in their ability to activate the classical pathway of complement.

Of 123 healthy Danish individuals investigated, 93 were homozygous (743.6%) for GGC, 28 heterozygous (22.8%), and 2 homozygous for GAC (1.6%). The gene frequency of the GAC allele was found to be 0.13. DNA sequencing of the cloned exon 1 from one GAC homozygous individual revealed no other substitution. The median MBP concentration in the group containing the GAC allele was 43.4 times lower than in the GGC homozygous group. However, the range of MBP in plasma was wide and overlapping between the groups. MBP protein was detected in both the GAC homozygotes. This study suggested that the GAC allele is able to produce a functional MBP protein which may be detected in serum at low concentrations (Garred et al. 1992).

The point mutation (GGA to GAA) involving codon 57 of exon 1 has been reported in Gambians from West Africa. In the Gambians the codon 57 mutation was remarkably common whereas the codon 54 mutation was very rare. In contrast, the codon 54 mutation was frequent in both the British Caucasian and the Hong Kong Chinese population. It was predicted that both homozygous and heterozygous individuals would have profoundly reduced serum levels of the protein and this was confirmed by immunoassay complement activation through lectin pathway. These two mutations have arisen independently because of the divergence of African and non-African populations.

Codon 52 Polymorphism Increases Risk of Premature Delivery: MBL2 gene polymorphisms are associated with an increased risk of neonatal infections. A relation between the maternal *MBL2* genotype and the risk of premature delivery has been indicated. Bodamer et al. (2006) suggested that the frequency of the codon 52 polymorphism was higher in the pre-term group compared to the term group (10.8% vs. 4.9%), while the frequency of codon 54 polymorphism was equal in both groups (11.3% vs. 11.8%). Data suggest that the fetal MBL2 genotype might be an additional genetic factor contributing to the risk of premature delivery.

SNPs at Codon 54 in MBL Are Associated with Increased Prevalence of Respiratory Infection: The SNPs of the innate immunity receptors CD14, MBL, and Toll-like receptor-2 with clinical phenotype in critically ill patients with systemic

inflammatory response syndrome are associated with increased prevalence of positive bacterial cultures and sepsis but not with altered prevalence of septic shock or decreased 28-day survival. Furthermore, CD14 SNPs were associated with Gram-negative bacteria and Toll-like receptor-2 with Gram-positive bacteria, whereas MBL was not associated with a particular organism class. The prevalence of the codon 54 mutation of the MBL gene in patients having repeated respiratory infections as well as the prevalence of the MBL mutant genotype among patients with diffuse panbronchiolitis was further supported (Gomi et al. 2004).

MBL Gene Polymorphisms in Gestational Diabetes Mellitus: Insulin resistance is a feature of gestational diabetes mellitus (GDM). A genetic predisposition to a pro-inflammatory state could favor the appearance of GDM during pregnancy. An association has been found between G54D and GDM. GDM patients carrying the G54D mutation require insulin therapy more frequently and have heavier infants than GDM women homozygous for the wild-type allele. An inverse correlation in GDM patients between neonatal weight and plasma MBL levels has been reported. Thus, pregnant women bearing the G54D MBL allele have a greater risk for developing GDM and having heavier infants (Megia et al. 2004).

MBL Genotypes in Acute Lymphoblastic Leukemia: Epidemiological studies show that acute lymphoblastic leukemia (ALL) can be induced by interactions between the immune system and early childhood infections. Since, certain types of childhood acute lymphoblastic leukemia develop as a multiple step process involving both pre- and postnatal genetic events, the MBL may play a critical role in the immune response in early childhood before specific immune protection develops. Schmiegelow et al. (2002) indicated that low-level MBL genotypes is associated with an increased risk of childhood ALL, particularly with early age at onset. Childhood ALL may often be initiated in utero. The prenatal origin of childhood leukemia was ascertened in children with B-precursor acute lymphoblastic leukemia carrying the chromosomal translocation t(12;21), the most common subtype of all childhood ALL. Study provided evidence that the development of t(12;21) B-precursor ALL may be initiated in utero. However, age at leukemia may be inversely correlated with the burden of cells with leukemia clonal markers, i.e. leukemia predisposed cells at birth (Hjalgrim et al. 2002).

MBL Genotypes in Viral Hepatitis: The prevalence of mutations in MBL gene was assessed in patients of hepatitis B. A mutation in codon 52 of the MBP gene was present in two (11%) of 19 Caucasian patients with acute hepatitis B and nine (27%) of 33 Caucasian patients with chronic hepatitis B, compared with four (4%) of 98 Caucasian controls. By contrast the prevalence of the mutation was similar in Asian patients with chronic hepatitis B and in Asian controls (one [5%] of 20 vs. two [2%] of 117). Mutations in codon 54 and codon 57 were found in similar proportions of patients and controls. These findings showed in Caucasian, but not Asian, patients an association of the codon 52 mutation of the MBL gene with persistent hepatitis B virus (HBV) infection. They suggest an important role for this gene, or a gene in linkage disequilibrium with MBL, in determining outcome after HBV infection in adult but not neonatal life (Thomas et al. 1996).

Bellamy et al. (1998) investigated the association between variant MBP alleles and malaria, tuberculosis, and HBV in adults and children in Gambia. Of the 2,041 Gambians screened for MBP mutations, 944 (46%) were homozygous for the wild-type allele, 922 (45%) were carriers of a single variant allele, and 175 (8.6%) possessed 2 mutant alleles. The most common mutation in Africans – the codon 57 variant allele – was weakly associated with resistance to tuberculosis in both patients and controls. Although MBP deficiency may predispose to recurrent infections, this study failed to provide evidence that such a deficiency is a major risk factor for infectious diseases (Bellamy et al. 1998).

The hepatitis C virus (HCV) envelope glycoprotein E2 binds the DC-SIGN and the related liver endothelial cell lectin through high-mannose N-glycans. Several high-mannose N-glycans in a structurally defined cluster on E2 bind to several subunits of the oligomeric lectin CRD and represent a strategy by which HCV targets to and concentrates in the liver and infects dendritic cells (Lozach et al. 2003). To determine the relevance of MBL polymorphism to hepatitis C virus infection, Matsushita et al. (1998) determined the MBL genotypes in 159 hepatitis C virus-infected chronic hepatitis patients and 218 healthy controls in Japan by looking at 4 polymorphic loci: 2 (H/L and X/Y) within the promoter region and 2 (P/Q and A/B) within exon-1 of the *MBL* gene. It indicated that the MBL-related innate immune system plays an important role in elimination of hepatitis C virus during interferon therapy (Matsushita et al. 1998). Matsushita et al. (2001) demonstrated that the MBL genotype can be significantly associated with increased risk for to primary biliary cirrhosis (PBC), and that increased production of MBL plays a critical role in immunopathogenesis.

42.2.1.2 Loss of Carbohydrate Binding and MASP-2 Auto-Activation in Mutated MBL

The mutations Gly25→Asp and Gly^{28}→Glu disrupt the disulfide-bonding arrangement of the protein and cause at least a fivefold increase in the half-time of secretion of MBP compared with wild-type rat serum MBP. A similar phenotype, including a threefold increase in the half-time of

secretion, disruption of the disulfide bonding arrangement, and inefficient complement fixation, was observed when nearby glucosylgalactosyl hydroxylysine residues at positions 27 and 30 were replaced with arginine residues. The results suggest that defective secretion resulting from structural changes in the collagen-like domain is likely to be a contributory factor for MBP immunodeficiency (Heise et al. 2000).

To investigate the molecular defects associated with heterozygosity, rat serum MBP polypeptides (MBP-A: 56% identical in sequence to human MBP) and rat MBP polypeptides containing mutations associated with human immunodeficiency were co-expressed in mammalian expression system. The resulting proteins are secreted almost exclusively as heterooligomers that were defective in activating the complement cascade. Functional defects were caused by structural changes to the N-terminal collagenous and cysteine-rich domains of MBP, disrupting interactions with associated serine proteases. These mutations demonstrated how a SNP gives rise to the molecular defects that lead to the disease phenotype in heterozygous individuals (Wallis 2002). Wallis et al. (2005) analyzed the molecular and functional defects associated with two variant proteins of lectin pathway. Mutations $Gly^{25} \rightarrow Asp$ and $Gly^{28} \rightarrow Glu$ created comparable structural changes in rat MBL but the G28E variant activated complement >10-fold less efficiently than the G25D variant, which in turn had approximately sevenfold lower activity than wild-type MBL. Analysis of mutant MBL-MASP-2 complexes formed from recombinant proteins showed that reduced complement activation by both mutant MBLs was due to failure of activation of MASP-2 efficiently on binding to a mannan-coated surface. Disruption of MBL-MASP-2 interactions as well as to changes in oligomeric structure and reduced binding to carbohydrate ligands compared with wild-type MBL probably account for the intermediate phenotype of the G25D variant. However, carbohydrate binding and -MASP-2 activation are ostensibly completely decoupled in complexes assembled from the G28E mutant, such that the rate of MASP-2 activation is no greater than the basal rate of zymogen MASP-2 autoactivation. Analogous molecular defects in human MBL probably combine to create the mutant phenotypes of immunodeficient individuals (Wallis et al. 2005).

Since it is difficult to evaluate MBL in patients blood on the only basis of protein contents, or in combination with MBL genotyping due to possible association of altered oligomeric state of MBL, Dumestre-Perard et al. (2002) purified MBL from human plasma and showed the presence of MBL in two different oligomeric forms. Data on the specific activity of these forms showed that the higher oligomeric forms of MBL had the ability to induce C4 cleavage more efficiently than the corresponding lower oligomers (Dumestre-Perard et al. 2002).

How to Define Abnormal MBL Pathway and Disease Associations: Although, the association between MBL deficiency and risk of infection with other common diseases and death during years of follow-up has established the role of MBL in innate immune system, yet, in a large ethnically homogeneous Caucasian population, there was no evidence for significant differences in infectious disease or mortality in MBL-deficient individuals versus controls and suggested that MBL deficiency is not a major risk factor for morbidity or death in the adult Caucasian population (Dahl et al. 2004; Eisen and Minchinton 2003; Kilpatrick 2002; Summerfield et al. 1997). While addressing possible correlation between MBL levels and clinical conditions an issue is how to define MBL deficiency. The physiologically relevant MBL level leading to clinical manifestations is likely to differ in different diseases. In the examples given below, a number of different levels have been used as cut-off values defining MBL deficiency. Judged from clinical trials it appears that at least 200 ng MBL/mL plasma is needed for reconstituting in vitro functional activity (C4b deposition) after MBL infusion in MBL deficient individuals (Valdimarsson et al. 2004). On the other hand, in leukaemic patients a cut-off level of 500 ng/mL or more has been suggested (Peterslund et al. 2001; Neth et al. 2001), and in the cases of obstetric problems even lower levels (100 ng/mL) have been indicated.

42.2.2 MBL and Viral Infections

42.2.2.1 MBL and HIV Interaction

A broad range of proteins binds high-mannose carbohydrates found on the surface of the envelope protein gp120 of the HIV and thus interfere with the viral life cycle, providing a new method of controlling HIV infection. While glycosylation of HIV gp120/gp41 provides a formidable barrier for development of strong antibody responses to the virus, it also provides a potential site of attack by the innate immune system through MBL/MBP. The MBL that binds to HIV depends on the high-mannose glycans present on gp120 while host cell glycans incorporated into virions do not contribute substantially to this interaction. The MBL has been shown to interact with all tested HIV strains. However, drugs that alter processing of carbohydrates enhance neutralization of HIV primary isolates by MBL. Complement activation on gp120 and opsonization of HIV due to MBL binding have also been observed but these immune mechanisms have not been studied in detail. MBL has also been shown to block the interaction between HIV and DC-SIGN. Clinical studies show that levels of MBL, an acute-phase protein, increase during HIV disease. Because of apparently universal reactivity with HIV strains, MBL clearly represents an important mechanism for recognition

of HIV by the immune system. However, further studies are needed to define the in vivo contribution of MBL to clearance and destruction of HIV (Botos and Wlodawer 2005; Ji et al. 2005). MBL that binds to high mannose glycans on HIV-1 (gp120), has been shown to neutralize the cell line-adapted strain HIV(IIIB). But HIV primary isolates (PI) are generally more resistant to neutralization by antibodies. Considering that PI are produced in primary cells that could alter the number of high mannose glycans on HIV relative to cell lines, Ying et al. (2004) showed that both PI and cell line-adapted HIV, despite binding of MBL, are relatively resistant to neutralization by levels of MBL normally present in serum. However, binding and opsonization of HIV by MBL may alter virus trafficking and viral-antigen presentation during HIV infection (Ying et al. 2004).

In search of the effect of MBL-2 polymorphisms on susceptibility and progression of HIV-1 infection in children, Dzwonek et al. (2006) observed MBL deficiency more frequently in patients with severe disease. The study suggested that MBL-2 variants may be less frequent in children classified as long-term non-progressors (LTNPs) and hence MBL analysis can be useful in identifying children with slow disease progression and, consequently, may not require immediate antiretroviral treatment (Dzwonek et al. 2006). Production of HIV in the presence of the mannosidase I inhibitor deoxymannojirimycin (dMM) significantly enhanced binding of HIV to MBL and increased MBL neutralization of an M-tropic HIV primary isolate. In contrast, HIV cultured in presence of alpha-glucosidase I and II inhibitors, castanospermine and deoxynojirimycin showed only slight effect on virus binding and neutralization by MBL. The study suggested that specific alterations of the N-linked carbohydrates on HIV gp120/gp41 can enhance MBL-mediated neutralization of virus by strengthening the interaction of HIV-1 with MBL (Hart et al. 2003).

42.2.2.2 Susceptibility to RSV, CoV and HTLV

Respiratory syncytial virus (RSV) is the most important microbiological cause of lower respiratory tract infection (LRTI) in infants. MBL deficiency is the most common immunodeficiency on the African Continent. MBL deficiency has an impact on the hospitalization for LRTI caused by RSV in infants from Soweto, South Africa. But in contrary to expectations, results suggested no association between low levels of MBL or carriage of variant alleles and LRTI caused by RSV (Kristensen et al. 2004).

Corona Virus Infection and Acute Respiratory Syndrome: Little is known about the innate immune response to severe acute respiratory syndrome (SARS) corona virus (CoV) infection. The MBL plays an important role in SARS-CoV infection. The distribution of MBL gene polymorphisms was significantly different between patients with SARS and normal subjects, with a higher frequency of haplotypes associated with low or deficient serum levels of MBL in patients with SARS. There was, however, no association between MBL genotypes, which are associated with low or deficient serum levels of MBL, and mortality related to SARS. MBL could bind SARS-CoV in calcium-dependent and mannan-inhibitable fashion in vitro, suggesting that binding is through the carbohydrate recognition domains of MBL. Furthermore, deposition of complement C4 on SARS-CoV was enhanced by MBL. Inhibition of the infectivity of SARS-CoV by MBL in fetal rhesus kidney cells suggested that MBL contributes to the first-line host defense against SARS-CoV and that MBL deficiency is a susceptibility factor for acquisition of SARS (Ip et al. 2005).

Susceptibility to T-cell Lymphotropic Virus: Pontes et al. (2005) investigated the association between MBL gene polymorphism and the susceptibility to human T-cell lymphotropic virus (HTLV) infection in 83 HTLV-infected asymptomatic subjects. Detection of MBL*A, MBL*B, and MBL*C was performed by amplifying a fragment of 349 bp (exon 1) and restriction fragment length polymorphism analysis with BanI and MboII endonucleases. A strong association has been demonstrated between MBL polymorphism and HTLV infection. Presence of genotype BB may be associated with the susceptibility to HTLV. Though further studies, with a larger number of individuals, are necessary, MBL polymorphism could have a possible impact on diseases associated with HTLV infection (Pontes et al. 2005).

42.3 Autoimmune and Inflammatory Diseases

Studies demonstrating the binding of MBL to the endothelium and causing excessive complement activation and subsequent tissue damage are known (Jordan et al. 2003), where as MBL deficiency may be advantageous in some circumstances since MBL may lead to an increased cytokine secretion by macrophages (Takahashi et al. 2002). There seems to be a delicate balance as to when MBL levels may be involved in harmful or in beneficial inflammation such as in the cardiovascular and other systems.

42.3.1 MBL Gene in Rheumatoid Arthritis

The etiology of autoimmune diseases is largely unknown. Studies on associations between MBL deficiency and rheumatoid arthritis (RA) have been discussed in reviews (Graudal 2004; Barton et al. 2004). Results depend on ethnic groups, type of

patients and the symptoms studied. Low levels of serum MBL are associated with a higher erythrocyte sedimentation rate (ESR), joint swelling score, limitation of joint motion score, and annual increase in radiographic destruction score. Despite this, indications are that low MBL levels may be linked with symptoms indicating a poor prognosis as well as an earlier debut. Whether variant alleles of the MBL gene causing low serum concentrations of MBL are associated with increased susceptibility to RA and erosive outcome in an inception cohort of patients with early polyarthritis was studied by Jacobsen et al. (2001). Jacobsen et al. (2001) suggested that MBL variant alleles appear to be weak susceptibility markers for RA, and patients with early polyarthritis and homozygous for MBL structural variant alleles have a higher risk of developing early erosive RA. These findings, together with the positive association between MBL variant alleles and the increased serum levels of IgM RF and CRP, point at the MBL gene as a relevant locus in the pathophysiology of RA. Graudal (2004) indicated that MBL-deficient patients have a relative risk of a severe radiographic event of 3.1 compared with the MBL competent group. The relative risk (RR) of early IL-1α auto-antibodies positive patients developing serious radiographic joint destruction was significantly lower than for IL-1α auto-antibodies-negative patients. Perhaps MBL and IL-1α auto-antibodies are predictors of prognosis of RA and play important roles in the pathogenesis of RA (Graudal 2004; Lee et al. 2005). Low levels of the protein have been related to a poor prognosis in rheumatoid arthritis perhaps due to the modulatory action that MBL exerts on the secretion of tumor necrosis factor α, a central molecule in the pathogenesis of rheumatoid arthritis (Graudal et al. 2000). Low MBL levels have also been associated with adult dermatomyositis and are probably related to a reduced clearance of apoptotic keratinocytes (Werth et al. 2002). Genotypes related to a lower production of MBL have also been linked to the development of systemic lupus erythematosus (Villarreal et al. 2001; Davies et al. 1995).

42.3.2 Systemic Lupus Erythematosus

Genetic factors play a major role in the development of SLE. More than 5% of cases are familial and the concordance rate between identical twins is 40%. Genetic studies in mice suggest a complex mechanism of transmission involving interactions among several susceptibility genes and, probably, protective genes. Genetic studies in humans have identified nearly 50 chromosomal areas possibly involved in lupus transmission. Significant linkage has been found for at least six regions, two on chromosome 1, one near the HLA region on chromosome 6, and three on chromosomes 2, 4, and 16, respectively. The genetic polymorphism of cytokines and, perhaps, of the T-cell receptor (TCR) may contribute to deregulate lymphocyte activity. The polymorphism of the Fc receptors of immunoglobulins may affect immune complex clearance, thereby promoting tissue damage. Further genetic studies are needed to enrich the fund of knowledge on lupus and to identify new targets for treatment (Perdriger et al. 2003).

From the several investigations on MBL and SLE the consensus is emerging that low levels of MBL predisposes to development of the disease. However, the connection is not like for C1q where SLE develops in almost all of the rare cases of deficiency. Rather, it seems that MBL deficiency aggravates the disease or the development (Ohlenschlaeger et al. 2004; Takahashi et al. 2005). In SLE patients, MBL deficiency increase the risk for respiratory tract infections (Garred et al. 2001; Takahashi et al. 2005) as well as the risk of developing arterial thromboses (Ohlenschlaeger et al. 2004).

As with infectious disease, there is some evidence that the risk of pathology increases if there is another co-existing immune defect. For example, in a cohort of Spanish patients, the odds ratio for developing SLE was 2.4 for individuals with MBL deficiency, but this increased to 3.2 when there was also a co-existing partial C4 deficiency (Davies et al. 1997). Studies in patients with SLE have reported that MBL deficiency also influences their risk of developing certain complications, which include arterial thromboses (Ohlenschlaeger et al. 2004) and respiratory tract infections (Garred et al. 2001; Takahashi et al. 2005).

42.3.2.1 MBL Polymorphisms in SLE

Whether dysfunctional or deficient MBP variants are found with increased frequency in black patients with SLE was determined (Sullivan et al. 1996). Two structural polymorphisms of MBP, associated with low serum levels of MBP, were found with significantly increased frequency in the SLE patient population. In contrast, a promoter haplotype associated with particularly high serum levels of MBP was negatively associated with SLE. Thus, it seemed that deficiencies of MBP predispose individuals to SLE (Sullivan et al. 1996). The distribution of promoter variants of the MBL gene and correlations between the promoter variants and serum MBL concentrations in Chinese patients with SLE were investigated. Significant differences in the distribution of the two pairs of promoter polymorphisms, H/L and Y/X, between SLE patients and controls, were observed. Analysis of the correlation between promoter haplotypes and serum MBL levels revealed HY as the highest-producing, LY as the intermediate-producing, and LX as the lowest-producing haplotypes. The LX haplotype was present at a frequency of 0.259 in SLE patients and 0.154 in controls and was significantly associated with SLE. The low-producing promoter polymorphism of the MBL gene is associated with SLE, and a low serum MBL level is a risk factor for SLE (Ip et al. 1998). Whether occurrence, characteristics, and progression of SLE are associated with polymorphism of the MBL gene and with serum MBL concentration, Takahashi et al. (2005) reported that MBL gene

polymorphism influences susceptibility to SLE, but has no direct effect on disease characteristics. Serum MBL levels fluctuate during the course of SLE in individual patients. MBL genotyping may be useful in assessing the risk of infection during treatment of SLE (Takahashi et al. 2005). MBL and FcγRII (CD32) polymorphisms have both been implicated as candidate susceptibility genes in SLE. These patients carried MBL codon 54 mutant allele more frequently than controls and the haplotype HY W52 W54 W57 was significantly lower in cases compared with controls. The MBL gene codon 54 mutant allele appears to be a risk factor for SLE, whilst haplotypes encoding for high levels of MBL are protective against the disease. However, differences between controls and patients were not significant when FcγRIIa polymorphisms were considered (Villarreal et al. 2001).

42.3.2.2 Lectin Pathway in Murine Lupus Nephritis

In SLE, hypocomplementaemia and complement deposition have been described both in man and in experimental models. In mice, MBL is expressed in two forms, MBL-A and MBL-C. In young and old MRL-lpr and control MRL+/+, the declining levels of MBL-A and MBL-C showed a high degree of correlation. In aged MRL-lpr mice in which autoimmunity is most pronounced, high auto-Ab titers and strong deposition of glomerular immune complexes were associated with deposition of C1q, C3, MBL-A and MBL-C. Thus, in addition to the classical pathway and the alternative pathway, the lectin pathway of complement activation is also involved in murine lupus nephritis (Trouw et al. 2005). SLE patients were associated with a reduced functional activity of the MBL pathway of complement, in relation to expression of MBL variant alleles, increased levels of autoantibodies against cardiolipin and C1q, but not against MBL. The enhanced production of autoantibodies may be related to disturbed clearance of apoptotic material due to impaired MBL function (Seelen et al. 2005). Cohorts of MRL-lpr mice, which are known to develop age-dependent SLE-like disease showed that at 2 months of age all mice already had elevated levels of anti-C1q autoantibodies, and elution of kidneys confirmed the presence of these antibodies in renal immune deposits in MRL-lpr mice and not in control MRL+/+ mice. Thus, anti-C1q antibodies are already present in serum and immune deposits of the kidney early in life and therefore can play a role in nephritis during experimental SLE-like disease in mice (Trouw et al. 2004).

Effect of (S)-Armepavine on Autoimmune Disease of MRL/MpJ-lpr/lpr Mice: (S)-Armepavine (C19H23O3N; MW313) from *Nelumbo nucifera* suppresses T cells proliferation. (S)-armepavine prevents lymphadenopathy and elongated life span of MRL/MpJ-lpr/lpr mice, which have disease features similar to human SLE. The action seemed to be mediated by inhibition of splenocytes proliferation, suppression of IL-2, IL-4, IL-10, and IFN-γ gene expressions, reduction of glomerular hypercellularity and immune complexes deposition, and decrease of urinary protein and anti-double stranded DNA autoantibody production. It has been suggested that (S)-armepavine is an immunomodulator for the management of autoimmune diseases like SLE (Liu et al. 2006a).

42.3.2.3 Autoantibodies to C1q and MBL in SLE

In SLE patients, there is an association between the occurrence of autoantibodies to C1q and MBL and renal involvement. The presence of autoantibodies to MBL, analogous to autoantibodies to C1q in patients with SLE, contributes to the disease development. Anti-MBL autoantibodies were of the IgG isotype and the binding site of IgG anti-MBL was located in the F(ab')2 portion. Anti-MBL are present in sera from SLE patients and influence the functional activity of MBL (Seelen et al. 2003). More SLE patients have IgG anti-MBL antibodies than normal controls. However, in SLE, these antibodies are neither sensitive nor specific for this condition. They occur more frequently in (proliferative) lupus nephritis, particularly during active disease. Furthermore, levels of anti-C1q rise, in many cases, prior to a relapse of lupus nephritis, suggesting a pathogenic role for the autoantibodies in immune complex-mediated renal disease. In addition, anti-C1q may interfere with the clearance of apoptotic cells, so influencing induction and expression of autoimmunity (Kallenberg 2008; Mok et al. 2004).

Cardiovascular Disease and SLE: Cardiovascular disease is an important complication in patients with SLE. Variant alleles of the MBL gene are associated with SLE as well as with severe atherosclerosis. Ohlenschlaeger et al. (2004) determined whether *MBL* variant alleles were associated with an increased risk of arterial thrombosis among Danish patients with SLE and suggested that among patients with SLE, homozygosity for *MBL* variant alleles is associated with an increased risk of arterial thrombosis. The risk of venous thrombosis is not increased, indicating that MBL has a specific role in providing protection against arterial thrombosis (Ohlenschlaeger et al. 2004).

42.3.3 Systemic Inflammatory Response Syndrome/Sepsis

A systemic inflammatory response syndrome (SIRS) is well known in patients after major surgery (Bone 1992). Clinical studies of critically ill patients requiring intensive care management have shown that individuals who are MBL deficient are more likely to develop the SIRS and progress to septic

shock and death (Garred et al. 2003b; Fidler et al. 2004), findings which may well relate to the proinflammatory cytokine response. In some cases, SIRS occurs in response to infection and "sepsis" is then used to describe the symptoms. More severely a septic shock may develop with multi-organ dysfunctions (MOD). The MBL levels and genotypes were investigated in 272 adults (197 with sepsis) prospectively admitted to the ICU. No difference was seen between genotype frequencies in patients with SIRS as compared to healthy controls. But the frequency of MBL variant genotypes was significantly higher in patients with sepsis compared with the patients without sepsis, and the risk ratios for the development of "severe sepsis" and "septic shock" ranged from 1.3 to 3.2 times higher in patients with A/O or O/O versus A/A genotype. MBL levels were inversely related to the severity of sepsis (Garred et al. 2003a, b). The SNP of MBL with clinical phenotype in critically ill Caucasians with SIRS are associated with increased prevalence of positive bacterial cultures at admission to the ICU. Patients in low MBL haplotype group did not have significantly increased rates of sepsis or septic shock at admission to the ICU. Survival at day 28 did not differ significantly between the low MBL haplotype and high MBL haplotype groups. Furthermore, MBL was not associated with a particular organism class. The prevalence of the codon 54 mutation of MBL gene in patients having repeated respiratory infections as well as the prevalence of MBL mutant genotype among patients with diffuse panbronchiolitis was further supported (Gomi et al. 2004).

In contrast, the prevalence of the MBL mutant genotype among patients with nontuberculous mycobacteria or Aspergillus chronic infection was not different from that in control subjects. Thus, SNPs in innate immunity receptors may alter recognition and clearance of bacteria without changing outcomes of critically ill adults with systemic inflammatory response syndrome (Sutherland et al. 2005). Polymorphisms of both codon 54 allele and promoter variants of the mannose MBL gene in patients with primary Sjogren's syndrome (SS) was suggested as one of the genetic factors that determines susceptibility to SS (Wang et al. 2001).

Fidler et al. (2004) analyzed the MBL levels and genotypes levels in 100 critically ill children admitted to ICU. A sevenfold greater risk of developing SIRS within 48 h of admission (60% of the patients) was observed for those carrying MBL variant alleles than those with wild type alleles (A/O + O/O vs. A/A). A significant relation was also found between severity of the systemic response to infection and the presence of an MBL mutation. If the severity of illness among the patients admitted with infections was divided into localized infection, sepsis, and septic shock, the median MBL levels were inversely related to severity, and the children with MBL levels below 1,000 ng/mL had a greater chance of developing SIRS. A study of the frequency of sepsis in very low birth weight infants did not reveal statistical significance in clinical data between infants with and without specific mutations in a number of genes, including MBL genotypes (Ahrens et al. 2004). Following surgery of 156 patients undergoing major elective gastrointestinal surgery for malignant disease, Siassi et al. (2003) reported that patients who developed sepsis or SIRS showed significantly lower mean post-operative MBL levels. In colorectal cancer patients, postoperative infection is associated with poor prognosis. Ytting et al. (2005) have reported significantly increased frequency of pneumonia after primary operation in colorectal carcinoma patients who were having low MBL levels. MBL deficiency appears to play an important role in susceptibility of critical ill patients to the development and progression of sepsis and septic shock, and confers a substantially increased risk of fatal outcome. There is clearly a need for improvement in defining which patient groups and which clinical data are relevant to examine.

42.3.4 MBL and Inflammatory Bowel Diseases

Inflammatory bowel disease (IBD) is a pathological spectrum encompassing ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis. The resultant IBD phenotype is the consequence of multiple interactions between environmental factors, particularly enteric flora, and the host response to this environment, determined by immunogenetic, epithelial, and other non-immune genetic factors. MBL, as an important component of innate immunity, has engendered considerable research interest. In an early study of 340 unrelated patients with IBD genotyped for *MBL2* exon 1 coding mutations, the frequency of deficient alleles was significantly lower in patients with UC than either the control group, or those with CD (Rector et al. 2001). The study by Rector et al. (2001) suggests that MBL deficiency could be protective against UC; alternatively, it could be interpreted that MBL deficiency, in individuals otherwise predisposed to IBD, may skew the phenotype away from the UC spectrum of disease towards CD. This concept is supported by another study that genotyped *MBL2* in patients with CD, UC, or healthy controls (Seibold et al. 2004). The study also assessed anti-*Saccharomyces cerevisiae* antibody (ASCA) and MBL levels within the same subsets of patients, albeit slightly different numbers within each group. CD patients with MBL deficiency were significantly more likely to be positive for ASCA and for their lymphocytes to proliferate in response to mannan. Thus, it appears that MBL deficiency could impair normal processing of mannan-expressing microbial antigens, such as those found on the cell surface of many common microorganisms. The accumulated antigens could then stimulate the immune system, and contribute to the production of ASCA and possibly the pathogenesis of Crohn's disease. Thus, MBL deficiency

might act primarily to influence IBD-specific phenotype in these patients. It should be noted, however, that a follow-up study, testing a larger cohort of CD patients ($n = 241$), failed to confirm the significant association between variant MBL genotypes and ASCA positivity (Joossens et al. 2006). Seibold et al. (2004) suggested that the frequency of homozygous and compound heterozygous for variant exon 1 alleles differed significantly between patients suffering from CD or UC and the healthy controls.

42.3.4.1 Celiac Disease and MBL

Celiac disease is a multi-factorial/auto-immune disorder caused in genetically susceptible patients, by the ingestion of dietary gluten. Though very little is known about the genetic factors, but there is a strong association of two HLA haplotypes (DQ2 or α1*05, β1*02 and DQ8 or α1*0301, β1*0302) with the disease. Boniotto et al. (2002) indicated an association between celiac disease and the presence of variant MBL alleles. Boniotto et al. (2005) and Iltanen et al. (2003) reported the frequency of homozygosity for variant MBL alleles to be higher in the patients. Among 149 Italian celiac patients, 116 showed the presence of DQ2 or DQ8. MBL2 allele and genotype frequencies varied significantly between celiac patients and healthy humans. It is likely that in those rare cases of celiac disease that are negative for DQ2 and DQ8, the non-HLA susceptibility genotypes would exert a greater effect. The study also analyzed apoptosis within small intestinal biopsy specimens, and showed that MBL tended to aggregate to areas of apoptosis within the epithelium. MBL has been implicated in the normal clearance of apoptotic bodies (Nauta et al. 2003; Ogden et al. 2001). The authors postulated that the association between MBL and celiac disease, and indeed other autoimmune conditions, could relate to impaired apoptosis, whereby MBL deficiency impairs the normal removal and clearance of apoptotic cells that may subsequently reveal previously hidden self-antigen, causing loss of self-tolerance, and spreading of autoimmunity (Boniotto et al. 2005). The association between variant *MBL2* alleles and coeliac disease has also been confirmed within the Finnish population (Iltanen et al. 2003) (Worthley et al. 2006). The low MBL genotypes were strongly associated with more celiac disease symptoms as well with increased frequency of secondary autoimmune diseases. By immunohistochemistry MBL was found to be present, together with apoptotic cells, in the basal lamina under the intestinal epithelium, where they had previously found mRNA for MBL. Boniotto et al. suggested that impaired removal of apoptotic cells due to MBL deficiency might predispose to the development of autoimmune symptoms. Mice lacking MBL have been shown to be less efficient in removal of apoptotic cells (Stuart et al. 2005). In vitro studies have also implicated MBL in removal of apoptotic cells (Ogden et al. 2001; Nauta et al. 2004). Alternative explanation could be that increased susceptibility to intestinal infections and diarrhea, associated with low MBL, may change the intestinal epithelia thus allowing for abnormal stimulation of anti-gliadin immune responses and triggering of the cascade leading to celiac disease. A role for MBL like in celiac disease could be easily applied to other autoimmune disorders.

42.3.4.2 MBL and Gastrointestinal Infection

Despite the well-established role of MBL in innate immunity, there have been relatively few studies describing the clinical effect of MBL deficiency in enteric infections. One notable exception is the association between MBL deficiency and risk of *Cryptosporidium parvum* enteritis. This study indicated that patients with biallelic coding mutations (*O*/*O*) had a significantly greater chance of cryptosporidiosis compared to those who were either wild-type or heterozygous for *MBL2* mutation (Kelly et al. 2000). The association between MBL deficiency and cryptosporidiosis was confirmed in young Haitian children by Kirkpatrick et al. (2006). However, the two combined studies present compelling evidence for the role of MBL in the host defense against *Cryptosporidium spp*. infection. However, MBL deficiency was not associated with an increased risk of *Escherichia coli* 0157: H7 colitis nor the complication of HUS (Proulx et al. 2003). *H. pylori* is one of the most common human bacterial infections, affecting approximately 50% of humans. Several immunogenetic polymorphisms are associated with clinical outcomes in *H. pylori* infection, as well as with the risk of infection itself. *H pylori* activates MBL in vitro (c/r Worthley et al. 2006). Studies demonstrated that *H. pylori*-related chronic gastritis causes an increase in gastric mucosal MBL expression, but no association was found between *MBL2* genotype and risk of chronic gastritis (Bak-Romaniszyn et al. 2006; Worthley et al. 2007).

MBL has been implicated in mediating gastrointestinal ischemia/reperfusion injury in mice (Hart et al. 2005). But MBL-null mice (deficient in the murine genes encoding MBL) developed only minor gut injury after induced ischemia/reperfusion insult compared to the wild-type mice. On the contrary, MBL has been implicated as a mediator of ischemia/reperfusion injury in both the myocardium (Walsh et al. 2005) and the kidney (Møller-Kristensen et al. 2005).

42.3.5 Rheumatic Heart Disease

Whereas MBL deficiency has been associated with rheumatic disorders, high MBL levels associated to disease has been reported by Hansen et al. (2003). Rheumatic fever (RF) is the most common cause of acquired valvular disease in

children and young adults. The pathogenic mechanisms responsible for the development of RF/RHD are associated to an abnormal host immune response (both at humoral and cellular level) to cross-reactive streptococcal antigens. The significantly elevated circulating MBL levels in patients with RHD together with the greater prevalence of MBL deficiency in controls suggest that MBL may cause undesirable complement activation contributing to the pathogenesis of RHD (Schafranski et al. 2004). Probably, MBL deficiency may represent an advantage against the development of rheumatic mitral stenosis and that increased MBL levels may be related to the development of RHD. Under normal conditions, MBL does not bind to the organism's own tissues, but in situations of cellular hypoxia, glycosylation of cell surfaces may occur, leading to the deposition of MBL followed by complement activation. The significantly elevated levels of MBL observed in chronic RHD suggest that MBL may represent a pathogenic factor in the complex physiopathology of the disease, whereas MBL deficient individuals might be less susceptible to develop chronic RHD. Studies demonstrating the binding of MBL to the endothelium and causing excessive complement activation and subsequent tissue damage are known (Jordan et al. 2003), where as MBL deficiency may be advantageous in some circumstances since MBL may lead to an increased cytokine secretion by macrophages (Takahashi et al. 2002). Under some other conditions, MBL is associated to disease severity in both infectious and autoimmune disease (Garred et al. 1997, 1999). Although MBL is an acute-phase protein produced by the liver, its level only shows a moderate increase and determined genetically in inflammatory diseases. The elevated MBL levels in patients with chronic RHD might corroborate the chronic inflammatory activity present in these individuals and contribute to valve injury through complement activation. In addition, MBL may act as an immunomodulatory molecule, inducing a higher secretion of cytokines by macrophages (Turner and Hamvas 2000).

42.3.6 MBL in Cardio-Vascular Complications

There seems to be a delicate balance as to when MBL levels may be involved in harmful or in beneficial inflammation in the cardiovascular system. For example, Kawasaki disease is a systemic vasculitis in childhood possibly caused by infections and in the developed world Kawasaki disease is the most common cause of acquired heart disease in children (Royle et al. 2005). MBL as an initiator of inflammation, Biezeveld et al. (2003) while studying the frequency of MBL genotypes with Kawasaki disease in Dutch patients found a higher frequency of MBL mutations as compared to the genotypes in controls. Children younger than 1 year were at higher risk of development of CAD. Kawasaki disease occurs more frequent in oriental children (10 times more frequent than in Caucasians) (Royle et al. 2005). In Chinese, Cheung et al. (2004) did not see a difference between the MBL genotypes of patients and controls. The recent observation of an association between a human coronavirus and Kawasaki disease (Esper et al. 2005) fits with the many indications of MBL having antiviral activity. Plaque material may be removed from inside of the carotid artery (e.g., by endarterectomy) to avoid cerebral attack. Rugonfalvi-Kiss et al. (2005) indicated that female patients with genotypes associated with lower MBL levels had a slower rate to early restenosis, suggesting that a high level of MBL may be part of the pathophysiology of this condition.

In a study of 76 patients with severe atherosclerosis, Madsen et al. (1998) found that there were more patients with myocardial infarcts among Norwegians with low MBL allotypes than in controls. Saevarsdottir et al. (2005) found in a cohort study in Iceland that the risk of developing myocardial infarction was higher in MBL deficient individuals. The relationship between markers of innate immunity and clinical outcomes in patients with heart failure (HF) after acute myocardial infarction (AMI) suggested that atherogenesis and heart failure are associated with the altered control of inflammation by innate immune defenses, which include TLRs and MBL. Circulating levels of MBL and sTLR2 may reflect different aspects of the innate immune response and the involvement of innate immunity responses in the pathogenesis of post-MI heart failure (Ueland et al. 2006).

Lectin Pathway in Myocardial Ischemia-Reperfusion: MBL plays a dual role in modifying inflammatory responses to sterile and infectious injury. Although complement is widely accepted as participating in the pathophysiology of ischemia-reperfusion injury, the specific role of the lectin pathway has been addressed by Jordan et al. (2001). Since, blockade of the lectin pathway with inhibitory mAbs protects the heart from ischemia-reperfusion by reducing neutrophil infiltration and attenuating pro-inflammatory gene expression, it appears that the lectin complement pathway is activated after myocardial ischemia-reperfusion and leads to tissue injury.

Mice devoid of MBL-dependent lectin pathway activation but fully active for alternative and classical complement pathways, are protected from cardiac reperfusion injury with resultant preservation of cardiac function. Significantly, mice that lack a major component of the classical complement pathway initiation complex (C1q) but have an intact MBL complement pathway are not protected from injury. Thus, the MBL-dependent pathway of complement activation is the key regulator of myocardial reperfusion ischemic injury (Walsh et al. 2005).

Diabetic Patients at High Risk for Diabetic-Nephropathy: Whether lectin pathway of complement activation plays a role in the pathogenesis of human glomerulonephritis, is not well known. It has been proposed that MBL may bind to altered self components, which may possibly be found in diabetic patients, and MBL could thus be a potential pathogenic factor for diabetic cardiovascular complications, e.g., nephropathy (Hansen et al. 2005; Saevarsdottir et al. 2005). Normo-albuminuric type 1 diabetics have been found to have higher MBL levels than non-diabetic controls, with a stepwise increase in circulating MBL levels with increasing levels of urinary albumin excretion (Hansen et al. 2003). In another study, a significantly larger proportion of patients with diabetic nephropathy presented a MBL genotype associated with higher MBL level, when compared to the group with MBL genotypes associated with low MBL levels (Hansen et al. 2004). The elevated serum MBL levels in type 1 diabetic patients with diabetic nephropathy were confirmed by Saraheimo et al. (2005). In a long follow up study on 270 type 1 diabetic patients, it was found that for patients with type 1 diabetes and MBL-levels below the median of 1.6 μg/mL the risk of developing micro or macro-albuminuria was 26%, while the patients with MBL-levels above the median had a risk of 41% of developing micro or macro-albuminuria (Hovind et al. 2005). These studies suggested that a high MBL geno- and phenotype is associated with an increased risk of developing diabetic kidney disease and that assessing MBL status may prove beneficial in identifying patients at risk for micro- and macro-vascular complications (c/r Thiel et al. 2006).

MBL has been detected in the glomeruli of patients with lupus nephropathy, membranous nephropathy, membranoproliferative glomerulonephritis type I and anti-GBM nephritis. It was proposed that MBL binds to agalactosyl oligosaccharides of IgG that terminate in N-acetylglucosamine (Lhotta et al. 1999). Elevated serum MBL levels of were implicated in the pathogenesis of renal manifestations of Henoch-Schönlein purpura (Endo et al. 2000), in IgA nephropathy (Endo et al. 1998), in other forms of human glomerulonephritis (Lhotta et al. 1999), and in vascular complications of diabetes mellitus type 1 (Hansen et al. 2003).

42.3.7 Other Inflammatory Disorders

42.3.7.1 Sarcoidosis and MBL Variants

Sarcoidosis is a chronic granulomatous disease of unknown aetiology. The causative agent may be an infectious micro-organism. MBL variants predisposed to sarcoidosis by increasing their susceptibility to micro-organisms among sarcoidosis patients showed that the frequencies of variants were similar regardless of severity of disease outcome. MBL gene variants did not indicate to influence susceptibility to sarcoidosis, age of disease onset, or severity of disease. The average patient ages at time of diagnosis were similar for all MBL genotypes (Foley et al. 2000). However, a 57-year-old woman patient with pulmonary sarcoidosis suggested interstitial nephritis without proteinuria and hematuria, whereas a renal biopsy showed granulomatous interstitial nephritis and mild mesangial proliferative glomerulonephritis. From this case of renal sarcoidosis, it was hypothesized that *P. acnes* might be involved in pathogenesis of granulomatous interstitial nephritis and that it plays a role in glomerular complement activation via the lectin pathway (Hagiwara et al. 2005).

42.3.7.2 Behcet's Disease

Behcet's disease (BD) is a multisystemic, recurrent inflammatory disease caused by the combination of genetic and environmental factors. The haplotypes of the MBL2 gene can influence therapeutic response in BD, thus affecting the clinical symptoms in BD patients. The promoter region, MBL2-550*C/*C (L/L) homozygote was found to have a lower frequency in BD patients than that in controls. No difference was observed in the allele frequencies of G-221 C (Y/X), C + 4 T (P/Q) or Gly54Asp (A/B) of the MBL2 gene in BD patients and in controls. The HYPA haplotype contributed to BD occurrence, whereas the LYPA haplotype was negatively associated with BD. BD patients with several symptoms and with an earlier disease-onset age had a higher HYPA haplotype frequency. BD patients showing poor response (S) to therapy had a higher HYPA frequency than those showing good response (M). It appeared that possessing HYPA increases the risk of BD and that the MBL2 HYPA haplotype plays a role in MBL levels and increases the susceptibility to BD (Park et al. 2005).

42.3.7.3 Cystic Fibrosis (CF) Lung Disease

The MBL deficiency has been associated with poor outcome in cystic fibrosis (CF) lung disease. A mutation in MASP-2 and higher serum levels of MBL than healthy controls belonging to the MBL pathway in serum have been described. Thus, MBL pathway function is affected both by MBL and by MASP-2 genotypes (Carlsson et al. 2005). Patients undergoing abdominal aortic aneurysm (AAA) repair are exposed to an ischaemia-reperfusion injury (IRI), which is in part mediated by complement activation. During IRI, the patients undergoing AAA repair experience a mean decrease in plasma MBL level of 41% representing significant lectin pathway activation. This indicated that consumption of MBL occurs during AAA repair, which suggested an important role for lectin pathway in IRI. Hence, specific transient inhibition of lectin pathway activity can be of significant therapeutic value in patients undergoing open surgical AAA repair (Norwood et al. 2006).

In Guillain-Barre syndrome (GBS), complement activation plays a crucial role in the induction and extent of the post-infectious immune-mediated peripheral nerve damage. The MBL2 genotype, serum MBL level, and MBL complex activity are associated with the development and severity of GBS. The MBL2 B allele was associated with functional deficiency and relatively mild weakness. Studies support the hypothesis that complement activation mediated by MBL contributes to the extent of nerve damage in GBS, which is codetermined by the MBL2 haplotype (Geleijns et al. 2006).

42.3.7.4 Experimental Polymicrobial Peritonitis

Peritonitis is the most common and major complication in the treatment modality of peritoneal dialysis (PD) for uraemic patients. The contribution of the different complement activation pathways was studied in the host defense against experimental polymicrobial peritonitis induced by cecal ligation and puncture by using mice deficient in either C1q or factors B and C2. The C1q-deficient mice lack the classical complement activation pathway. Mice with a deficiency of both factors B and C2 lack complement activation via the classical, the alternative, and the lectin pathways and exhibited the maximum mortality of 92%, indicating a significant contribution of the lectin and alternative pathways of complement activation to survival (Windbichler et al. 2004). While examining the role of serum MBL concentration and point mutations in MBL gene in PD-related peritonitis, Lam et al. (2005) found that both homozygous and heterozygous patients had profoundly reduced serum level of MBL. Thus, dialysis patients having lower MBL levels may increase the susceptibility of infection.

42.4 Significance of MBL Gene in Transplantation

MBL Replacement Therapy Following Stem Cell Transplantation: Life-threatening complications such as graft versus host disease and infection remain major barriers to the success of allogeneic hemopoietic stem cell transplantation (SCT). Among various factors, MBL deficiency is a risk factor for infection in other situations where immunity is compromised. MBL2 coding mutations were associated with an increased risk of major infection following transplantation. MBL2 promoter variants were also associated with major infection. The high-producing haplotype HYA was associated with a markedly reduced risk of infection. Donor MBL2 coding mutations and recipient HYA haplotype were independently associated with infection in multivariate analysis. Thus, these results suggest that MBL2 genotype influences the risk of infection following allogeneic SCT and that both donor and recipient MBL2 genotype are important (Mullighan et al. 2002).

A retrospective study examining associations between polymorphisms in the gene encoding MBL, MBL2 and risk of major infection post-SCT was conducted in 96 related myeloablative transplants. The study showed that "low-producing" MBL2 coding alleles, when present in the donor, were significantly associated with increased risk of major infection in the recipient following neutrophil count recovery. Furthermore, a "high-producing" MBL2 haplotype, HYA, when present in the recipient, was protective against infection. Since MBL is under development as a therapeutic agent, findings suggest that administration of MBL may reduce the risk of infection post-transplant. Further work is required to confirm these results. These results indicate a report of a genetic determinant of risk of infection post-SCT, and highlight the importance of non-HLA genetic factors in determining the risk of transplant complications (Mullighan and Bardy 2004).

Genetic MBL variants are frequent and associated with low MBL serum levels. Higher MBL levels may be associated with more complement-mediated damage resulting in inferior graft survival. Berger et al. (2005) showed no significant difference in incidence of delayed graft function in recipients with a low MBL level ($<$ or $=$ 400 ng/mL) compared to those with a higher MBL level ($>$400 ng/mL). If these data can be confirmed, pre-transplant MBL levels may provide additional information for risk stratification prior to kidney transplantation (Berger et al. 2005).

Complement Activation Is Harmful for the Allograft Endothelium: In heart transplant recipients, Fiane et al. (2005) recorded transplant-associated coronary artery disease and observed an association with MBL deficiency. They also recorded that acute rejection of the transplant was seen in 6 out of 6 with MBL deficiency as compared to 15 out of 32 with higher MBL levels. Assuming that MBL may interact with the transplanted tissue and initiate complement activation, this study added to the list of studies, which suggested that complement activation is harmful for the endothelium in general, and possibly for the allograft endothelium in particular.

MBL Pathway and SPKT Graft Survival: Simultaneous pancreas-kidney transplantation (SPKT) is the treatment of choice for patients with type 1 diabetes and renal failure. However, this procedure is characterized by a high rate of postoperative infections, acute rejection episodes, and cardiovascular mortality. The lectin pathway of complement activation contributes to cardiovascular disease in diabetes and may play an important role in inflammatory damage after organ transplantation. MBL serum levels and MBL genotypes in patients who received an SPKT from 1990 through 2000 and related graft survivals revealed that survival was significantly better in recipients with MBL gene polymorphisms associated with low MBL levels. Thus,

MBL is a potential risk factor for graft and patient survival in SPKT. It is hypothesized that MBL contributes to the pathogenesis of inflammation-induced vascular damage both in the transplanted organs and in the recipient's native blood vessels (Berger et al. 2007). To address further the role of MBL deficiency, Verschuren et al. (2008) showed that high levels of serum MBL are associated with protection against urinary tract infections and, more specifically, against urosepsis after SPKT. These results indicate an important role for the lectin pathway of complement activation in antimicrobial defense in these transplant recipients (Verschuren et al. 2008).

42.5 MBL in Tumorigenesis

42.5.1 Polymorphisms in the Promoter

In paediatric oncology patients with febrile neutropenia, MBL levels are correlated to clinical and laboratory parameters. Structural exon-1 MBL2 mutations and the LX promoter polymorphism were related to deficient MBL levels. The capacity to increase MBL concentrations during febrile neutropenia was associated with MBL2 genotype. Infectious parameters did not differ between MBL-deficient and MBL-sufficient neutropenic children. However, most patients (61%) were severely neutropenic, compromising the opsonophagocytic effector function of MBL. MBL substitution might still be beneficial in patients with phagocytic activity (Frakking et al. 2003).

Five polymorphisms in the promoter and first exon of the MBL2 gene alter the expression and function of MBL in humans and are associated with inflammation-related disease susceptibility. These five polymorphisms create six well-characterized haplotypes that result in lower (i.e., LYB, LYC, HYD, and LXA) or higher (i.e., HYA and LYA) serum MBL concentrations. A statistically significant association was found between the X allele of the promoter Y/X polymorphism (which results in a lower serum MBL concentration) and improved lung cancer survival among white patients but not among African American patients. The functional Y/X polymorphism of the innate-immunity gene MBL2 and MBL2 haplotypes and diplotypes appear to be associated with lung cancer survival among white patients (Pine et al. 2007). A significantly higher incidence of MBL deficiency/insufficiency-associated genotypes was found among patients with malignant disease. Findings reflecting anti-tumorigenic activity of MBL protein suggest potential therapeutic application. However, it cannot be excluded that mbl-2 mutant alleles may be in linkage disequilibrium with an unidentified tumor susceptibility gene(s) (Swierzko et al. 2007).

The genetic variations in key genes of MBL2 could influence the risk for breast cancer. A preliminary analysis of SNPs in MBL2 in breast cancer [166 African-American (AA) patients vs. 180 controls and 127 Caucasian (CAU) patients vs. 137 controls] presents evidence that common genetic variants in the 3′-UTR of MBL2 might influence the risk for breast cancer in AA women, probably in interaction with the 5′ secretor haplotypes that are associated with high concentrations of MBL (Bernig et al. 2007).

42.5.2 MBL Binding with Tumor Cells

Changes in cell surface structures during oncogenic transformation appear to promote binding of MBL to cancer cells (Hakomori 2001) where the protein can mediate cytotoxic effects including MBL-dependent cell mediated cytotoxicity (Ma et al. 1999; Nakagawa et al. 2003). Experimental studies suggest that MBL (both wild-type and the mutant *B* allele) may possess anti-colorectal cancer tumor activity (Ma et al. 1999). The MBP/MBL binds specifically to oligosaccharides expressed on the surface of human colorectal carcinoma cells, SW1116. MBL binding occurs in colon adenocarcinoma cell lines (Colo205, Colo201 and DLD-1), but not in any of the leukemic cell lines. The binding of MBL to these cell lines was sugar-specific and calcium-dependent. The degree of MBL binding was correlated with the expression of Lewis A and Lewis B antigens on these cell lines (Muto et al. 1999). Intra-tumoral administration of the recombinant vaccinia virus carrying a *MBL2* gene significantly reduced tumor size as compared to controls, along with prolonged survival of mice (Ma et al. 1999). However, these results were not reflected in clinical trials. In fact, patients with colorectal cancer have increased activation of the lectin-complement pathway and increased levels of serum MBL (Ytting et al. 2004). In patients undergoing surgery for colorectal cancer, however, low preoperative levels of serum MBL have been linked to an increased risk of developing post-colectomy pneumonia (Ytting et al. 2005). Further studies may clarify the role of the lectin-complement pathway in colorectal cancer.

42.6 Complications Associated with Chemotherapy

42.6.1 Neutropenia

Secondary immunodeficiencies due to disease or treatment have provided interesting patient populations within which to study the role of MBL. One such group comprises those receiving chemotherapy for malignancy. In these patients,

chemotherapy induces neutropenia and increased risk of infection. Studies have thus attempted to analyze the correlation between MBL deficiency and infections in such patients. It is difficult to compare these studies since these studies include patients with a variety of underlying malignancies and variety of chemotherapy, different combinations of antimicrobial agents and various other factors (Klein and Kilpatrick 2004). However, there is clear evidence of the importance of MBL for protection in leukemia patients. MBL genotypes and MBL levels were correlated to the causes, frequency and duration of febrile neutropenic periods in children receiving chemotherapy (Neth et al. 2001). The majority of children were patients of acute lymphoblastic leukemia (ALL). Children with variant MBL alleles exhibited twice as many days of febrile neutropenia as children with wild type genotypes. Analysis by MBL quantification supported this as children with less than 1 μg MBL/mL had a higher number of days with febrile neutropenia. Peterslund et al. (2001) described infections defined as bacteremia, pneumonia or both in hematological malignancies of 54 adults treated with chemotherapy. All patients with the infections, except one, showed MBL levels below 0.5 μg/mL. Vekemans et al. (2005) conducted a prospective observational study focusing on assessment of MBL as a risk factor for infection during chemotherapy induced neutropenia in adult hematological cancer patients. They included 255 patients and determined MBL levels as well as MBL genotypes. A higher rate of severe infection was seen in MBL deficient patients. The impact was further increased when acute leukaemic patients were excluded. In a contrasting study, Bergmann et al. (2003) followed 80 adults undergoing therapy for acute myeloid leukaemia (AML), which involves intense highly myelosuppressive treatment. They found no effect of MBL deficiency on frequency, severity or duration of fever and suggested that the severe immunosuppression induced by the combination of the myeloid cancer and chemotherapy may obscure the normal effector functions of MBL, though, Kilpatrick et al. (2003) failed to see anything but a modest effect of MBL levels below 100 ng/mL in a retrospective study on 128 patients, most of whom were prepared for bone marrow transfer and more than half presented with AML. Results cast doubt on the potential value of MBL replacement therapy in this clinical context (Kilpatrick et al. 2003). A growing body of evidence indicates that genetic factors are involved in an increased risk of infection. MBL gene polymorphisms that cause low levels of MBL are associated with the occurrence of major infections in patients, mainly bearing hematological malignancies, after high-dose chemotherapy (HDT) rescued by autologous peripheral blood stem cell transplantation (auto-PBSCT). A retrospective examination of 113 patients treated with HDT and auto-PBSCT revealed that the low-producing genotypes, B/B and B/LXA, were associated with major bacterial infection. A nation-wide study, conducted to assess the allele frequency of the MBL coding mutation in a total of 2,623 healthy individuals in Japan, revealed the frequency of allele B as 0.2, almost the same in seven different areas of Japan. This common occurrence suggested that MBL deficiency may play an important role in the clinical settings of immune-suppression (Horiuchi et al. 2005). Studies by Aittoniemi et al. (1999) in patients with chronic lymphocytic leukemia did not observe any effect of MBL on infections. A possible association between MBL genotypes and severe infections in patients with multiple myeloma receiving moderate strength induction chemotherapy has been studied. From the MBP genotypes, identified in bone marrow biopsies, the study concluded that during induction chemotherapy in patients with multiple myeloma, a general protective effect of wild-type MBL2 against chemotherapy-related infections was not apparent. However, indications were there of a reduced occurrence of septicaemia in patients with wild-type compared with variant MBL2. Further studies in larger cohorts of patients are relevant (Molle et al. 2006). Thus, further studies are required to describe the patients particularly at risk when being MBL deficient.

42.6.2 Animal Studies

The MBL knock-out mice have made possible experimental investigations of the effect of MBL deficiency. The mouse has two genes encoding different MBL molecules (MBL-A and -C) compared to one in humans. Both MBLs in mice are able to bind to carbohydrate surfaces and activate the complement system. A slight difference in carbohydrate specificity has been reported for the two mouse MBLs. Mice with only MBL-A knocked-out were first produced, but only mice with both MBL-A and -C knocked out (MBL DKO) are suitable as animal model of human MBL deficiency. In 2004, Shi et al. demonstrated that MBL DKO sepsis model mice were highly susceptible to intravenous inoculation via tail vein with *Staphylococcus aureus*, all dying within 48 h, compared with 55% survival of MBL wild-type mice. Infusion of recombinant MBL reversed the phenotype. No difference was seen when the bacteria were injected intra-peritoneally. However, if the mice were treated with cyclophosphamide, simulating chemotherapy-induced neutropenia, before the intra peritoneal infection, the MBL DKO had more abscesses than the wild type. The MBL DKO mice were also more susceptible to challenge with herpes simplex virus type 2 (Gadjeva et al. 2004). In line with the suggested involvement of MBL in autoimmune diseases the MBL DKO mice were examined for autoimmune symptoms when 18-month-old (Stuart et al. 2005). No such signs were observed. On the other hand, the ability to clear apoptotic

cells was less efficient in the MBL knock-outs. It has been hypothesized that while MBL does not bind significantly to healthy tissue, changes due to abnormal conditions might reveal MBL ligands. Indeed, MBL is expressed by some tumor cell lines, and gene therapy with an MBL-vaccinia construct was found protective in nude mice transplanted with a human colorectal cancer cell line (Ma et al. 1999). In vitro studies have indicated binding of MBL to cells exposed to hypoxia-reoxygenation (simulating ischemia/reperfusion) and subsequently it was shown that infusion of a blocking anti-MBL antibody would protect against myocardial destruction following ischemia/reperfusion in a rat model (Jordan et al. 2001). Using MBL DKO mice Møller-Kristensen et al. (2005) found, in a model of kidney ischemia reperfusion (I/R) injury, that the MBL DKO were partially protected as evidenced by a better kidney function in these mice after ischemia/reperfusion. Increased deposition of the complement factor C3 was seen in wild type mice, and binding of MBL to sections of kidney could be inhibited with mannose. In agreement with this, deVries et al. (2004) found MBL-A and -C deposited in the kidneys after ischemia/reperfusion in MBL wild type mice. The recombinant vaccinia virus carrying human MBP gene possesses a potent growth-inhibiting activity against human colorectal carcinoma cells transplanted in KSN nude mice. The treatment resulted into a prolonged life span of tumor-bearing mice. Local production of MBP had a cytotoxic activity, which was proposed as MBP dependent cell-mediated cytotoxicity (MDCC). This study offers a model for the development of an effective and specific host defense factor for cancer gene therapy (Ma et al. 1999; Thiel et al. 2006).

42.7 MBL: A Reconstitution Therapy

Since genetically determined MBL deficiency is very common and can be associated with increased susceptibility to a variety of infections, the potential benefits of MBL reconstitution therapy need to be evaluated. In a phase I trial on 20 MBL-deficient healthy adult volunteers receiving a total of 18 mg of MBL in three 6 mg doses given (i.v.), once a week for a period of 3 weeks did not show adverse clinical changes or any sign of infusion-associated complement activation. Study suggested that infusion of purified MBL as prepared by Statens Serum Institut (SSI) is safe. However, adults have to be given at least 6 mg twice or thrice weekly for maintaining protective MBL levels assumed to be about 1,000 ng/mL (Valdimarsson et al. 2004). Considerations of MBL genotyping and association with infection opens the possibility of producing clinical grade recombinant MBL that resulted to establishing a company having this aim (Jensenius et al. 2003). The treatment of chronic disorders may possibly also be considered on the longer term. The invention led to use of at least MBL oligomer comprising at least one MBL subunit, for the manufacture of a medicament for prophylaxis and/or treatment of infection (Thiel and Jensenius 2007).

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Pulmonary Collectins in Diagnosis and Prevention of Lung Diseases

43

Anita Gupta

43.1 Pulmonary Surfactant

Pulmonary surfactant is a complex mixture of lipids and proteins, and is synthesized and secreted by alveolar type II epithelial cells and bronchiolar Clara cells. It acts to keep alveoli from collapsing during the expiratory phase of the respiratory cycle. After its secretion, lung surfactant forms a lattice structure on the alveolar surface, known as tubular myelin. Surfactant proteins (SP)-A, B, C and D make up to 10% of the total surfactant. SP-B and SPC are relatively small hydrophobic proteins, and are involved in the reduction of surface-tension at the air-liquid interface. SP-A and SP-D, on the other hand, are large oligomeric, hydrophilic proteins that belong to the collagenous Ca^{2+}-dependent C-type lectin family (known as "Collectins"), and play an important role in host defense and in the recycling and transport of lung surfactant (Awasthi 2010) (Fig. 43.1). In particular, there is increasing evidence that surfactant-associated proteins A and -D (SP-A and SP-D, respectively) contribute to the host defense against inhaled microorganisms (see Chaps. 24 and 25). Based on their ability to recognize pathogens and to regulate the host defense, SP-A and SP-D have been recently categorized as "Secretory Pathogen Recognition Receptors". While SP-A and SP-D were first identified in the lung, the expression of these proteins has also been observed at other mucosal surfaces, such as lacrimal glands, gastrointestinal mucosa, genitourinary epithelium and periodontal surfaces. SP-A is the most prominent among four proteins in the pulmonary surfactant-system. The expression of both SP-A and SP-D is complexly regulated on the transcriptional and the chromosomal level. SP-A is a major player in the pulmonary cytokine-network and has been described to act in the pulmonary host defense. This chapter gives an overview on the understanding of role of SP-A and SP-D in for human pulmonary disorders and points out the importance for pathology-orientated research to further elucidate the role of these molecules in adult lung diseases. As an outlook, it will become an issue of pulmonary pathology which might provide promising perspectives for applications in research, diagnosis and therapy (Awasthi 2010).

43.2 SP-A and SP-D in Interstitial Lung Disease

SP-A and SP-D appear in the circulation in specific lung diseases. Interstitial lung disease (ILD), also known as diffuse parenchymal lung disease (DPLD), refers to a group of lung diseases affecting the interstitium of lung: alveolar epithelium, pulmonary capillary endothelium, basement membrane, perivascular and perilymphatic tissues. The term ILD is used to distinguish these diseases from obstructive airways diseases. Most types of ILD involve fibrosis, but this is not essential; indeed fibrosis is often a later feature. The phrase "pulmonary fibrosis" is no longer considered a synonym, but the term is still used to denote ILD involving fibrosis. The term is commonly combined with idiopathic in "idiopathic pulmonary fibrosis", denoting fibrotic ILD that cannot be ascribed to a distinct primary cause.

43.2.1 Pneumonitis

Chronic hypersensitivity pneumonitis (HP) eventually ensues to extensive lung fibrosis when exposure to causative antigen continues. Klebs von den Lungen (KL)-6, a mucin-like glycoprotein and SP-D are elevated in most cases. Correct diagnosis in the early stage is crucial, since chronic summer-type HP can result in a fatal outcome after continuous exposure to the causative antigen (Inase et al. 2007). In pulmonary tissues of collagen vascular disease-associated interstitial pneumonia (CVD-IP) and hypersensitivity pneumonitis (HP), SP-D can be a marker for maturity of regenerating epithelial cells. SP-A along with KL-6 is detected in intimate relationship to the stage of regeneration

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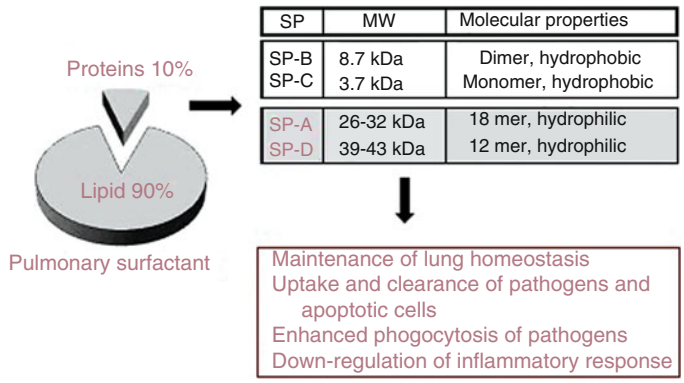

Fig. 43.1 Presence of surfactant proteins (SP) in lung surfactant, their properties and major functions of SP-A and SP-D

of alveolar epithelial cells and expressed before SP-D (Ohtsuki et al. 2007). Radiation pneumonitis (RP) is most common complication of radiotherapy for thoracic tumors. Both SP-A and SP-D concentrations in sera from patients with RP were significantly higher than those from patients without RP. Serum SP-A and SP-D may be of diagnostic value for detection of RP, even when radiographic change is faint (Takahashi et al. 2001). Despite the rise of SP-D and KL-6 in serum in adult patients with various types of interstitial pneumonia (IP) and collagen diseases with interstitial pneumonia, KL-6 may be superior in sensitivity of IP, where as SP-D may be more specific for IP than KL-6. Early decrease of SP-D contrasts with the transient increase of KL-6 levels after prednisolone pulse therapy (Arai et al. 2001). High serum KL-6 value is an indicator of ILD of Wilson-Mikity syndrome and better than SP-D and LDH levels (Takami et al. 2003). Thus serum SP-A and SP-D monitoring along with KL-6 is useful indicator for estimating RP (Matsuno et al. 2006).

43.2.2 Interstitial Pneumonia (IP)

SP-A and SP-D in BAL as Indicator of Pneumonia in Children

SP-A and SP-D in serum significantly increase in patients with pulmonary alveolar proteinosis (PAP), idiopathic pulmonary fibrosis (IPF) and interstitial pneumonia with collagen vascular diseases (IPCD) (Kuroki et al. 1998; Takahashi et al. 2006b). The concentrations of SP-A and SP-D in BAL fluids from patients with IPF and IPCD are rather lower than those in healthy controls; and the SP-A/phospholipid ratio may be a useful marker of survival prediction. SP-D-deficient patients have more frequently pneumonias and their long-term outcome is worse than those with detectable SP-D. Among children with recurrent bronchitis and SP-D detectable in bronchoalveolar lavage (BAL), patients with allergic asthma had threefold levels of SP-D compared with controls. In contrast, SP-D deficiency due to consumption or failure to up-regulate SP-D may be linked to pulmonary morbidity in children (Griese et al. 2008).

SP-A Levels can Differentiate Usual Interstitial Pneumonia with Non-Specific Interstitial Pneumonia (NSIP)

There is a need to use serum markers for differentiating usual interstitial pneumonia (UIP) from other ILD. Serum levels of SP-A and SP-D in patients with UIP and nonspecific interstitial pneumonia (NSIP) are significantly higher than in healthy volunteers. In particular, serum SP-A levels in patients with UIP are significantly higher than in patients with NSIP, where as SP-D in BAL fluid in UIP patients were significantly lower than in patients with NSIP. Thus, serum SP-A level seems useful marker to differentiate UIP from NSIP (Ishii et al. 2003).

Abnormal tracheal aspirate surfactant phospholipids and SP-A are noted in children with bacterial pneumonia, viral pneumonitis, and ARDS, but not in children on cardiopulmonary bypass (Baughman et al. 1993; LeVine et al. 1996). SP-A in pneumonia group is significantly reduced and the reduction was better indicator in the Gm^{+}-pneumonia group than in Gm^{-}-pneumonia group patients (Baughman et al. 1993). Fulminant early-onset neonatal pneumonia is associated with ascending intrauterine infection (IUI) and alveolar M showed significantly less nitric oxide synthase 2 (NOS2) isoform than in the controls. In the airway samples, the infants with fulminant pneumonia after birth had low intracellular NOS2 and significantly low IL-1β and SP-A than noninfected IUI infants (Aikio et al. 2000).

Foster et al. (2002) suggested that signaling of EGF axis and differential regulation of SPs persist during postnatal lung development, and SP-A and SP-D may modulate post-pneumonectomy (PNX) lung growth in dogs. SP-D in patients, hospitalized for community-acquired pneumonia of suspected bacterial origin, indicates significant changes during pulmonary infection (Daimon et al. 2005; Leth-Larsen et al. 2003). The SA-A and SP-D in sera are useful

for identification of the clinical condition of horses with bacterial pneumonia (Hobo et al. 2007).

43.2.3 ILD Due to Inhaled Substances

Cigarette smoke may alter component and function of pulmonary surfactant. Alterations in serum levels of SP-A may reflect smoking habits since serum SP-A was higher in active smokers than in nonsmokers (Nomori et al. 1998). However, SP-A is not a sensitive discriminating factor to separate smokers from nonsmokers. The contents of SP-A and SP-D in BAL fluids were significantly decreased in smokers compared to those in nonsmokers, although there was no significant difference of total phospholipid content between two groups (Honda et al. 1996). SP-A may decrease due to the cumulative effects of long-term smoking and development of emphysema, while SP-D decreases due to long-term smoking (Betsuyaku et al. 2004; Shijubo et al. 1998). Emphysema can be induced in mice by chronic cigarette smoke exposure with increase of SP-D in emphysema lungs. While accumulation of foamy alveolar macrophages may play a key role in the development of smoking-induced emphysema, increased SP-D may play a protective role in the development of smoking-induced emphysema, in part by preventing alveolar cell death (Hirama et al. 2007).

Although effects of maternal smoking on fetal growth and viability are overwhelmingly negative, there is a paradoxical enhancement of lung maturation as evidenced, in part, by a lower incidence of RDS in infants of smoking mothers. Epidemiologic and experimental evidence further support the view that a tobacco smoke constituent, possibly nicotine, affects the development of the lung in utero. The murine embryonic lungs explanted at 11 days gestation showed a 32% increase in branching after 4 days in culture in presence of 1 μM nicotine and 7–15-fold increases in mRNAs encoding SP-A and SP-C after 11 days. The nicotine-induced stimulation of surfactant gene expression could, in part, account for the effect of maternal smoking on the incidence of RDS (Wuenschell et al. 1998).

Intratracheal administration of crystalline silica to rats elicits a marked increase in alveolar accumulation of surfactant lipids and SP-A. The extracellular accumulation of SP-D is markedly increased in silica-induced lipoproteinosis, and that SP-D is associated with amorphous components identified by electron microscopy. SP-D may be useful biomarkers for early diagnosis and serum SP-D concentration may associate with the pathogenesis of silicosis (Barbaro et al. 2002; Wang et al. 2007b). Alcohol consumption at high levels during pregnancy is associated with immuno-modulation and premature birth. Chronic maternal ethanol consumption during the third trimester of pregnancy alters SP-A gene expression in fetal lung. These alterations may underlie increased susceptibility of preterm infants, exposed to ethanol in utero, to RSV and other microbial agents (Lazic et al. 2007). The exposure to moderate and high occupational levels of Diesel exhaust (DE) causes an increase in lung injury and inflammation, and a decrease in host defense molecules, which could result in increased severity of infectious and allergic lung disease. Several inflammatory and immune cytokines are upregulated at various time points and concentrations, in contrast to SP-A and SP-D which were significantly decreased at protein level. (Gowdy et al. 2008).

43.2.4 Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive disease of lung characterized by an inflammatory infiltrate, alveolar type II cell hypertrophy and hyperplasia, and ultimate parenchymal scarring. The phospholipid composition of the surface-active material recovered by BAL is abnormal in this disease. The content of SP-A in lavage was reduced, even when normalized for the total amount of surface-active material (SP-A/total phospholipids (PL)) recovered. The reduction in SP-A was not specific to IPF but also occurred in other interstitial lung diseases. Despite this, SP-A/PL in BAL is a biochemical marker that predicts survival in patients with IPF (McCormack et al. 1995; Phelps et al. 2004).

The serum SP-A and SP-D levels are significantly elevated in patients with IPF and systemic sclerosis compared to sarcoidosis, beryllium disease and normal controls, and correlated with radiographic abnormalities in patients with IPF. Dohmoto et al. (2000) hypothesized that regenerated or premature bronchoepithelial cells may circulate in the blood in patients with IPF. RT-PCR for cytokeratin 19 (CK19) and pulmonary SP-A in peripheral blood in patients with IPF and pulmonary fibrosis (PF) associated with collagen vascular disorders suggests that there were some circulating bronchoepithelial cells expressing mRNA for SP-A in peripheral blood of patients associated with collagen vascular disorders. Thus, both serum SP-A and SP-D levels are highly predictive of survival in patients with IPF (Greene et al. 2002; Takahashi et al. 2006b) and the measurement of SP-D in sera can provide an easily identifiable and useful clinical marker for the diagnosis of IPF, IPCD, and PAP, and can predict the disease activity of IPF and IPCD and the disease severity of PAP (Honda et al. 1995). However, KL-6 is the best serum marker for ILD (Ohnishi et al. 2002). Serum KL-6 and SP-D were also prognostic markers in acute exacerbation of IPF after treatment with Sivelestat (Endo et al. 2006; Nakamura et al. 2007). High levels of SP-D in BAL fluids are associated in patients with PAP, but not with IPF and IPCD.

Selman et al. (2003) examined associations between IPF and genetic polymorphic variants of SP-A1, SP-A2, SP-B, SP-C, and SP-D. One SP-A1 ($6A^4$) allele and SNPs that characterize the $6A^4$ allele and one SP-B (B1580_C) were found with higher in nonsmoker and smoker IPF subgroups, respectively, compared with healthy controls. To explore whether a tryptophan (in $6A^4$) or an arginine (in other SP-A1 alleles and in all SP-A2 alleles) at amino acid 219 alters protein behavior, two truncated proteins that varied only at amino acid 219 were oxidized by exposure to ozone. Differences in the absorption spectra (310–350 nm) between the two truncated rSP-A proteins, before and after protein oxidation, suggested allele-specific aggregation attributable to amino acid 2143. The SP-B SNP B1580_C, to be a risk factor for IPF smokers, was also shown to be a risk factor for other pulmonary diseases. The SP-C and SP-D SNPs and SP-B-linked microsatellite markers did not associate with IPF. These findings indicated that surfactant protein variants may serve as markers to identify subgroups of patients at risk. The observed alleles of SP-A and SP-D in association with various diseases are summarized in Table 43.1. Different alleles of these genes seem to predispose the individuals to various diseases. A logical explanation seems to be that different SNPs lead to different alterations in function or expression. However, common SNPs predispose Caucasians to RDS and Mexicans to TB. Similarly, common SNPs predispose the Indian population to ABPA and TB. Furthermore, Met11 SP-D allele is predisposing Mexicans to TB and Finns to RSV infection. It is also interesting to note that some of the alleles of SP-A interact with other alleles of SP-A and SP-B and thus increase the susceptibility of subjects to a disease (Kishore et al. 2005).

43.2.5 Cystic Fibrosis

Cystic fibrosis (CF) is an inherited disorder of CFTR gene, a chloride ion channel. The lack of this channel causes reduced water content of secretions. This affects the mucus secreted as part of the lung's defence and creates sticky, viscous mucus. In patients with CF, neutrophils are recruited in excess to the airways yet pathogens are not cleared and the patients suffer from chronic infections. In CF, the disease-causing gene has been clearly identified as the CF transmembrane conductance regulator gene, but genetic variants of the MBP and SP-A have been associated with disease severity in CF.

Table 43.1 Broad range of pathogens interacting with surfactant protein (SP)-A and SP-D

Type	Name of pathogen	Surfactant protein	Reference(s)
Bacteria	*E. coli*	SP-D	Kuan et al. (1992)
	Salmonela minnesota	SP-D	Kuan et al. (1992)
	H. pylori	SP-D	Appelmelk et al. (2005)
	Klebsiella pneumoniae	SP-D	Keisari et al. (2001), Ofek et al. (2001)
	Mycoplasma pneumoniae and *Histoplasma capsulatum*	SP-A and SP-D	Ernst (1998), Chiba et al. (2002), Gaynor et al. (1995)
	Haemophilus influenzae	Minimal effects of SP-D	Tino and Wright (1996), Restrepo et al. (1999)
	Pseudomonas aeruginosa *Stenotrophomonas maltophilia*	SP-D, not SP-A	Malloy et al. (2005), Giannoni et al. (2006), Bufler et al. (2004)
	Mycobacterium tuberculosis	Virulent and attenuated M. tuberculosis strains bind best	Ferguson et al. (1999, 2002), Hall-Stoodley et al. (2006)
	Mycobacterium avium	SP-A and SP-D	Kudo et al. (2004)
	Group B streptococcus' (*Streptococcus agalactiae*) and *S. pneumoniae*	SP-A and SP-D	Jounblat et al. (2004), Kuronuma et al. (2004)
	B. bronchiseptica (LPS); *Ruminant bronchopneumonia*	SP-D	Schaeffer et al. (2004), Grubor et al. (2004)
	Alloiococcus otitidis	SP-A	Konishi et al. (2006)
Yeast and fungi	*Cryptococcus neoformans*	SP-A and SP-D	Schelenz et al. (1995), Walenkamp et al. (1999), van de Wetering et al. (2004)
	Aspergillus fumigatus	SP-A and SP-D	Allen et al. (1999), Madan et al. (1997a, b)
	Coccidioides posadasii	SP-A and SP-D	Awasthi et al. (2004), Awasthi (2010)
	Candida albicans	SP-D	Van Rozendaal et al. (2000)
	Pneumocystis carinii	SP-D	O'Riordan et al. (1995), Vuk-Pavlovic et al. (2001), Atochina et al. (2004a), Yong et al. (2003)
Viruses	Influenza A virus	SP-A and SP-D	Malhotra et al. (1994), Hartshorn et al. (1994, 1997), Levine et al. (2001), Tecle et al. (2007b)
	HIV	SP-D binds HIV – gp120	Meschi et al. (2005)
	Respiratory syncytial virus	SP-A and SP-D	Ghildyal et al. (1999), Hickling et al. (1999), Griese (2002)

Allele associations and allele interaction of surfactant protein genes in relation to RDS have been discussed (Floros and Fan 2001). Studies have shown a deficiency of SP-A in airway fluids from patients with CF and other inflammatory pulmonary conditions. Findings suggest that the neutrophil serine proteases cathepsin G and/or elastase and/or proteinase-3 may contribute to degradation of SP-A and SP-D, thereby diminishing innate pulmonary antimicrobial defence (Rubio et al. 2004; von Bredow et al. 2001, 2003).

The dramatic decrease of SP-A and SP-D in the presence of normal surfactant phospholipid may be a mechanism underlying the relative ineffectiveness of cellular inflammatory response in killing invading bacteria in lungs of patients with CF. In bronchoalveolar lavage fluids (BALFs), although SP-A levels tend to decline in CF patients compared with non-CF, and the decline was only significant in presence of bacterial infection. Among CF patients, SP-A concentrations in BALF were inversely related to inflammation and age (Hull et al. 1997; Noah et al. 2003). Reports suggest that decreasing protease activity and increasing collectin activity may be beneficial in early CF (Alexis et al. 2006; Baker et al. 1999).

However, both, SP-D and TNF-α, are significantly increased in CF patients compared with patients of allergic fungal rhinosinusitis (AFRS), suggesting activation of both innate immunity and Th1-mediated inflammation and potential correlation between SPs and downstream adaptive immune responses (Skinner et al. 2007). Rat SP-D is highly resistant to degradation by a wide range of proteolytic enzymes. Patients with CF and chronic rhinosinusitis (CRS) with nasal polyposis demonstrated elevated SP-A1, -A2, and -D. While in patients with AFS, SP-A1, SP-A2, and SP-D, were not significantly different, these proteins are upregulated in various forms of CRS, particularly in CF-CRS (Woodworth et al. 2007).

43.2.6 Familial Interstitial Lung Disease

Amin et al. (2001) studied the development of chronic lung injury in a familial form of ILD. An 11-year-old girl, her sister, and their mother who were diagnosed with chronic ILD were negative for SP-C and decreased levels of SP-A and SP-B in BALF. Lung biopsy from both children demonstrated a marked decrease of pro-SP-C in the alveolar epithelial cells but strong staining for pro-SP-B, SP-B, SP-A, and SP-D. The apparent absence of SP-C and a decrease in the levels of SP-A and SP-B were related to familial ILD. Several linkage and association studies have been done using SPs genes as markers to locate pulmonary disease susceptibility genes, but few have studied markers systematically in different ethnic groups.

43.3 Connective Tissue Disorders

43.3.1 Systemic Sclerosis

Significant progress is being made in terms of understanding the pathogenesis and various options for therapy of systemic sclerosis patients whose disease course is complicated by ILD. The significance of serum SP-A, SP-D and KL-6 for diagnosis and treatment of ILD in connective tissue disorders has been evaluated by different workers. Serum KL-6 and SP-D levels are more specific and useful markers for diagnosis and evaluation of ILD compared with serum LDH in connective tissue disorders (Ogawa et al. 2003; Suematsu et al. 2003). Characteristics or disease activity of early ILD has been evaluated in subjects. In abnormal group, curvilinear subpleural lines or thickened interlobular and intralobular lines were observed more frequently in lower lung fields and SP-A and SP-D were higher in true abnormalities group than in control group. True parenchymal abnormalities in posterior subpleural aspect of lung may indicate early ILD activity (Al-Salmi et al. 2005; Kashiwabara 2006). Since higher levels of SP-A and SP-D are associated with more severe lung function impairment at presentation, and better recovery over time, Janssen et al. (2005) suggested that SP-A, SP-D and KL-6 are especial markers of disease activity. Nevertheless, serum pulmonary and activation-regulated chemokine (PARC) levels may be more useful marker for active PF in systemic sclerosis (SSc) (Kodera et al. 2005) since elevated PARC values correlated more sensitively reflecting the PF activity than serum KL-6 or SP-D levels.

In lung fibrosis in patients with SSc and inflammatory myopathies, KL-6, von Willebrandt factor (vWF), soluble E-selectin (sES), SP-D are good surrogate factors of PF but cannot replace conventional diagnostic procedures. However, these markers are suitable for the assessment of progression and severity of PF in systemic autoimmune disorders once the diagnosis is established (Kumánovics et al. 2008). Takahashi et al. (2006b) indicated that elevated levels of serum SP-A and SP-D reflect the presence of ILD and the combination of SP-D and X-ray contributes to reduce the risk of clinicians overlooking ILD complicated by SSc (Highland and Silver 2005; Yanaba et al. 2004).

Maeda et al. (2001) compared serum SP-D in collagen diseases such as systemic scleroderma (SSd), scleroderma spectrum disorders (SSD), systemic lupus erythematodes (SLE), Sjogren syndrome (Sjs), dermatomyositis (DM), rheumatoid arthritis (RA), and dermatitis (DE) as a control. Patients with SSc possess higher levels of SP-D than those with other collagen diseases and dermatitis, which may correspond to severity of pulmonary fibrosis (Maeda et al. 2001). The basic and clinical studies of SSc patients with ILD are yielding promising data that may be translated in to

more effective diagnostic and therapeutic strategies Although the SP-D level in sera of patients with polymyositis/dermatomyositis (PM/DM) is significantly elevated, the serum SP-D in patients with ILD was still higher than those without ILD, suggesting that serum SP-D level is a useful marker for ILD in patients with PM/DM (Ihn et al. 2002). However, there is a need to investigate whether another connective tissue disease has developed when laboratory findings cannot be explained by usual clinical course of an existing connective tissue disease (Ishiguro et al. 2007).

43.3.2 Sarcoidosis

Sarcoidosis also called sarcoid, Besnier-Boeck disease or Besnier-Boeck-Schaumann disease, is a disease in which abnormal collections of chronic inflammatory cells form as nodules in multiple organs. KL-6, SP-A and SP-D levels in BALF were increased in pulmonary sarcoidosis. Since these markers are specifically derived from epithelial cells, it is considered that KL-6 and SP-D levels are reflecting damage or release of these markers from epithelial cells due to the inflammatory response. Among serum Clara cell 16 (CC16), KL-6, and SP-D as markers of ILD, and their ability to reflect pulmonary disease severity and prognosis in sarcoidosis, KL-6 is the best marker in differentiating patients from healthy controls (Günther et al. 1999; Hamm et al. 1994; Janssen et al. 2003; Kunitake et al. 2001). The median amounts of SP-A in BAL fluid in control subjects was 2.82 mg/L (range, 0.92–5.17 mg/L). In comparison to control, SP-A in patients with asthma had a lower value of SP-A, which remained unchanged in patients with pulmonary sarcoidosis (van de Graaf et al. 1992). In contrast, SP-A levels in BAL fluids from patients with sarcoidosis were markedly higher than in control subjects and it was comparable with patients of hypersensitivity pneumonitis (HP). In both conditions, SP-A^+ alveolar macrophages were increased (Günther et al. 1999; Hamm et al. 1994).

The serum levels of SP-A in patients with IPF (205 ± 23 ng/mL) and PAP (285 ± 23 ng/mL) were significantly higher than those in healthy controls (45 ± 3 ng/mL). In patients of sarcoidosis, pneumonia, and tuberculosis SP-A values were 52 ± 27 ng/mL, 65 ± 11 ng/mL, and 49 ± 23 ng/mL, respectively. The SP-A appears to circulate in the bloodstream as a complex with Ig in IPF and in PAP (Kuroki et al. 1993).

43.4 Pulmonary Alveolar Proteinosis

A diffuse lung process of unknown etiology is characterized by the presence of alveolar spaces filled with amorphous eosinophilic (but sometimes basophilic) PAS-positive material of predominantly phospholipid nature in alveolar lumina. It is generally regarded as type of response to alveolar injury and results from accumulation of surfactant apoprotein through either: increased secretion by granular pneumocytes, or abnormal uptake and handling by alveolar macrophages. The prominent increase of SP-A and SP-D in BAL fluids and sputum is diagnostic for pulmonary alveolar proteinosis (PAP) (Kuroki et al. 1998; Brasch and Müller 2004; Takahashi et al. 2006a). There are reports about polymorphisms and mutations on the surfactant protein genes, especially SP-B that may be associated with congenital alveolar proteinosis.

43.4.1 Idiopathic Pulmonary Alveolar Proteinosis

SP-A in BALF of PAPs patients is significantly increased in comparison to normal volunteers and hence can be used as a diagnostic tool in the clinical laboratory (Brasch et al. 2004; Honda et al. 1996). PAP is a rare lung disorder and can be caused by inactivation of either granulocyte-macrophage colony-stimulating factor (GM-CSF) or GM receptor common β-chain (βc) genes in mice [$GM^{-/-}$, $\beta c^{-/-}$], demonstrating a critical role of GM-CSF signaling in surfactant homeostasis. Studies demonstrate abnormal accumulation of SP-A and SP-D in air spaces of patients with PAP (Crouch et al. 1993) and the precursors of SP-B, SP-B and SP-C. Although lung histology in $\beta c^{-/-}$ and $GM^{-/-}$ mice was indistinguishable, distinct differences were observed in surfactant phospholipid and surfactant protein concentrations in lungs of $\beta c^{-/-}$ and $GM^{-/-}$ mice. The defect in clearance was significantly more severe in $GM^{-/-}$ than in $\beta c^{-/-}$ mice. GM-CSF concentrations, increased in BALF but not in serum of $\beta c^{-/-}$ mice, were consistent with a pulmonary response to the lack of GM-CSF signaling. The observed differences in surfactant metabolism suggest the presence of alternative clearance mechanisms regulating surfactant homeostasis in mice and may provide a molecular basis for the range in severity of PAP symptoms (Reed et al. 2000). In a young patient with idiopathic PAP, the enhanced serum anti-GM-CSF antibody level demonstrated a striking difference in the distribution of SP-A and SP-D in intra-alveolar substance with idiopathic PAP (Ohtsuki et al. 2008; Kobayashi et al. 2008b).

Evidence suggests that not only an impairment of surfactant clearance by alveolar macrophages, but also an abnormal secretion of transport vesicles containing precursors of SP-B (but not SP-C) and an insufficient palmitoylation of SP-C, which may lead to the formation of di- and oligomeric SP-C forms, play a role in the pathogenesis of pulmonary alveolar proteinosis.

43.4.2 Structural Changes in SPs in PAP

The primary structures of human pulmonary SPs isolated from lung lavage of patients with alveolar proteinosis demonstrate significant differences from lung surfactant proteins isolated from lungs of healthy individuals. In contrast to SP-A from normal lungs, PAP-SP-A was shown to contain large amounts of non-reducable cross-linked β chains, where as proteinosis SP-B showed a significantly increased molecular weight by approx. 500 Da for the unreduced protein dimer. In contrast, SP-C from proteinosis patients was modified by (1) partial or even complete removal of palmitate residues and (2) additional N-terminal proteolytic degradation (Voss et al. 1992).

Pathophysiological structural modifications in SP-A seemed to occur in the alveolar space, and may lead to a reduced surfactant function (Voss et al. 1992). Multimerized form of SP-A oligomer (alveolar proteinosis protein-I, APP-I) has been detected besides the normal-sized octadecamer (APP-II) in SP-As isolated from PAP patients. Analysis of APP revealed that it was composed of two proteins. The M_r of APP-I and APP-II were 1.65 MDa and 0.93 MDa, respectively. APP-I and APP-II showed almost identical amino acid compositions. Electron microscopy revealed that APP-II was a hexameric particle, presumably consisting mainly of octadecamers whose diameter was approximately 30 nm. In contrast, APP-I was made of multimerized larger aggregates whose diameter appeared to be about 70–90 nm. Both APP-I and APP-II retained the abilities to bind DPPC. Reconstitution experiments with porcine SP-B and phospholipids revealed that multilamellated membranes in structures formed from APP-I consisted of several layers of doubled unit membranes. APP-I failed to form tubular myelin structures. In contrast, APP-II formed well-formed lattice structures seen in tubular myelin The multimerized form of human SP-A oligomer exhibits the reduced capacity to regulate phospholipid secretion from type II cells, and lower affinity to bind to type II cells. It is to be reminded that the integrity of a flower-bouquet-like octadecameric structure of SP-A oligomer is important for the expression of full activity of this protein, indicating the importance of the oligomeric structure of mammalian lectins with collagenous domains. Thus there exists an abnormal multimerized form of SP-A oligomer in the alveoli of patients with PAP that exhibits abnormal function on phospholipid membrane organization (Hattori et al. 1996a, b).

In alveolar proteinosis, cholesterol/disaturated phospholipid ratios (CHOL/DSP) are invariably elevated, whereas the SP-A/DSP and SP-B/DSP ratios are generally elevated. Because the SP-B/SP-A ratio was normal in all cases, it was suggested that structural changes to the proteins occurred secondarily and that caution must be used in comparing functional data derived using SP-A obtained from patients with PAP (Doyle et al. 1998). The major part of SP-A from a proteinosis patient consisted of SP-A2 gene product while SP-A1 gene product was present in only a small amount. The disulfide bridges in the carbohydrate recognition domain were identified to be in the 1–4, 2–3 pattern common for collectins. Interchain disulfide bridges were discovered between two Cys-48 residues and cysteine residues in the N-terminal region. However, the exact disulfide bridge connections within the bouquet-like ultrastructure could not be established (Berg et al. 2000).

43.5 Respiratory-Distress Syndrome and Acute Lung Injury

43.5.1 ARDS and Acute Lung Injury

Acute respiratory distress syndrome (ARDS), also known as respiratory distress syndrome (RDS) or adult respiratory distress syndrome (in contrast with IRDS) is a serious reaction to various forms of injuries to lung. ARDS is caused by a variety of direct and indirect issues. It is characterized by inflammation of lung parenchyma leading to impaired gas exchange with concomitant systemic release of inflammatory mediators causing inflammation, hypoxemia and frequently resulting in multiple organ failure. A less severe form is called acute lung injury (ALI). Clinical and biochemical evidences suggest that the etiology of RDS is multifactorial with a significant genetic component. There are reports about polymorphisms and mutations on the surfactant protein genes, especially surfactant proteins-B that may be associated with RDS, ARDS, and congenital alveolar proteinosis. The measurement of SP-A and SP-D in amniotic fluids and tracheal aspirates reflects lung maturity and the production level of the lung surfactant in infants with RDS. The SP-A concentrations in BAL fluids are significantly reduced in patients with ARDS and also in patients at risk to develop ARDS (Kuroki et al. 1998; Takahashi et al. 2006a). Patients with low concentrations of SP-A and SP-B in the BAL are at risk for ARDS before onset of clinically defined lung injury, though the SP-D concentrations remain in normal range. Thus, SP abnormalities occur before and after the onset of ARDS, and the responses of SP-A, SP-B, and SP-D differ in important ways. However, plasma SP-D is a valuable biomarker in ALI/ARDS and SP-A increases during the early phase of ARDS, including some molecular alteration followed by decrease during the late phase (Endo et al. 2002; Kuroki et al. 1998; Takahashi et al. 2006b; Zhu et al. 2001).

Elevated level of SP-A has also been reported in the sera of patients with acute cardiogenic pulmonary edema (APE) and in patients with ARDS relative to healthy subjects and ventilated patients with no cardio-respiratory disease. Serum SP-A was inversely related to blood oxygenation and to

static respiratory system compliance both at the time of patient's entry into the study and during the course of admission. Since SP-B is synthesized as a precursor smaller than alveolar SP-A, Doyle et al. (1995, 1997) suggested that immunoreactive SP-B that enters more readily than SP-A, is cleared acutely, and provides a better indicator of lung trauma (Shimura et al. 1996).

Prematurely born infants can develop the neonatal RDS because of a deficiency of pulmonary surfactant. At autopsy RDS lungs lacked tubular myelin and had decreased immunoreactivity for antisera to SP-A, an important component of tubular myelin. Therefore, a role for SP-A in the conversion of lamellar bodies to tubular myelin and in the pathogenesis of RDS was proposed. It was postulated that if SP-A is indeed necessary for the conversion of lamellar bodies to tubular myelin, in RDS either there is a deficiency of adequate amounts of functional SP-A, or some other important component of surfactant is missing (deMello et al. 1993). Mechanical ventilation is the main modality of treatment of ARDS. On mechanical ventilation, there is a progressive increase in SP-A levels in patients with ARDS, and may be one of the contributors for recovery in ARDS. A significant increase within the first 4 days was found in those infants who survived, whereas no such change was found in those infants who died (Balamugesh et al. 2003; Stevens et al. 1992). Intratracheal aerosolization of LPS in rats produces typical features of human ARDS. The SP-D binds inhaled LPS-endotoxin in vivo, which may help to protect the lung from endotoxin-induced disease (van Rozendaal et al. 1999). The SP-D was reduced in lung of young rats following ALI at early stage and early administration of Dex could reverse the SP-D content (Shu et al. 2007). SP-A in sera of cord blood from infants born at gestational ages <32 weeks with RDS was 15.1 ng/mL compared to without RDS (5.8 ng/mL) and significantly related to the non-RDS outcome (Cho et al. 2000). Shimoya et al. (2000) suggested that IL-6 elevation in fetuses with chorioamnionitis promotes fetal lung maturation by inducing SP-A synthesis, thereby decreasing the incidence of RDS in the preterm neonates.

Acute Lung Injury (ALI): Plasma SP-A, but not SP-D, was higher in patients with fewer days of unassisted ventilation and in patients with an absence of intact alveolar fluid clearance. In contrast, pulmonary edema fluid SP-D, but not SP-A, was lower in patients with worse oxygenation. Reduced pulmonary edema fluid SP-D and elevated plasma SP-A concentrations at the onset of ALI may be associated with more severe disease and worse clinical outcome and may serve as valuable biochemical markers of prognosis (Cheng et al. 2003). The BALF proteome analysis showed the presence of several isoforms of SP-A, in which an N-non-glycosylierte form and several proline hydroxylations were identified (Bai et al. 2007). In the plasma and edema fluid, protein profile of ALI patients showed multiple qualitative changes. Nearly all ALI patients also had protein spots that indicated truncation or other posttranslational modifications (Bowler et al. 2004).

43.5.2 Bronchopulmonary Dysplasia (BPD)

The pathophysiology of bronchopulmonary dysplasia (BPD) as an inflammatory disorder, secondary to neonatal RDS represents a major complication of prematurity. Maximum SP-A and anti-SP-A antibodies (SAS) immune complex values between 2 and 4 weeks after birth correlate with subsequent development of BPD independently and may be useful in analyzing the course and outcome of neonatal RDS, in particular the likelihood of subsequent development of BPD (Strayer et al. 1995). Weber et al. (2000) investigated an association of polymorphisms of SP-A1 and SP-A2 encoding genes and the risk of BPD in Caucasian preterm infants below 32 weeks of gestation matched for immaturity and year of birth. BPD was defined as oxygen dependency or need for mechanical ventilation at day 243. A significantly increased frequency of SP-A1 polymorphism $6A^6$ in infants was associated with BPD compared with controls. In addition to established risk factors for BPD, $6A^6$ polymorphism for SP-A1 gene is an independent co-factor.

BPD_28D (O2 dependency at 28 days of life) and BPD_36W (O2 dependency at 36 week post-menstrual age) are diseases of prematurely born infants exposed to mechanical ventilation and/or oxygen supplementation. Genetic variants of SP-A, B, C, and D and SP-B-linked microsatellite markers are risk factors in BPD. Significant associations were observed for alleles of SP-B and SP-B-linked microsatellite markers, and haplotypes of SP-A, SP-D, and SP-B. Unlike SP-A, SP-D does not contribute to lowering surface tension. SP-D-deficient mice have no respiratory abnormalities at birth, but it causes development of emphysema and predisposition to specific infections. No human infant or child with respiratory distress and mutation in the SP-D gene has been identified (Yurdakök 2004). Studies in larger sample size are warranted to confirm these observations and delineate genetic background of BPD subgroups (Pavlovic et al. 2006).

43.5.2.1 SP-A Deficiency in Primate Model of BPD with Infection

In a baboon model of hyperoxia-induced BPD and superimposed infection, animals constituting a group- pro re nata (PRN) were delivered by hysterotomy at 140 days gestational age and ventilated on clinically appropriate

oxygen for a 16-day experimental period and served as controls. Immunostaining with SP-A, SP-B, and SP-C antibodies showed variable staining patterns. The study demonstrated that a deficiency of SP-A mRNA expression persists in chronic lung injury and variable protein staining patterns are manifested depending upon the underlying pathology (Coalson et al. 1995; King et al. 1995).

Awasthi et al. (1999) measured SP-A and SP-D levels and their mRNAs in three groups of animals: (1) nonventilated premature baboon fetuses; (2) neonatal baboons delivered prematurely at 140 d gestation age (ga) and ventilated with PRN O_2; (3) animals of same age ventilated with 100% O_2 to induce chronic lung injury. In chronic lung injury, SP-A is significantly reduced in alveolar space. SP-D concentration in lavage was nearly equal to that in normal adults, but the total collectin pool in lavage was still significantly reduced. Because these collectins may bind and opsonize bacteria and viruses, decrements in their amounts may present additional risk to those premature infants who require prolonged periods of ventilatory support (Awasthi et al. 1999). Reduced SP-D expression in BAL fluid was associated with progression of bronchial dysplasia in heavy smokers. SP-D levels in BAL fluid may serve a potential biomarker to identify smokers who are at risk of early lung cancer (Sin et al. 2008b). Cheng et al. (2003) proved the hypothesis that reduced pulmonary edema fluid SP-D and elevated plasma SP-A concentrations at onset of ALI may be associated with more severe disease and worse clinical outcome and may serve as valuable biochemical markers of prognosis (Cheng et al. 2003).

43.6 Chronic Obstructive Pulmonary Disease (COPD)

43.6.1 COPD as a Group of Diseases

Obstructive lung disease is a category of respiratory disease characterized by airway obstruction. Chronic obstructive pulmonary disease (COPD), also known as chronic obstructive airways disease (COAD) or chronic airflow limitation (CAL) is a group of illnesses characterised by airflow limitation that is not fully reversible. The flow of air into and out of the lungs is impaired. The COPD is characterized by chronic inflammation. It is most likely the result of complex interactions of environmental and genetic factors. Term COPD includes the conditions of emphysema and chronic bronchitis although most patients with COPD have characteristics of both conditions to varying degrees. Asthma being a reversible obstruction of airways is often considered separately, but many COPD patients also have some degree of reversibility in their airways. The most common cause of COPD is cigarette smoking. COPD may also be caused by breathing in other particles and gases. Diagnosis of COPD is established through spirometry and chest X-ray although other pulmonary function tests can be helpful. Emphysema can only be seen on CT scan. COPD is generally irreversible although lung function can partially recover if the patient stops smoking. α1-antitrypsin deficiency is a rare genetic condition that results in COPD (particularly emphysema) due to lack of antitrypsin protein which protects fragile alveolar walls from protease enzymes released by inflammatory processes.

The prevalence of COPD is age-dependent, suggesting an intimate relationship between the pathogenesis of COPD and aging. Genetic polymorphism in SP-A is associated with the development of COPD in Chinese Hans. The genotypes of patients with COPD and healthy smoking subjects as controls for SP-A gene showed that in COPD group, the frequencies of +186 locus genotypes AA, AG and GG were 86.4%, 12.5% and 1.1%i respectively; compared to 66.7%, 27.6% and 5.7% in control group. The frequencies of polymorphic genotypes at +655 locus and +667 loci showed no significant difference between the COPD group and control group (Xie et al. 2005).

43.6.1.1 Serum SP-A in COPD and Its Relation to Smoking

SP-A occurs physiologically in small amounts in blood. Tobacco smoke induces increased alveolo-capillary leakage of SPs into blood and its level in blood may help in the assessment of lung injury caused by smoke. SP-A is occasionally elevated in non-ILD pulmonary patients. Serum SP-A increased in current smokers than in never- or ex-smokers and in COPD and pulmonary thromboembolism than in other diseases. Serum SP-D and KL-6 were unaffected by smoking. Therefore, different baseline levels of serum SP-A need to be established for smokers and non-smokers. Serum SP-A may be a useful marker for predicting COPD in the preclinical stage (Behera et al. 2005; Kobayashi et al. 2008a). Different alleles of SP-A and SP-D associated with various diseases have been summarized by Kishore et al. (2005) and given in Table 43.2. Analysis between COPD and smokers revealed several COPD susceptibility alleles (AA62_A, B1580_C, D2S388_5), based on an odds ratio (OR $>$ 2.5). Results indicate that surfactant protein alleles may be useful in COPD by either predicting the disease in a subgroup and/or by identifying disease subgroups that may be used for therapeutic intervention (Guo et al. 2001).

Proteome research revealed increased levels of SP-A in COPD but not in normal or fibrotic lung. Furthermore, elevated SP-A protein levels were detected from the induced sputum supernatants of COPD patients. The levels of other surfactant proteins (SP-B, SP-C, SP-D) were not altered. It is suggested that SP-A is linked to the pathogenesis of COPD and can be considered as a potential COPD biomarker (Ohlmeier et al. 2008). Toxic metals and transition elements

are detectable in exhaled breath condensate (EBC) of studied subjects (Mutti et al. 2006).

43.6.1.2 SP-D Is an Ideal Biomarker in COPD

In COPD, SP-D is an ideal biomarker that is produced mostly in lungs and can be measured in the peripheral circulation. It changes with the clinical status of the patient and has inherent functional attributes that suggest a possible causal role in pathogenesis of disease (Sin et al. 2008b, c).

In a multivariable linear regression model, COPD was independently associated with lower SP-D levels. Given the importance of this molecule in lung, low levels may play a role in the pathogenesis and/or progression of COPD (Sims et al. 2008). Inhaled corticosteroids alone or in combination exhibited partial systemic anti-inflammatory effects, reducing significantly only SP-D serum levels. ICS in conjunction with long-acting β2-adrenergic agonist significantly reduced serum SP-D levels. These drugs reduce lung-specific but not generalized biomarkers of systemic inflammation in COPD. Hydrofluoroalkane-beclomethasone dipropionate (HFA-BDP) controls eosinophilic inflammation, including in distal airways, more effectively than fluticasone propionate (FP) Diskus (Ohbayashi and Adachi 2008; Sin et al. 2008a).

43.6.2 Emphysema

Emphysema is a chronic pulmonary disease marked by an abnormal increase in size of air spaces. Pulmonary emphysema, a major component of COPD, is pathologically characterized by destructive alterations in pulmonary architectures as a result of persistent inflammation. Emphysema may be a dynamic disease process in which alveolar wall cell death and proliferation are repeated. The decrease of surfactant protein secreted by the alveolar type II cell is one of the important causes of limiting air of pulmonary emphysema and the changes of SP-A may be related to emphysematous changes in the lung. Cigarette smoke and LPS alter lung SP-A gene activity and protein homeostasis (Hu et al. 2008). Mice deficient in SP-D$^{-/-}$ develop progressive emphysema with age. SP-D gene-targeted mice develop severe pulmonary lipidosis, and foamy macrophage infiltrations. By lowering surface tension at the air-water interface in the surfactant deficient premature lung, exogenous surfactant replacement therapy for neonatal RDS has been highly successful in decreasing mortality after preterm birth. It has emerged that SP-A and SP-D have additional roles in host defence distinct from the surface tension lowering effects of surfactant. Recombinant forms of SP-D could be useful therapeutically in attenuating inflammatory processes in neonatal chronic lung disease, cystic fibrosis, and emphysema (Clark and Reid 2003).

43.6.3 Allergic Disorders

43.6.3.1 Allergic Inflammation in Asthma

Asthma is an obstructive lung disease where the bronchial tubes (airways) are extra sensitive (hyperresponsive). The airways become inflamed and produce excess mucus and muscles around the airways tighten making the airways narrower. Asthma is usually triggered by breathing in things present in air such as dust or pollen that produces an allergic reaction. It may be triggered by other things such as an upper respiratory tract infection, cold air, exercise or smoke. Asthma is diagnosed by the characteristic pattern of symptoms. A peak flow meter can record variations in the severity of asthma over time. Spirometry can provide an assessment of the severity, reversibility, and variability of airflow limitation, and help confirm the diagnosis of asthma. Significant changes occur in levels of SP-A and SP-D during the asthmatic response in animal models as well as in asthmatic patients. The impact of the SP-A and SP-D on asthmatic allergic inflammation and vice versa has been reviewed (Hohlfeld et al. 2002). Serum SP-D concentrations are affected in allergic patients and correlate with changes in allergic airway inflammation. Serum SP-D levels may give additional information, beside bronchial hyperresponsiveness (BHR) and sputum eosinophils, about the degree of bronchial inflammation in allergic patients (Koopmans et al. 2004).

Immunoregulatory Roles of SP-A and SP-D

Studies on allergen-sensitized murine models and asthmatic patients show that SP-A and SP-D can: specifically bind to aero-allergens; inhibit mast cell degranulation and histamine release; and modulate the activation of alveolar macrophages and DCs during the acute hypersensitive phase of allergic response (Erpenbeck et al. 2005; Wang et al. 1998). They also can alleviate chronic allergic inflammation by inhibiting T-lymphocyte proliferation as well as increasing phagocytosis of DNA fragments and clearance of apoptotic cell debris. Furthermore, it has emerged, from the studies on SP-D-deficient mice, that, when these mice are challenged with allergen, they develop increased eosinophil infiltration, and abnormal activation of lymphocytes, leading to the production of Th2 cytokines. Intranasal administration of SP-D significantly attenuated the asthmatic-like symptoms seen in allergen-sensitized wild-type, and SP-D-deficient, mice. These findings provide a new insight of role that surfactant proteins play in handling environmental stimuli and in their immunoregulation of airway inflammatory disease (Wang and Reid 2007).

Both SP-A and SP-D can inhibit histamine release in the early phase of allergen provocation and suppress lymphocyte proliferation in the late phase of bronchial inflammation, the two essential steps in the development of asthmatic

symptoms (Wang et al. 1998). Studies suggest that the increased levels of SP-A and D may play a protective role in an allergic inflammation in the pathogenesis of bronchial asthma. Structural remodelling of airways in asthma that follows inflammation may be affected by SP-D-mediated effects on immune response. SP-D accumulation is increased in this model of allergen-induced eosinophilia, both in upper and lower airways (Cheng et al. 2000; Kasper et al. 2002). SP-D gene-deficient mice (*Sftpd*$^{-/-}$) have an impaired systemic Th-2 response at baseline and reduced inflammation and airway responses after allergen exposure. Translational studies revealed that a polymorphism in *SFTPD* gene was associated with lower atopy and possibly asthma susceptibility. Thus, SP-D-dependent innate immunity influences atopy and asthma (Brandt et al. 2008). Dex significantly down-regulates SP-D in allergic airways and lavage fluid. In addition, Dex promoted airway expression of vitamin D-binding protein, heptoglobin and α1-antitrypsin (Zhao et al. 2007).

Serum SP-D is increased in acute and chronic inflammation in mice. Profiles of SP-A and SP-D in acute and chronic inflammation indicated that serum SP-D can serve as a biomarker of lung inflammation in both acute and chronic lung injury in mice (Fujita et al. 2005). Because of their capability to directly inhibit T-cell activation and T-cell-dependent allergic inflammatory events, SP-A and SP-D may be significant contributors to the local control of Th-2 type inflammation in the airways. SP-D is able to reduce the immediate allergen-induced mediator release and the early bronchial obstruction in addition to its effects on airway inflammation and bronchial hyperresponsiveness in an *A. fumigatus* mouse asthma model. Thus, SP-D not only reduces allergen-induced eosinophilic inflammation and airway hyper-responsiveness but also provides protection against early airway obstruction by inhibition of early mediator release (Erpenbeck et al. 2006; Takeda et al. 2003). However, mice sensitized and challenged with either *A. fumigatus* or OVA increased SP-D levels in their lung. Allergen exposure induced elevation in SP-D protein levels in an IL-4/IL-13-dependent manner, which in turn, prevents further activation of sensitized T cells. This negative feedback regulatory circuit could be essential in protecting the airways from inflammatory damage after allergen inhalation (Haczku et al. 2006). Haczku (2006) support the hypothesis that SP-A and SP-D have a role in regulation of allergic airway sensitization.

Murine Model of Asthma

Dust mite allergens can directly activate alveolar macrophages (AΦs), induce inflammatory cytokines, and enhance T-helper type 2 cytokine production. The SP-D is able to bind mite allergens and alleviates allergen-induced airway inflammation and may be an important modulator of allergen-induced pulmonary inflammation (Liu et al. 2005a). There is marked reduction in SP-A and SP-D levels in the BALF of dust mite (*Dermatophagoides pteronyssinus*, Der p)-sensitized BALB/c mice after allergen challenge. Both SP-A and SP-D were able to suppress Der p-stimulated intrapulmonary lymphocyte proliferation of naïve mice with saline or allergen challenge, or of Der p-sensitized mice with saline challenge. On the contrary, this suppressive effect was mild on lymphocytes from sensitized mice after allergen challenge. These results indicated the involvement of pulmonary surfactant proteins in the allergic bronchial inflammation of sensitized mice (Wang et al. 1996, 2001). Both SP-A and SP-D down-regulate the eosinophilic inflammation in murine asthma models and shift the cytokine profile towards a T helper cell type 1 response. In addition, they are effective at alleviating bronchial hyperresponsiveness. There is evidence of activation of innate immune system in asthma which results in the production of pro-inflammatory cytokines and may contribute to the pathogenesis of neutrophilic asthma (Simpson et al. 2007).

43.6.3.2 Chronic Sialadenitis and Chronic Rhinosinusitis

SP-A and mRNA and protein were detected in glands of patients with chronic sialadenitis. The expression in salivary glands of patients with chronic sialadenitis was significantly higher than from healthy salivary glands. SP-A immunoreactivity, localized in the epithelial cells and submucosal glands of paranasal sinus mucosa in normal and chronic sinusitis patients, was enhanced in chronic rhinosinusitis mucosa as compared with normal paranasal sinus mucosa (Lee et al. 2004, 2006). SP-A expression in human nasal tissue was correlated with symptoms suggestive of allergic rhinitis. (Wootten et al. 2006).

43.6.4 Interactions of SP-A and SP-D with Pathogens and Infectious Diseases

Microbial targets for SP-D include both Gram-positive and Gram-negative respiratory pathogens, influenza, and respiratory syncytial viruses, *Cryptococcus neoformans, Pneumocystis carinii*, and *Aspergillus fumigatus*. Both monocytes/macrophages and neutrophils express surface receptors that can interact with SP-D. The interactions between SP-D and microorganisms and in many instances immune cells promote both microbial aggregation and enhanced phagocytosis. SP-D has been shown to bind to a variety of bacteria, including rough strains of *Salmonella Minnesota* and *E. coli* as well as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Lim et al. 1994). SP-D also stimulates the phagocytosis of *Pseudomonas aeruginosa* (Restrepo et al. 1999). The interaction of SP-D with bacteria

often results in CRD-dependent bacterial aggregation or agglutination. Unlike SP-A (van Iwaarden et al. 1994), SP-D does not bind to lipid A. It interacts with *E. coli* through the core polysaccharides and/or the O-specific antigens. The core region of the LPS of other gram-negative bacteria is broadly recognized by SP-D as well (Kuan et al. 1992). SP-D can be used as a biomarker for chronic periodontitis. As no significant associations of SFTPD gene polymorphisms could be detected, other mechanisms influencing SP-D serum/plasma expression might exist (Glas et al. 2008).

SP-D has been shown to bind to the influenza A virus, resulting in aggregation of the target (Hartshorn et al. 1996a). The binding and inhibition of hemagglutination was inhibited by chelation of calcium and by carbohydrates, suggesting that the interaction of SP-D with the virus was mediated via the CRD. SP-D also enhances the neutrophil uptake of the virus in a calcium-dependent manner (Hartshorn et al. 1997). Further enhanced antiviral and opsonic activity for influenza A virus was obtained by making a human MBP and SP-D chimera (White et al. 2000) (Table 43.1). The degree of multimerization of SP-D also appears to be important for its interactions with viruses (Brown-Augsburger et al. 1996; Hartshorn et al. 1996b). SP-D induces massive aggregation of influenza A virus particles (Hartshorn et al. 1996a). This massive agglutination of organisms could contribute to lung host defence by promoting airway mucociliary clearance, but it could also promote internalization by phagocytic cells. Recombinant SP-D inhibited RSV infectivity both in vitro and in vivo (Hickling et al. 1999; Le Vine et al. 2004), and reduced SP-D protein levels have been detected in RSV infection (Kerr and Paton 1999). A direct interaction between the yeast *Candida albicans* and SP-D confirms the importance of SP-D in innate immunity (van Rozendaal et al. 2000).

43.6.4.1 Distinct Effects of SP-A or -D Deficiency During Bacterial Infection

Surfactant proteins A and D expressed in respiratory tract bind bacterial, fungal and viral pathogens, enhancing their opsonization and killing by phagocytic cells. Clearance of bacterial pathogens including group *B streptococci, Haemophilus influenza, Pseudomonas aeruginosa* and viral pathogens, *respiratory syncytial virus, adenovirus and influenza A virus*, was deficient in SP-A$^{-/-}$ mice (Table 43.1). Mice lacking SP-A (SP-A$^{-/-}$) or SP-D (SP-D$^{-/-}$) and wild-type mice, infected with group *B streptococcus* or *Haemophilus influenzae*, are associated with increased inflammation and inflammatory cell recruitment in lung after infection. Although, decreased killing of group B *streptococcus* and *H. influenzae* was observed only in SP-A$^{-/-}$ mice but not in SP-D$^{-/-}$ mice, bacterial uptake by alveolar macrophages was reduced in both SP-A- and SP-D-deficient mice. Isolated alveolar macrophages from SP-A$^{-/-}$ mice generated significantly less, whereas those from SP-D$^{-/-}$ mice generated significantly greater superoxide and H_2O_2 compared with wild-type alveolar macrophages.

In SP-D$^{-/-}$ mice, bacterial killing was associated with increased lung inflammation and increased oxidant production. Where as, bacterial killing was decreased and associated with increased lung inflammation and decreased oxidant production in SP-A$^{-/-}$, macrophage phagocytosis was decreased in both SP-A and SP-D deficient mice. SP-A deficiency was associated with enhanced inflammation and synthesis of pro-inflammatory cytokines. SP-D$^{-/-}$ mice cleared these bacteria as efficiently as wild-type mice; however, clearance of viral pathogens was deficient in SP-D$^{-/-}$ mice and associated with increased inflammation. Study suggests that SP-A and SP-D play distinct roles during bacterial infection of lung (LeVine et al. 2000, 2001).

Alloiococcus otitidis has been found to be associated with otitis media with effusion. SP-A and MBL interact with *A. otitidis* in Ca^{2+}-dependent manner. Results demonstrate that *A. otitidis* is a ligand for SP-A and TLR2, and that the collectins enhance the phagocytosis of *A. otitidis* by macrophages, suggesting important roles of collectins and TLR2 in the innate immunity of the middle ear against *A. otitidis* infection (Konishi et al. 2006). Meningococcal disease occurs after colonization of nasopharynx with *Neisseria meningitidis*. Variation in genes of surfactant proteins affects the expression and function of SPs. Gene polymorphism resulting in substitution of glutamine with lysine at residue 223 in the CRD of SP-A2 increases susceptibility to meningococcal disease, as well as the risk of death (Jack et al. 2006). In contrast to defensive function, SP-D in BALF binds β-glucan onB. *Dermatitidis and*, blocks BAM access to β-glucan, thereby inhibiting TNF-α production. Thus, whereas BALF constituents commonly mediate antimicrobial activity, *B. dermatitidis* may utilize BALF constituents, such as SP-D, to blunt the host defensive reaction; this effect could reduce inflammation and tissue destruction but could also promote disease (Lekkala et al. 2006)

43.7 Pulmonary Tuberculosis

43.7.1 Enhanced Phagocytosis of *M. tuberculosis* by SP-A

During initial infection with *M. tuberculosis*, bacteria that reach the distal airspaces of lung are phagocytosed by AMΦs in presence of pulmonary surfactant. Studies indicated a direct interaction between SP-A and macrophage in mediating enhanced adherence of *M. tuberculosis* (Gaynor et al. 1995). Since, SP-A binds mannose, it was hypothesized that SP-A attaches to *M. tuberculosis* and serves as a ligand

between *M. tuberculosis* and AΦs. Stokes et al. (1998) demonstrated that explanted alveolar AΦs do not efficiently bind *M. tuberculosis* in a serum-free system, although a small subpopulation of these AΦs could bind mycobacteria. In contrast, almost 100% of peritoneal AΦs bind mycobacteria under similar conditions. Evidence suggests that opsonic binding of *M. tuberculosis* by differentiated alveolar Ms is mediated by complement and CR3, and that the poor binding by resident alveolar AΦs is due to their poor expression of CR3. Thus, attachment of *M. tuberculosis* to AΦs is an essential early event in primary pulmonary tuberculosis and SP-A helps in early capture and phagocytosis of *M. tuberculosis* by AΦs. Ferguson et al. (2002) provided evidence for specific binding of SP-D to *M. tuberculosis* and indicated that SP-D and SP-A serve different roles in the innate host response to this pathogen in lung.

43.7.1.1 Lipomannan and ManLAM are Major Mycobacterial Lipoglycans as Potential Ligands

The SP-A binds to *M. bovis Bacillus Calmette-Guerin* (BCG), the vaccinating strain of pathogenic mycobacteria, and also to a lesser extent to *M. smegmatis*, which indicates that SP-A does not discriminate virulent from nonpathogenic strains. Lipomannan and mannosylated lipoarabinomannan (ManLAM) are two major mycobacterial cell-wall lipoglycans, which act as potential ligands for binding of SP-A. Both the terminal mannose residues and the fatty acids are critical for binding. It appears that recognition of carbohydrate epitopes on lipoglycans by SP-A is dependent on the presence of fatty acids (Sidobre et al. 2000, 2002).

Rivière et al, (2004) claim that the hydrophobic aglycon part of ManLAM is associated to a supra-molecular organization of these complex molecules. Furthermore, the deacylated ManLAMs or the lipid-free mannosylated arabinomannans, which do not exhibit characteristic ManLAM activities, do not display this supra-molecularorganization. These observations suggest that the ManLAMs immunomodulatory activities might be associated to their particular organization. The critical micellar concentration of ManLAMs obviously supports the notion that this supra-molecularorganization may be responsible for the specific biological activities of these complex molecules (Rivière et al. 2004).

As indicated, the molecular recognition of ManLAM terminal mannose units by CRDs of SP-A depends on the presence of lipid moiety of ManLAMs associated to a characteristic supra-molecular organization of ManLAM complex. On the other hand, the deacylated ManLAM or the lipid-free mannosylated arabinomannans, which do not exhibit characteristic ManLAM activities, do not display this supra-molecular organization. Therefore the ManLAM immunomodulatory activities might be associated to their particular organization. The critical micellar concentration of ManLAM supports the notion that this supra-molecular organization is responsible for specific biological activities of these complex molecules.

Apa Glycoprotein on *M.* tuberculosis: A Potential Adhesion to SP-A: Although lipoglycan ManLAM is considered as the major C-type lectin target on mycobacterial surface, Ragas et al. (2007) identified Apa (alanine- and proline-rich antigenic) glycoprotein as new potential target for SP-A, which binds to purified Apa. Apa is associated to the cell wall for a long time to aid in the attachment of SP-A. Because, Apa seems to be restricted to the *M. tuberculosis* complex strains, it was proposed that it may account for selective recognition of complex strains by SP-A containing homologous functional domains.

SP-A Enhances *M. avium* Ingestion by Macrophages: Tuberculosis leads to immune activation and increased HIV-1 replication in lung. SP-A promotes attachment of *M. tuberculosis* to AΦs during infection with HIV. SP-A levels and attachment of *M. tuberculosis* to AΦs inversely correlate with peripheral blood CD4 lymphocyte counts (Downing et al. 1995). *M. avium* complex (MAC) is a significant cause of opportunistic infection in patients with AIDS. Once in lung, MAC can interact with SP-A. Work on pulmonary pathogens including *M. bovis* BCG suggests that SP-A participates in promoting efficient clearance of these organisms by AMs. Lopez et al. (2003) reported that SP-A can bind to and enhance the uptake of MAC by AΦs, similar to BCG and *M. tuberculosis*. However, unlike BCG and other pulmonary pathogens that are cleared in presence of SP-A via a NO-dependent pathway, macrophage-mediated clearance of MAC is not enhanced by SP-A.

Suppression of Reactive Nitrogen Intermediates by SP-A in AMs in Response to *M. tuberculosis:* Reactive nitrogen intermediates (RNIs) play a significant role in the killing of *mycobacteria*. RNI levels generated by AΦs were significantly increased when IFNγ-primed AΦs were incubated with *M. tuberculosis*. However, the RNI levels were significantly suppressed in presence of SP-A. Furthermore, incubation of deglycosylated SP-A with *M. tuberculosis* failed to suppress RNI by AΦs, suggesting that the oligosaccharide of SP-A, which binds to *M. tuberculosis*, is necessary for this effect. Pasula et al. (1999) showed that SP-A-mediated binding of *M. tuberculosis* to AΦs and decreased RNI levels may be one mechanism by which *M. tuberculosis* diminishes the cytotoxic response of activated AΦs.

43.7.2 SP-A Modulates Inflammatory Response in AΦs During Tuberculosis

There is a severe reduction in SP-A levels in BAL during tuberculosis only in the radiographically involved lung

segments, and the levels returned to normal after 1 month of treatment. The SP-A levels were inversely correlated with the percentage of neutrophils in BAL fluid, suggesting that low SP-A levels were associated with increased inflammation in the lung. SP-A has pleiotropic effects even at low concentrations found in tuberculosis patients. This protein augments inflammation in presence of infection and inhibits inflammation in uninfected macrophages, protecting uninvolved lung segments from the deleterious effects of inflammation (Gold et al. 2004).

SP-A modulates phenotypic and functional properties of cells of adaptive immune response such as DCs and lymphocytes. Bone marrow-derived DCs generated in presence of SP-A fail to increase LPS-induced up-regulation of MHC class II and CD86 co-stimulatory molecule on DCs surface and behaves like "tolerogenic DCs". SP-A may also induce tolerance by suppressing the proliferation of activated T lymphocytes (Hussain 2004). SP-A suppresses lymphocyte proliferation and IL-2 secretion, in part, by binding to its receptor, SP-R210. However, the mechanisms underlying this effect are not well understood. The effects of antibodies against the SP-A-binding (neck) domain (α-SP-R210n) or nonbinding C-terminal domain (α-SP-R210ct) of SP-R210 on human peripheral blood T cell immune responses against *M. tuberculosis* support the hypothesis that SP-A, via SP-R210, suppresses cell-mediated immunity against *M. tuberculosis* via a mechanism that up-regulates secretion of IL-10 and TGF-β1 (Samten et al. 2008). Role of SP-A and SP-D in linking innate and adaptive immunity to regulate host defense has been suggested by Wright (2005). Although both SP-A and SP-D can bind to T cells and directly inhibit proliferation, SP-A can also indirectly inhibit T-cell proliferation via suppression of dendritic cell (DC) maturation. SP-D has been shown to enhance antigen uptake and presentation. Taken together, these in vitro results suggest that the combined role of SP-A and SP-D is to modulate the immunologic environment of the lung so as to protect the host, yet thwart an overzealous inflammatory response that could potentially damage the lung and impair gas exchange (Wright 2005)

43.7.3 Marker Alleles in *M. tuberculosis*

Regression analyses of tuberculosis and tuberculin-skin test positive groups, on the basis of odds ratios, revealed tuberculosis susceptibility (DA11_C and GATA_3) and protective (AAGG_2) marker alleles. Similarly, between tuberculosis patients and general population control subjects, susceptibility $1A^3$, $6A^4$, and B1013_A and protective AAGG_1, and AAGG_7 marker alleles were observed. Moreover, interactions were seen between alleles $6A^2$ and $1A^3$ and between $1A^3$ and B1013_A. Studies indicate a possible involvement of SP alleles in tuberculosis pathogenesis (Floros et al. 2000). Malik et al. (2006) investigated polymorphisms in the *SFTPA1* and *SFTPA2* genes for association with tuberculosis in 181 Ethiopian families comprising 226 tuberculosis cases. Four polymorphisms, SFTPA1 307A, SFTPA1 776T, SFTPA2 355C, and SFTPA2 751C, were associated with tuberculosis. Additional subgroup analysis in male, female and more severely affected patients provided evidence for SFTPA1/2-covariate interaction. Among five intragenic haplotypes identified in SFTPA1 gene and nine identified in SFTPA2 gene, $1A^3$ was most significantly associated with tuberculosis susceptibility (Table 43.2).

SNPs in Collagen Region of SP-A2 as a Contributing Factor: Relation exists between polymorphisms in the collagen regions of SP-A2 genes and pulmonary tuberculosis. Seven SNPs (4 exonic and 3 intronic) were identified in collagen regions of SP-A1 and SP-A2 genes in Indian population. Two intronic polymorphisms, SP-A1C1416T and SP-A2C1382G showed significant association with pulmonary tuberculosis. A redundant SNPA1660G of SP-A2 gene showed significant association with pulmonary tuberculosis. This polymorphism, when existing along with a non-redundant polymorphism, SP-A2G1649C (Ala91Pro) resulted in a stronger association with pulmonary tuberculosis. The SNPs in collagen region of SP-A2 may be one of the contributing factors to the genetic predisposition to pulmonary tuberculosis (Madan et al. 2002).

43.7.4 Interaction of SP-D with *M. tuberculosis*

Since many mycobacteria are facultative intracellular pathogens, their ability to cause disease involves entry, survival and replication within host cells. Although much progress has been made in our understanding of entry by mycobacteria, we anticipate that clarification of role of entry in pathogenesis will require further application of newly developed molecular tools to dissect each of the proposed mechanisms.

SP-D is known to bind *M. tuberculosis*. Binding of SP-D to *M. tuberculosis* is calcium dependent, and carbohydrate inhibitable. The binding of SP-D to Erdman lipoarabinomannan is mediated by terminal mannosyl oligosaccharides of this lipoglycan. Incubation of *M. tuberculosis* with subagglutinating concentrations of SP-D leads to reduced adherence of bacteria to macrophages, whereas incubation of

Table 43.2 SP-A and SP-D alleles associated with various diseases (Kishore et al. 2005).

Polymorphism		Disease association, population, type of study
Gene	Allele	
SP-A2	1A0	Susceptibility, RDS, Caucasian
SP-A1	6A2	Susceptibility, RDS, Caucasian
SP-A1	6A3	Protection, RDS, Caucasian
SP-A2	1A2	Protection, RDS, Caucasian
SP-A1	6A3	Protection, RDS, Negroids
SP-A1, SP-A2	6A2-1A0	Susceptibility, RDS, Caucasian, family
SP-A1	6A2	Protection, RDS, Caucasian, twins
SP-A1, SP-A2	6A2-1A0	Protection, RDS, Caucasian, twins
SP-A1	6A6	Susceptibility, BPD, Caucasian
SP-A	AA62_A	Susceptibility, COPD, Mexican
SP-D	D2S388_5	Susceptibility, COPD, Mexican
SP-A1	6A1	Susceptibility, IPF, Mexican
SP-D	DA11_C	Susceptibility, TB, Mexican
SP-A2	1A3	Susceptibility, TB, Mexican
SP-A1	6A4	Susceptibility, TB, Mexican
SP-A1, SP-A2	6A2-1A0	Susceptibility, TB, Mexican
SP-A1	C1416T	Susceptibility, TB, Indian
SP-A2	C1382G	Susceptibility, TB, Indian
SP-A2	A1660G-G1649C	Susceptibility, TB, Indian
SP-D	G459A	Susceptibility, TB, Indian
SP-D	T3130G	Susceptibility, TB, Indian
SP-D	Met 11	Susceptibility, RSV, Finnish
SP-A2	A1660G-G1649C	Susceptibility, ABPA, Indian

RDS respiratory distress syndrome, *BPD* bronchopulmonary dysplasia, *COPD* chronic obstructive pulmonary disease, *IPF* idiopathic pulmonary fibrosis, *TB* tuberculosis, *RSV* respiratory syncy–tial virus, *ABPA* allergic bronchopulmonary aspergillosis (Adapted with permission from Kishore et al. 2005 © Springer)

bacteria with SP-A leads to significantly increased adherence to monocyte-derived macrophages. Ferguson et al. (2002) provided evidence for specific binding of SP-D to *M. tuberculosis* and indicated that SP-D and SP-A serve different roles in the innate host response to this pathogen in lung. Further studies provide direct evidence that inhibition of phagocytosis of *M. tuberculosis* affected by SP-D occurs independently of aggregation process. SP-D limits the intracellular growth of bacilli in macrophages by increasing phagosome-lysosome fusion but not by generating a respiratory burst (Ferguson et al. 2006). Results also provide evidence that SP-A and SP-D enhance mannose receptor-mediated phagocytosis of *M. avium* by macrophages (Kudo et al. 2004). Virulent and attenuated *M. tuberculosis* strains bind best to immobilized SP-A (Hall-Stoodley et al. 2006). *Mycobacterium avium* has developed numerous mechanisms for entering mononuclear phagocytes. The SP-A, and SP-D, exhibit a concentration-dependent binding to *M. avium*. Studies provide evidence that SP-A and SP-D enhance mannose receptor-mediated phagocytosis of *M. avium* by macrophages (Kudo et al. 2004).

43.7.5 Association of SPs with Diabetes

Insulin decreased SP-A gene transcription in human lung epithelial cells (Miakotina et al. 2002). Alveolar type II cells and nonciliated bronchiolar epithelial (Clara) cells in lungs of rats with diabetes have decreased SP-A, but increased mRNA. This is on account of differential expression in the level of SP-A, SP-B, and SP-C mRNAs in both alveolar and bronchiolar epithelial cells from diabetic lungs in comparison to control lungs (Sugahara et al. 1994). Nonetheless, Fernández-Real et al. (2008) reported circulating SP-A significantly higher among patients with glucose intolerance and type 2 diabetes than in subjects with normal glucose tolerance, even after adjustment for BMI, age, and smoking status. In amniotic fluid from diabetic women, SP-A levels were significantly less than in nondiabetic pregnancies. Hypertension did not modify SP-A in diabetic women. Although Snyder et al. (1988) suggested that the concentration of amniotic fluid SP-A is decreased in diabetic pregnancies, McMahan et al. (1987) concluded that in well controlled diabetic pregnancies fetal lung maturation is not adversely affected. SP-A and SP-B were significantly elevated in amniotic fluid from black mothers and in amniotic fluid from mothers who smoked during pregnancy (Pryhuber et al. 1991).

43.8 Expression of SPs in Lung Cancer

43.8.1 Non-Small-Cell Lung Carcinoma (NSCLC)

Molecular mechanisms underlying carcinogenesis of non-small cell lung cancer(NSCLC) may provide gene targets in critical pathways valuable for improving the efficacy of therapy and survival of patients with NSCLC (Chong et al. 2006). SP-A is described for a portion of NSCLC facilitating a diagnostic marker for these carcinomas (Goldmann et al. 2009). Studies in human lung carcinoma reported positive staining of tumor cells for SP-A, especially in peripheral airway cell carcinoma, which include bronchioloalveolar carcinoma and in some reports also papillary subtypes. The SP-A gene is expressed at higher levels in hyperplastic cells; the expression occurs predominantly, but not exclusively, in adenocarcinomas (Broers et al. 1992; Linnoila et al. 1992). The determination of SP-A in malignant effusions may help in distinguishing primary lung adenocarcinoma from adenocarcinomas of

miscellaneous origin. Analysis of SP-A gene transcript in pleural effusion is useful for diagnosis of primary lung adenocarcinoma (Saitoh et al. 1997; Shijubo et al. 1992). Gene expression of SP-A and SP-C was restricted to metastatic pulmonary adenocarcinomas (Betz et al. 1995). Camilo et al. (2006) suggested that all adenocarcinomas were negative for p63 where as 4 (26.6%) of 15 were positive for SP-A.

Uzaslan et al. (2005) studied 169 primary adenocarcinomas of lung (109 acinar, 32 solid with mucin, 24 papillary and 4 mucinous) for SP-A expression. Twenty-five percent of acinar, 38% of papillary and 3% of solid adenocarcinoma with mucin showed a positive intracytoplasmic SP-A reaction of the tumor cells. Results support the theory that SP-A-producing cells may generate not only bronchioloalveolar and papillary carcinoma, but also other subtypes of lung adenocarcinoma (Stoffers et al. 2004; Uzaslan et al. 2005). Tsutsumida et al. (2007) advocate that high MUC1 expression on the surface is an important characteristic of a micropapillary pattern, where as reduced surfactant apoprotein A expression in the micropapillary pattern may be an excellent indicator for poor prognosis in small-size lung adenocarcinoma.

43.8.1.1 Genetic Factors as Lung Cancer Risk

Deletions of the SP-A gene are specific genomic aberrations in bronchial epithelial cells adjacent to and within NSCLC, and are associated with tumor progression and a history of smoking. SP-A deletions might be a useful biomarker to identify poor prognoses in patients with NSCLC who might therefore benefit from adjuvant treatment (Jiang et al. 2005). Seifart et al. (2005) genotyped for SP-A1, -A2, -B, and -D marker alleles in lung cancer subgroups, which included 99 patients with small cell lung carcinoma (SCLC), or non-SCLC (NSCLC, n = 68) consisting of squamous cell carcinoma (SCC), and adenocarcinoma (AC); controls and healthy individuals (population control). Seifart et al. (2005) found (a) no significant marker associations with SCLC, (b) rare SP-A2 ($1A^9$) and SP-A1 ($6A^{11}$) alleles associate with NSCLC risk when compared with population control, (c) the same alleles (1A9, $6A^{11}$) associate with risk for AC when compared with population ($6A^{11}$) or clinical control ($1A^9$), and (d) the SP-A1-$6A^4$ allele (found in ~10% of the population) associates with SCC, when compared with control. A correlation between SP-A variants and lung cancer susceptibility appears to exist, indicating that SP-A alleles may be useful markers of lung cancer risk.

The SP mRNAs with SP-A, B, and C were coexpressed in 10/12 (83%) of adenomas and 4/5 (80%) of carcinomas in both solid and tubulopapillary areas. SP-D mRNA signals were not noted in normal or neoplastic lung. ISH for SP A, B, or C mRNA was a helpful aid in the diagnosis of proliferative lesions of the murine lung (Pilling et al. 1999). In ovine pulmonary adenocarcinoma, caused by jaagsiekte sheep retrovirus, SP-A and C were expressed in 70% and 80% of tumor cells, respectively, whereas Clara cell 10-kDa protein was expressed in 17% of tumor cells (Platt et al. 2002).

43.8.1.2 TTF-1 and SP-A in Differential Diagnosis

Results suggest that TTF-1 can play an important role for the maintenance and/or differentiation process in bronchiolar and alveolar cells (Nakamura et al. 2002). TTF-1 is frequently expressed in human lung cancer, especially in adenocarcinoma and small cell lung cancer, and TTF-1 expression is closely related to the expression of surfactant protein. Zamecnik and Kodet (2002) described positive results for TTF-1 and SP-A in 75% and 46% of pulmonary adenocarcinomas and in 50% and 25% of pulmonary non-neuroendocrine large cell carcinomas (LCCs), respectively. Small cell lung carcinomas were TTF-1 positive in 89% of cases and completely negative for SP-A. Squamous cell carcinomas and carcinoid tumors were negative for both proteins. The frequency of TTF-1 expression in the nucleus was very low in human lung cancer cell lines; however, their cytoplasmic positivities should be further investigated (Fujita et al. 2003. Rossi et al. (2003) (1) support the metaplastic histogenetic theory for pulmonary carcinomas group of tumors; (2) show that cytokeratin 7 and TTF-1, but not SP-A, are useful immunohistochemical markers in this setting, and (3) suggest that this group of tumors has a worse prognosis than conventional NSCL carcinoma at surgically curable stages I, justifying their segregation as an independent histologic type. Lu et al. (2006) suggested that nuclear inclusions positive for SP-A antibody staining in adenocarcinomas of lung were derived from accumulated content in the perinuclear cistern resembling pseudoinclusion processes and composed of proteins antigenically cross-reactive with SP-A. Because of its diagnostic utility TTF-1 should be added to a panel of antibodies used for assessing tumors of unknown origin. The combination of anti-TTF-1 with anti-SP-A does not increase the diagnostic usefulness of TTF-1 alone (Lu et al. 2006). Suzuki et al. (2005) and Ueno et al. (2003) reported that Napsin is better marker than SP-A for diagnosis of lung adenocarcinoma. Napsin A is an aspartic proteinase expressed in lung and kidney. Napsin A is expressed in type II pneumocytes and in adenocarcinomas of lung.

43.8.1.3 SPs as a Tool for Diagnosis of Lung Tumors

Most bronchioloalveolar carcinomas of lung react positively for SP-A. Positive SP-A staining of large cell carcinoma of the lung could indicate that at least part of these tumors have the same cellular origin or differentiation as bronchioloalveolar carcinoma. Twenty of 63 (32%) tumors stained positive for SP-A. This may imply that about one third of large cell

carcinomas of lung have a similar cellular origin or differentiation as bronchioloalveolar carcinoma (Uzaslan et al. 2006).

SP-A, a marker for lung adenocarcinomas, can be used to differentiate lung adenocarcinomas from other types and metastatic cancers of other origins (Kuroki et al. 1998; Takahashi et al. 2006b). RT-PCR and primers specific for SP-A, SP-B, SP-C and SP-D genes were used to detect nodal metastases and occult tumor spread of pulmonary adenocarcinomas. A combination of SP-A and SP-D may help to establish a differential prognosis in patients with gefitinib-induced ILD (Kitajima et al. 2006). ILD is a serious adverse event in lung cancer patients treated with gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI). Pretreatment with gefitinib exacerbated LPS-induced lung EGFR-TKI by reducing SP-A expression in lung. EGFR- TKI may reduce SP-A expression in lungs of lung cancer patients and thus patients treated with EGFR tyrosine kinase inhibitor may be susceptible to pathogens (Inoue et al. 2008).

SP mRNAs were present in all lung tumors, with SPs A, B, and C being co-expressed in 83% of adenomas and 80% of carcinomas in both solid and tubulopapillary areas. No signals for SP D mRNA were noted in normal or neoplastic lung. Additionally, no staining for any SP transcript was observed in the hepatocellular carcinoma metastases. In situ hybridization for SP A, B, or C mRNA was helpful in diagnosis of proliferative lesions of the murine lung, enabling differentiation from hepatocellular metastases (Pilling et al. 1999; Qi et al. 2002).

43.9 Other Inflammatory Disorders

43.9.1 Airway Inflammation in Children with Tracheostomy

The long-term tracheostomy in infants and children may perpetuate chronic airway inflammation and airway remodeling due to easier access to the lungs for microorganisms. The SP-A and SP-D may directly interact with invading microorganisms and also modulate the activity of local immune cells. Children with tracheostomy had an increased total number of cells, increased neutrophils, and more frequently bacteria, but no viruses were recovered. SP-D concentration was reduced to half, though SP-A, SP-B, and SP-C were not different from controls. SP-D was inversely correlated to neutrophils, and high numbers of bacteria were associated with lower SP-D concentrations. It was suggested that bacteria and low SP-D support neutrophilic inflammation in the lower respiratory tract of nonsymptomatic with children with tracheostomy (Griese et al. 2004). BAL fluids from patients carrying a chronic tracheostoma agglutinated *P. aeruginosa*, which was completely inhibited by maltose. The agglutination of *P. aeruginosa* by BAL fluid was related in part to the concentration of SP-D. Additional factors, such as the multimeric organization of SP-D, are likely to contribute to the agglutination of microorganisms by BAL or other body fluids (Griese and Starosta 2005).

Pulmonary Alveolar Microlithiasis (PAM): Pulmonary alveolar microlithiasis (PAM) is an uncommon chronic disease characterized by calcifications within the alveoli and a paucity of symptoms in contrast to image findings. PAM occurs in the absence of any known disorder of calcium metabolism. Takahashi et al. (2006a) reported two cases of PAM, with markedly elevated sera concentrations of SP-A and SP-D, which showed a tendency to increase as the disease progressed. Therefore, SP-A and SP-D may function as serum markers to monitor the disease activity and progression of PAM.

Gastroesophageal Reflux Disease: Children with gastroesophageal reflux often suffer from chronic, severe lung damage and recurrent infections. The mechanisms may involve reflux induced lung injury with alterations of the SP-A and SP-D, which bind specifically to various microbes and increase their elimination by granular leukocytes and macrophages. In children with gastroesophageal reflux disease (GERD), the macromolecular organization of SP-A and SP-D were significantly reduced. The more active SP-A and especially those of SP-D were diminished, whereas the smaller sized forms of SP-D were markedly increased. Reduced amounts of SP-A and SP-D and an altered structural organization of the surfactant proteins may contribute to pathogenesis of chronic lung disease commonly observed in these children (Griese et al. 2002).

43.9.2 Surfactant Proteins in Non-ILD Pulmonary Conditions

Infants with increased pulmonary blood flow secondary to congenital heart disease suffer from tachypnea, dyspnea, and recurrent pulmonary infections. In congenital heart disease with pulmonary hypertension secondary to increased pulmonary blood flow, there is a decrease in SP-A gene expression as well as a decrease in SP-A and SP-B protein contents (Gutierrez et al. 2001). In an experiment involving 4-week-old lambs with pulmonary hypertension secondary to increased pulmonary blood flow following an in utero placement of an aortopulmonary vascular graft, Lee et al. (2004) found a decrease in SP-A gene expression as well as a decrease in SP-A and SP-B protein contents. But in a lamb model of congenital heart disease with pulmonary hypertension and increased pulmonary blood flow, the effect of the shunt on SP gene expression and protein content was not

apparent within first week of life (Lee et al. 2004). No significant association between the common genetic variants of SP-A and SP-D and victims of sudden infant death syndrome (SIDS) was disclosed by Stray-Pedersen et al. (2009). However, low SP-A protein expression may possibly be determined by the 6A2/1A0 SP-A haplotype, which should be a subject for further investigation.

The SP-A level decreases significantly in acute pulmonary embolism, which may play an important role in hypoxemia in pulmonary embolism (Xie et al. 2005). Although an immunohistochemical investigation of pulmonary SP-A suggested a characteristic increase in fatal asphyxiation, no particular change was observed in the total amount of SP-A mRNA. The analysis of the SP-A1/A2 ratio may assist interpretation of the molecular alterations of SP-A related to acute asphyxial death (Ishida et al. 2002).

In hyperpnea there is a significant increase in lamellar bodies (LB) SP-A, lysozyme, and phospholipid (PL) but no change in the protein-to-prolonged hyperpnea ratios. It was suggested that (1) surfactant-associated lysozyme is secreted with LB, (2) the majority of SP-A is linked to lipid secretion but not necessarily with LB, and (3) the majority of SP-B secretion is independent of PL secretion. (4) Hyperpnea did not alter the mRNA expression of SP-A, SP-B, SP-C, or lysozyme in alveolar type II cells, but expression of SP-A and SP-B mRNA was significantly increased in lung tissue (Yogalingam et al. 1996).

43.10 DNA Polymorphisms in SPs and Pulmonary Diseases

Though the genes underlying susceptibility to RDS are insufficiently known, genes coding for SP-A and B have been assigned as the most likely genes in the etiology of RDS. Acute-RDS (ARDS) develops in association with many serious medical disorders. Mortality is at least 40%, and there is no specific therapy. The deficiency in SP-A level has been implicated in the pathophysiology of ARDS. Associations between single nucleotide polymorphisms (SNPs) of human gene coding *SFTPA1, SFTPA2,* and *SFTPD* and infectious pulmonary diseases have been established by several groups.

43.10.1 Association Between SP-A Gene Polymorphisms and RDS

Evidences suggest that the etiology of RDS is multifactorial with a significant genetic component. There are reports about polymorphisms and mutations on the surfactant protein genes, especially surfactant proteins-B, that may be associated with RDS, ARDS, and congenital alveolar proteinosis. The human SP-A gene locus includes two functional genes, *SFTPA1* and *SFTPA2* which are expressed independently, and a pseudo gene. SP-A polymorphisms play a role in respiratory distress syndrome, allergic bronchopulmonary aspergillosis and idiopathic pulmonary fibrosis. The levels of SP-A are decreased in lungs of patients with CF, RDS and chronic lung diseases (Heinrich et al. 2006).

Both low levels of SP-A and SP-A alleles have been associated with RDS. Floros et al. characterized four allelic variants of SP-A1 gene (6A, $6A^2$, $6A^3$, and $6A^4$) and five allelic variants of the SP-A2 gene (1A, $1A^0$, $1A^1$, $1A^2$, and $1A^3$) and hypothesized that specific SP-A alleles/genotypes are associated with increased risk of RDS. Because race, gestational age (**GA**), and sex are risk factors for RDS, Kala et al. (1998) studied the distribution and frequencies of SP-A alleles/genotypes while adjusting for these factors as confounders or effect modifiers in control and RDS populations with GAs ranging from 24 week to term. Although the odds ratios of several alleles and genotypes were in opposite directions for black and white subjects, the homogeneity of odds ratio reached statistical significance only in case of $6A^3/6A^3$. Although differences were observed in subgroups with different GAs of RDS white population, definitive conclusions could not be made regarding the effect of modification by GA or as a function of sex. Study suggested that (1) the genetic analyses of RDS and SP-A locus should be performed separately for black and white populations and (2) SP-A alleles/genotypes and SP-B variant may contribute to the etiology of RDS and/or may serve as markers for disease subgroups. In a genetically homogeneous Finnish population, Rämet et al. (2000) showed that certain SP-A1 alleles ($6A^2$ and $6A^3$) and an SP-A1/SP-A2 haplotype ($6A^2/1A^0$) were associated with RDS. The $6A^2$ allele was over-represented and the $6A^3$ allele was under-represented in infants with RDS. According to results, diseases associated with premature birth did not explain the association between the odds of a particular homozygous SP-A1 genotype ($6A^2/6A^2$ and $6A^3/6A^3$) and RDS. In the population evaluated, SP-B intron 4 variant frequencies were low and had no association with RDS. Thus, SP-A gene locus is an important determinant for predisposition to RDS in premature infants.

Floros et al. (2001b), in family-based linkage studies to discern linkage of SP-A to RDS, showed a link between SP-A and RDS; certain SP-A alleles/haplotypes are susceptibil ($1A^0$, $6A^2$, $1A^0/6A^2$) or protective ($1A^5$, $6A^4$, $1A^5/6A^4$) for RDS. Some differences between blacks and whites with regard to SP-A alleles may exist. In a 107 father-mother-offspring trios, divided into two sets according to proband's phenotype, Haataja et al. (2001) evaluated familial segregation of candidate gene polymorphisms by the transmission disequilibrium test. A set of 76 trios were analyzed for transmission disequilibrium from parents to affected offspring. Another set of

31 trios were studied for allele transmission from parents to hypernormal offspring born very prematurely before GA of 32 weeks. SP-A1-A2 haplotype $6A^2$-$1A^0$ showed significant excess transmission to affected infants and SP-A1 allele $6A^2$ decreased transmission to the hypernormals. Study provides a support for a role of SP-A alleles as genetic predisposers to RDS in premature infants.

43.10.2 SP-A and SP-B as Interactive Genetic Determinants of Neonatal RDS

Haataja et al. (2000) investigated if SP-B gene or interaction between SP-A and SP-B genes has a role in genetic susceptibility to RDS. Of the two SP-B polymorphisms genotyped, the Ile131Thr variation, a putative N-terminal N:-linked glycosylation site of proSP-B and length variation of intron 4 have been suggested to associate with RDS. Neither of the two SP-B polymorphisms associated directly with RDS or with prematurity. Instead, results showed that known association between SP-A alleles and RDS was dependent on the SP-B Ile131Thr genotype. Hence, the SP-B Ile131Thr polymorphism is a determinant for certain SP-A alleles as factors causing genetic susceptibility to RDS ($6A^2$, $1A^0$) or protection against it ($6A^3$, $1A^2$).

Floros et al. (2001a) studied genotypes for SP-B intron 4 size variants and for four SNPs [−18 (A/C), 1013 (A/C), 1580 (C/T), 9306 (A/G)] in SP-B in black and white subjects. Based on odds ratio: (1) the SP-B intron 4 deletion variant in white subjects is more of an RDS risk factor for males and for subjects of 28 weeks < gestational age (GA) <33 weeks; (2) the SP-B intron 4 insertion variant in black subjects is more of an RDS risk factor in females; (3) in white subjects, SP-A1 ($6A^2/6A^2$) or SP-A2 ($1A^0/1A^0$ or $1A^0$/*) genotypes in subjects of certain GA and with a specific SP-B genotype (9306 (A/G) or deletion/*) are associated with an enhanced risk for RDS; (4) in black subjects, SP-A1 ($6A3/6A^3$ or $6A^3$/*) genotypes in subjects of 31 weeks < or = GA < or = 35 weeks and with the SP-B (1580 (T/T)) genotype are associated with a reduced risk for RDS. The SP-B polymorphisms are important determinants for RDS. These may identify differences between black and white subjects, as well as, between males and females regarding the risk for RDS. Moreover, SP-A susceptibility or protective alleles, in specific SP-B background, are associated with an increased or reduced risk for RDS.

43.10.3 RDS in Premature Infants

DNA samples from 441 premature singleton infants and 480 twin or multiple infants were genotyped for SP-A1, SP-A2, and SP-B exon 4 polymorphisms and intron 4 size variants in a homogeneous white population. Distribution of SP-A and SP-B gene variants between RDS and no-RDS infants were determined alone and in combination. The SP-A1 allele 6A2 and homozygous genotype 6A2/6A2 are over-represented in RDS of singletons when SP-B exon 4 genotype was Thr/Thr, and under represented in RDS of multiples when the SP-B genotype was Ile/Thr. The SP-A 6A2 allele in SP-B Thr131 background predisposed the smallest singleton infants to RDS, whereas near-term multiples were protected from RDS. There was a continuous association between fetal mass and risk of RDS, defined by SP-A and SP-B variants. Labeled lung explants with the Thr/Thr genotype showed proSP-B amino-terminal glycosylation, which was absent in Ile/Ile samples. Hence, Genetic and environmental variation may influence intracellular processing of surfactant complex and the susceptibility to RDS (Marttila et al. 2003b). However, the association between SP-A polymorphisms and RDS may not be applicable to entire population of premature infants. In twins, the association between SP-A polymorphism and RDS is different from that seen in premature singleton infants. The factor associated with SP-A genotype-specific susceptibility to RDS appears to be related to the size of uterus and length of gestation at birth (Marttila et al. 2003a). Zhai et al. (2008) reported that the frequency of SP-A1 allele $6A^2$ and $6A^3$ expression of SP-A in Chinese premature infants was low in neonatal RDS. In contrast, the frequency of SP-A2 allele $1A^0$ and $1A^1$ was high in normal Chinese premature infants. It supports that SP-A1 allele $6A^2$ may be a susceptible gene for RDS.

43.10.4 Gene Polymorphism in Patients of High-altitude Pulmonary Edema

A pathogenetic cofactor for development of high-altitude pulmonary edema (HAPE) is an increase in capillary permeability, which could occur as a result of an inflammatory reaction and/or free-radical-mediated injury to lung. Pulmonary SP-A has potent antioxidant properties and protects unsaturated phospholipids and growing cells from oxidative injury (Swenson et al. 2002). In view of protective role of SP-A against oxidative damage, Saxena et al. (2005) examined the association of constitutional susceptibility to HAPE with polymorphisms in SP-A1 and SP-A2. Allele frequencies of three loci in SP-A1 and one in SP-A2 were significantly different between low-altitude native (LAN) HAPE patients and LAN control subjects. Heterozygous individuals, with respect to SP-A1 C1101T and SP-A2 A3265C, showed less severity in oxidative damage in comparison with homozygous subjects (SP-A1 T1101 and SP-A2 C3265). The polymorphisms in SP-A1 might be one of the genetic factors contributing to susceptibility to HAPE (Saxena et al. 2005).

43.10.5 SNPs in Pulmonary Diseases

Four validated SNPs were genotyped with sequence-specific probes (TaqMan 7000) in 284 newborn infants below 32 weeks of GA. The finding of an association of a variant of the *Sftpd* gene, that has previously been shown to be associated with increased SP-D serum levels in adult patients with RDS in preterm infants, may provide a basis for the initial risk assessment of RDS and modification of surfactant treatment strategies. A role for SP-D in neonatal pulmonary adaptation has to be postulated. Genotyping for three SNP altering amino acids in the mature protein in codon 11 ($Met^{11}Thr$), 160 ($Ala^{160}Thr$), and 270 ($Ser^{270}Thr$) of the SP-D gene was performed and related to the SP-D levels in serum. Individuals with Thr/Thr-11-encoding genotype had significantly lower SP-D serum levels than individuals with Met/Met (11) genotype. Polymorphic variation in the N-terminal domain of the SP-D molecule influences oligomerization, function, and the concentration of the molecule in serum (Hilgendorff et al. 2009; Leth-Larsen et al. 2005; Sorensen et al. 2007).

Studies on twins indicated very strong genetic dependence for serum levels of SP-D. Sequencing of 5′ untranslated region (5′UTR), the coding region and the 3′ region of *Sftpd* gene of 32 randomly selected blood donors indicated one single *Sftpd* haplotype (allele frequency 13.53%) that showed a negative association with serum SP-D levels. The discovery of a frequent negative variant of *Sftpd* gene provides a basis for genetic analysis of function of SP-D in resistance against pulmonary infections and inflammatory disorders in humans (Heidinger et al. 2005). The presence of SP-D in non-pulmonary tissues, such as gastrointestinal tract and genital organs, suggest additional functions located to other mucosal surfaces. Sorensen et al. (2007) summarized studies on genetic polymorphisms, structural variants, and serum levels of human SP-A and SP-D and their associations with human pulmonary disease.

Polymorphisms of genes are transmitted together in haplotypes, which can be used in study of development of complex diseases such as RDS. Genetic haplotypes of these SP genes are associated with the development of RDS. Studies identify protective haplotypes against RDS and support findings related to SP genetic differences in children who develop RDS. An allele association study of 19 polymorphisms in SP-A1, SP-A2, SP-B, and SP-D genes in ARDS was carried out. Analysis revealed differences in frequency of alleles for some of the microsatellite markers flanking SP-B, and for one polymorphism (C/T) at nucleotide 1580 [C/T (1580)], within codon 131 ($Thr^{131}Ile$) of the SP-B gene. The latter determines the presence or absence of a potential N-linked glycosylation site. Based on the odds ratio, the C allele may be viewed as a susceptibility factor for ARDS. These data suggest that SP-B or a linked gene contributes to susceptibility to ARDS (Lin et al. 2000; Thomas et al. 2007).

Amino Acid Variants in SP-D Are Not Associated with Bronchial Asthma: As SP-D binds and neutralizes common allergens like house dust mites it is especially important in allergic asthma. Levels of SP-D are elevated in serum and alveolar lavage of asthmatic patients. Three common amino acid variants have been identified in SP-D and association of first variant has been described to severe infection with respiratory syncytial virus. The three polymorphisms leading to amino acid exchanges ($Met^{11}Thr$, $Ala^{160}Thr$, and $Ser^{270}Thr$) were typed in 322 asthmatic children and none of these polymorphisms was associated with bronchial asthma. Haplotype analyses revealed four major haplotypes all of which were evenly distributed between the populations. Functional amino acid variants in SP-D do not seem to play a major role in the genetic pre-disposition to bronchial asthma in children (Krueger et al. 2006).

Following allergen exposure in vivo, $SP\text{-}D^{-/-}$ mice expressed higher bronchoalveolar lavage (BAL) eosinophils and IL-13 and lower FN-γ expression at early time points compared with wild mice. IL-10 expression was increased at early time points in $SP\text{-}D^{-/-}$ compared with wild mice. SP-D may be critical for the modulation of early stages of allergic inflammation in vivo (Schaub et al. 2004).

Pettigrew et al. (2006, 2007) evaluated gene polymorphisms in loci encoding SP-A and risk of otitis media during first year of life among a cohort of infants at risk for developing asthma in white infants. Polymorphisms at codons 19, 62, and 133 in SP-A1, and 223 in SP-A2 were associated with race/ethnicity. In regression models incorporating estimates of uncertainty in haplotype assignment, the $6A^4/1A^5$ haplotype was protective for otitis media among white infants. On similar line, analyses suggested that polymorphisms within *SFTPA* loci may be associated with wheeze and persistent cough in white infants at risk for asthma. These associations require replication and exploration in other ethnic/racial groups.

43.10.6 Allergic Bronchopulmonary Aspergillosis and Chronic Cavitary Pulmonary Aspergillosis (CCPA)

Individuals with any structural or functional defects in SP-A and SP-D due to genetic variations might be susceptible to aspergillosis. Single nucleotide polymorphism in genes of collagen region of SP-A1 and SP-A2 has been associated with allergic bronchopulmonary aspergillosis (ABPA) and its clinical markers. SP-A2 G1649C and SP-A2 A1660G, polymorphisms in the collagen region of SP-A2, might be one of the contributing factors to genetic predisposition and

severity of clinical markers of ABPA. SNPs in SP-A2 and MBL genes showed significant associations with patients of ABPA in an Indian population. Patients carrying either one or both of GCT and AGG alleles of SP-A2 and patients with A allele at position 1011 of MBL had markedly higher eosinophilia, total IgE antibodies and lower FEV1. Therapeutic administration of SP-D and MBL proteins in a murine model of pulmonary invasive aspergillosis rescued mice from death. In mice mimicking human ABPA, SP-A and SP-D suppressed IgE levels, eosinophilia, pulmonary cellular infiltration and cause a marked shift from a pathogenic Th2 to a protective Th1 cytokine profile. Thus, collectins play an important role in Aspergillus mediated allergies and infections (Madan et al. 2005; Saxena et al. 2003).

Patients with CCPA or ABPA of Caucasian origin were screened for SNPs in collagen region of SP-A1 and SP-A2 and MBL. The T allele at T1492C and G allele at G1649C of SP-A2 were observed at slightly higher frequencies in ABPA patients (86% and 93%) than in controls (63% and 83%), and the C alleles at position 1492 and 1649 were found in higher frequencies in CCPA patients (33% and 25%) than in ABPA patients (14% and 7%). However, the CC genotype at position 1649 of SP-A2 was significantly associated with CCPA. Similarly, ABPA patients showed a higher frequency of TT genotype (71%) at 1492 of SP-A2 than controls (43%) and CCPA patients (41%). In case of MBL, the T allele and CT genotype at position 868 (codon 52) were significantly associated with CCPA, but not with ABPA. Further analysis of genotype combinations at position 1649 of SP-A2 and at 868 of MBL between patient groups showed that both CC/CC and CC/CT SP-A2/MBL were found only in CCPA patients, while GG/CT SP-A2/MBL was significantly higher in CCPA patients in comparison to ABPA patients. SNPs in SP-A1 did not differ between patients and controls. Distinct alleles, genotypes and genotype combinations of SP-A2 and MBL may contribute to differential susceptibility of the host to CCPA or ABPA (Vaid et al. 2007).

Allergic Airway Inflammation: The SP-A has potent immunomodulatory activities. SP-A protein levels in the BAL fluid showed a rapid, transient decline that reached the lowest values (25% of controls) 12 h after intranasal Af provocation of sensitized mice. It was speculated that a transient lack of SP-A following allergen exposure of airways may significantly contribute to the development of a T-cell dependent allergic immune response (Scanlon et al. 2005). After acute ovalbumin-induced allergic airway inflammation (1) alveolar epithelial type II cells (AEII) but not Clara cells show a significantly higher expression of SP-A and SP-D in rats leading also to higher amounts of both SPs in BALF and (2) macrophages gather predominantly SP-A (Schmiedl et al. 2008).

43.10.7 Autoreactivity Against SP-A and Rheumatoid Arthritis

Circulating SP-D is decreased in early rheumatoid arthritis and SP-A and SP-D levels in synovial fluid from patients correlated with rheumatoid factor, CRP, IgA, IgM, and IgG, and total lipid content. SP-A and SP-D seem to participate in initiation of immune system and joint inflammation within the joint (Kankavi 2006) and may be an additional RA disease modifier like MBL. The $Met^{11}Thr$ polymorphism in the N-terminal part of SP-D is important determinant in serum SP-D. But this polymorphism is also essential to the function and assembly into oligomers. SP-D levels did not correlate with traditional disease activity measures. The Thr^{11}/Thr^{11} genotype and the Thr^{11} allele tended to be more frequent in RA patients. Therefore, the low serum level of SP-D and the lack of correlation with traditional disease activity measures indicate that SP-D reflects a distinctive aspect in the RA pathogenesis (Hoegh et al. 2008; Miyata et al. 2002). Trinder et al. (2000) were able to show autoreactivity to SP-A, as expressed by IgG and IgM autoantibodies, and present in synovial fluid (SF) from patients with RA. There was no cross-reactivity between autoantibodies reactive with type II collagen (CII) and those reactive with SP-A or C1q; However, autoantibodies reacted with polymeric (dimers and larger) SP-A, but not with monomeric SP-A subunits, indicating that a degree of quaternary structure is required for antibody binding.

43.11 Inhibition of SP-A Function by Oxidation Intermediates of Nitrite

43.11.1 Protein Oxidation by Chronic Pulmonary Diseases

The oxidation of proteins may play an important role in the pathogenesis of chronic inflammatory lung diseases, and may contribute to lung damage. Higher levels of protein oxidation than in healthy controls were observed in patients with interstitial lung disease, gastro-esophageal reflux disease, and PAP. The proteins most sensitive to oxidation were serum albumin, SP-A, and α1-antitrypsin. Abundance of reactive oxygen species produced during neutrophilic inflammation may be a deleterious factor that leads to pulmonary damage in these patients (Starosta and Griese 2006). Primary chain and quaternary structure of SP-D in BALFs

showed significant changes under oxidative conditions in vitro and in vivo and functional capacity to agglutinate bacteria was impaired by oxidation. Free radicals generated in lungs resulting in oxidation of SP-D may impair host defense and may contribute to the suppurative lung diseases like cystic fibrosis (Starosta and Griese 2006).

43.11.2 Oxidation Intermediates of Nitrite

Nitration of protein tyrosine residues by peroxynitrite ($ONOO^-$) has been implicated in a variety of inflammatory diseases such as ARDS. A mixture of hypochlorous acid (HOCl) and nitrite (NO_2^-) induces nitration, oxidation, and chlorination of tyrosine residues in human SP-A, and inhibits SP-A's ability to aggregate lipids and bind mannose. Nitration and oxidation of SP-A was not altered by the presence of lipids, suggesting that proteins are preferred targets in lipid-rich mixtures such as pulmonary surfactant. Moreover, both horseradish peroxidase and myeloperoxidase (MPO) can utilize NO_2^- and H_2O_2 as substrates to catalyze tyrosine nitration in SP-A, and inhibit its lipid aggregation function. SP-A nitration and oxidation by MPO is markedly enhanced in presence of Cl^- and the lipid aggregation function of SP-A is completely abolished. Studies suggest that MPO released by activated neutrophils during inflammation utilizes physiological or pathological levels of NO_2^- to nitrate proteins, and may provide an additional mechanism in addition to ONOO – formation, for tissue injury in ARDS and other inflammatory diseases associated with upregulated NO* and oxidant production. The oxidant-mediated tissue injury is likely to be important in the pathogenesis of ARDS/ALI (Davis et al. 2002; Lang et al. 2002; Narasaraju et al. 2003).

In vitro and in vivo data suggest that NO alters surfactant protein gene expression. The role of NO in ALI remains controversial. Although inhaled NO increases oxygenation in clinical trials, inhibiting NOS can be protective. However, inhalation of NO may not be indicated in sepsis because of excessive NO production. Aikio et al. (2003) indicated that inhaled NO is effective in a select group of small premature infants and that the responsiveness to NO is associated with low NOS2 enzyme. Very low birth-weight infants (birthweight <1,500 g), infants with progressive respiratory failure and infection at birth have deficient pulmonary NOS2 and cytokine response. After surfactant therapy, these infants responded strikingly to inhaled NO. An acute pulmonary inflammatory response may contribute to respiratory adaptation in early-onset pneumonia. In intact lambs inhaled NO increased SP-A and SP-B mRNA and protein content with no change in DNA content. The mechanisms and physiological effects of these findings warrant further investigation (Stuart et al. 2003; Hu et al. 2007). Exposure of rats to NO2 showed impairment of SP-A and a higher alveolar pool size after in vivo exposure. The NO2-induced alterations of SP-A may contribute to the pulmonary toxicity of this oxidant (Müller et al. 1992). NO production from NOS2 expressed in lung parenchymal cells in a murine model of ARDS correlates with abnormal surfactant function and reduced SP-B expression. $NOS2^{-/-}$ null mice exhibit significantly less physiologic lung dysfunction and loss of SP-B expression. Study indicated that the expression of NOS2 in lung epithelial cells is critical for the development of lung injury and mediates surfactant dysfunction independent of NOS2 inflammatory cell expression and cytokine production (Baron et al. 2004).

43.11.3 BPD Treatment with Inhaled NO

Inhaled NO is used to treat a number of disease processes. BPD is characterized by arrested alveolar and vascular development of immature lung. The increased expression of SP-A mRNA under hyperoxia can be attributed, at least in part, to an induction of mRNA and protein expression in bronchial Clara cells. The expanded role of Clara cells in the defence against hyperoxic injury suggests that they support alveolar type 2 cell function and may play an important role in the supply of surfactant proteins to the lower airways (ter Horst et al. 2006). The inhaled nitric oxide treatment of premature infants at risk for bronchopulmonary dysplasia does not adversely affect endogenous surfactant function or composition and may improve surfactant function transiently (Ballard et al. 2007). Chorioamnionitis is a risk factor for the development of bronchopulmonary dysplasia. Endotoxin-induced oxidative stress to the fetus in the uniquely hypoxic intrauterine environment has been reported. SP-A and B mRNAs were highest at Day 2, suggesting that oxidative stress did not contribute to the lung maturation response. A modest lung oxidative stress in chorioamnionitis could contribute to bronchopulmonary dysplasia (Cheah et al. 2008).

43.12 Congenital Diaphragmatic Hernia

Pulmonary hypoplasia is one of the main causes for high mortality rate in patients with congenital diaphragmatic hernia (CDH). The expression of SP-A in hypoplastic CDH lung is reduced, and its concentration is decreased in amniotic fluid of pregnancies complicated by CDH. In animal models, surfactant deficiency contributes to the pathophysiology of the disease. In humans surfactant disaturated phosphatidylcholine (DSPC) synthesis and SP-A were significantly lower in infants with CDH than in control subjects (Cogo et al. 2002).

SP-A is altered in developing lungs from rat fetuses with CDH induced by maternal ingestion of Nitrofen on Day 9 of

gestation. There is decreased expression of SP-A in rat fetuses with CDH secondary to Nitrofen exposure (Mysore et al. 1998). In rat CDH model, induced in pregnant rats following administration of nitrofen, SP-A, SP-B, and SP-D mRNA expression in CDH lung were significantly decreased compared to controls at birth and 6 h after ventilation. The inability of O2 to increase SP mRNA expression in hypoplastic CDH lung suggests that the hypoplastic lung is not responsive to increased oxygenation for synthesis of SP (Shima et al. 2000). Though, SP's deficiency appears to be a common feature among various CDH models, TTF-1 expression was not altered in surgical model in contrast to nitrofen model, indicating different molecular mechanisms in two models (Benachi et al. 2002).

43.13 Protective Effects of SP-A and SP-D on Transplants

Surfactant treatment has been shown to improve lung transplant function, but the effect is variable. Erasmus et al. (2002) indicated that SP-A enrichment of surfactant improves the efficacy of surfactant in lung transplantation. After instillation of SP-A-enriched surfactant, P_{O2} values were reached to control values, whereas after SP-A-deficient surfactant treatment, the P_{O2} values did not improve (Erasmus et al. 2002). The impairment of surfactant adsorption from transplanted lungs may be correlated with decreased levels of SP-A, and increased levels of serum acute-phase protein C-reactive protein (CRP). The elevated levels of CRP in BAL can be a very sensitive marker of lung injury (Casals et al. 1998).

Bronchiolitis obliterans syndrome (BOS) affects long-term survival of lung transplant recipients (LTRs). Among 11 differentially expressed proteins in BALF, peroxiredoxin 2 (Prdx2) exclusively expressed in BOS; and SP-A expressed consistently less in BOS patients than in stable LTRs. The reduction of SP-A in BALF was detectable early after lung transplant, preceding BOS onset in four of five patients and indicated that SP-A levels in BALF could predict LTR patients who are at higher risk of BOS development (Meloni et al. 2007) BOS and IPS cause high mortality and impaired survival after allogeneic hematopoietic stem-cell transplantation (allo-HSCT). The pretransplant serum SP-D levels but not SP-A, KL-6 in BOS/IPS patients were lower than those in non-BOS/IPS patients. However, the patients with lower pretransplant serum SP-D level had a trend toward frequent development of BOS/IPS. Constitutive serum SP-D level before allo-HSCT may be a useful, noninvasive predictor for the development of BOS/IPS (Nakane et al. 2008).

Keratinocyte growth factor (KGF) given before bone marrow transplantation (BMT) can prevent allogeneic T cell-dependent lung inflammation, but the antiinflammatory effects of KGF were impaired in mice injected with both T cells and conditioning regimen of cyclophosphamide. Yang et al. (2000, 2002) demonstrated that addition of cyclophosphamide interferes with the ability of KGF to enhance SP-A production. The systemic pre-BMT injection of KGF in recipients of allogeneic T cells up-regulates SP-A, which may contribute to the early antiinflammatory effects of KGF. Exogenous and basal endogenous SP-A can suppress donor T-cell-dependent inflammation that occurs during the generation of idiopathic pneumonia syndrome after BMT. Wild-type and SP-A-deficient mice, given allogeneic donor bone marrow plus inflammation-inducing spleen T cells, showed that basal endogenous SP-A, and enhanced alveolar SP-A level modulate donor T-cell-dependent immune responses and prolong survival after allogeneic BMT.

43.14 Therapeutic Effects of SP-A, SP-D and Their Chimeras

43.14.1 SP-A Effects on Inflammation of Mite-sensitized Mice

SP-A and SP-D interact with a wide range of inhaled allergens, competing for their binding to cell-sequestered IgE resulting in inhibition of mast cell degranulation. SP-D interacts with glycoprotein allergens of house dust mite (*Dermatophagoides pteronyssinus*, Derp) via its CRDs and thus inhibits specific IgE, isolated from mite-sensitive asthmatic patients, from binding these allergens, and blocking subsequent histamine release from sensitized basophils. Exogenous administration of SP-A and SP-D diminishes allergic hypersensitivity in vivo. A fragment of recombinant human SP-D (rfh SP-D) has a therapeutic effect on allergen-induced bronchial inflammation through its inhibitory effect on NO and TNF-α production by AΦs, and thus preventing the development of Th-2 type cytokine response (Liu et al. 2005b; Singh et al. 2003). The rfhSP-D that is effective in diminishing allergic hypersensitivity in mouse models of dust mite allergy was more susceptible to degradation than the native full-length protein. The degradation and consequent inactivation of SP-A and SP-D may be a mechanism to account for the potent allergenicity of these common dust mite allergens (Deb et al. 2007). Evidence suggests for an antiinflammatory role for SP-D in response to noninfectious, subacute lung injury via modulation of oxidative-nitrative stress (Casey et al. 2005).

43.14.2 SP-D Increases Apoptosis in Eosinophils of Asthmatics

The effect of exogenous rfhSP-D on protection of adult mouse lung from LPS-induced and lipoteichoic acid (LTA)-induced injury was assessed in *Sftpd*$^{+/+}$ and *Sftpd*$^{-/-}$

mice. Intratracheal rhSP-D inhibited inflammation induced by intratracheal LPS and LTA instillation in lung. The antiinflammatory effects of rhSP-D were enhanced by addition of pulmonary surfactant, providing a potential therapy for the treatment of lung inflammation (Ikegami et al. 2007). In view of therapeutic effects of exogenous SP-D or rfhSP-D (composed of eight Gly-X-Y collagen repeat sequences, homotrimeric neck and lectin domains) in murine models of lung allergy and hypereosinophilic SP-D gene-deficient mice, Mahajan et al. (2008) suggested that rfhSP-D mediated preferential increase of apoptosis of primed eosinophils while not affecting the normal eosinophils. The increased phagocytosis of apoptotic eosinophils may be important mechanisms of rfhSP-D and SP-D-mediated resolution of allergic eosinophilic inflammation in vivo.

43.14.3 Targeting of Pathogens to Neutrophils Via Chimeric SP-D/Anti-CD89 Protein

Intratracheal rfhSP-D prevents shock caused by endotoxin released from the lung during ventilation in the premature newborn (Ikegami et al. 2006). In lambs, preterm infants experience enhanced susceptibility and severity to respiratory syncytial virus (RSV) infection. This was observed when SP-A, -D and TLR4 mRNA expression increased from late gestation to term birth, where as in preterm lungs, studies showed reduced SP-A, -D, and TLR4 expression and enhanced RSV susceptibility (Meyerholz et al. 2006).

A chimeric protein, consisting of a recombinant fragment of human SP-D coupled to a Fab' fragment directed against human Fc α receptor (CD89) (chimeric rfSP-D/anti-Fc), effectively targets pathogens recognized by SP-D to human neutrophils. A recombinant trimeric fragment of SP-D (rfSP-D), consisting of CRD and neck domain of human SP-D, cross-linked to the Fab' of an Ab directed against the human Fc α RI (CD89) (chimeric rfSP-D/anti-CD89 protein) enhanced uptake of *E. coli*, *C. albicans*, and *influenza A virus* by human neutrophils (Tacken et al. 2004). Both chimeric rfSP-D/anti-Fc receptor proteins increased internalization of *E. coli* by human promonocytic cell line U937, but only after induction of monocytic differentiation. Both CD64 and CD89 on U937 cells proved suitable for targeting by rfSP-D/anti-Fc receptor proteins (Tacken and Batenburg 2006). Collectin-based chimeric proteins may thus offer promise for therapy of infectious disease.

43.14.4 Anti-IAV and Opsonic Activity of Multimerized Chimeras of rSP-D

A recombinant human SP-D, consisting of a short collagen region (two repeats of Gly-Xaa-Yaa amino acid sequences), the neck domain and CRD can form a trimeric structure owing to neck domain and exhibits sugar-binding activity and specificity similar to those of native human SP-D. Though the truncated SP-D could bind to IAV, like native SP-D, but the truncated human SP-D was less effective in agglutinating bacteria than the native structure and failed to inhibit haemagglutination by IAV (Eda et al. 1997). On the other hand, chimeric collectin containing N-terminus and collagen domain of human SP-D and CRD of MBL showed greater anti-IAV activity than similarly multimerized preparations of SP-D or incompletely oligomerized preparations of the chimera. Highly multimerized preparations of chimera also caused greater increases in uptake of IAV by neutrophils. These studies may be useful for development of collectins as therapeutic agents against IAV infection (Hartshorn et al. 2000b; White et al. 2000).

Bovine serum conglutinin has greater ability to inhibit IAV infectivity than other collectins. Altering the carbohydrate binding properties of SP-D [e.g., by replacing its CRD with that of either MBL or conglutinin] can increase its activity against IAV. Hence, recombinant conglutinin and a chimeric protein containing NH_2 terminus and collagen domain of rat SP-D (rSP-D) fused to neck region and CRD of conglutinin (termed SP-D/Cong(neck + CRD)) have markedly greater ability to inhibit infectivity of IAV than wild-type recombinant rSP-D, confirming that potent IAV-neutralizing activity of conglutinin resides in its neck region and CRD. Furthermore, SP-D/Cong(neck + CRD) also caused substantially greater enhancement of neutrophil binding and H_2O_2 responses to IAV than r-conglutinin or rSP-D. Hence, chimeric SP-D/Cong(neck + CRD) protein showed favorable antiviral and opsonic properties of conglutinin and SP-D (Hartshorn et al. 2000a). Thus, the SP-D N-terminal and/or collagen domains contribute to the enhanced bacterial binding and aggregating activities of SP-D. Although replacement of neck recognition domains and CRDs of SP-D with those of MBL and conglutinin confer increased viral binding activity, it does not favorably affect bacterial binding activity, suggesting that requirements for optimal collectin binding to influenza virus and bacteria differ (Hartshorn et al. 2007).

Chimera of Trimeric Neck + CRDs of Human SP-D: The recombinant trimeric neck + CRDs of human SP-D (NCRD) retains binding activity for some ligands and mediates some functional activities. In comparison to strong neutralizing activity of lung SP-D for IAVs in vitro and in vivo, the NCRD derived from SP-D has weak viral-binding ability and lacks neutralizing activity. Using a panel of mAbs against NCRD, Tecle et al. (2008) showed that antiviral activities of SP-D can be reproduced without the N-terminal and collagen domains and that cross-linking of NCRDs is

essential for antiviral activity of SP-D with respect to IAV (Tecle et al. 2008).

Incubation of native SP-D or NCRDs with peroxynitrite results into nitration and nondisulfide cross-linking. Modifications could be blocked by peroxynitrite scavengers or pH inactivation of peroxynitrite. Abnormal cross-linking leads to defective aggregation. Thus, modification of SP-D by reactive oxygen-nitrogen species could contribute to alterations in the structure and function of SP-D at sites of inflammation in vivo (Matalon et al. 2009). In contrast, a trimeric neck and CRD construct of bovine serum collectin CL-46 induces aggregation of IAV and potently increases IAV uptake by neutrophils. CL-46-NCRD showed calcium-dependent and sugar-sensitive binding to both neutrophils and IAV. Results indicate that collectins can act as opsonins for IAV even in the absence of the collagen domain or higher order multimerization. This may involve increased affinity of individual CRDs for glycoconjugates displayed on host cells or the viral envelope (Hartshorn et al. 2010).

Insertion of Arg-Ala-Lys in NCRD Increases Inhibitory Activity: Arg-Ala-Lys (RAK) (immediately N-terminal to the first motif) in CL-43 contributes to differences in saccharide selectivity and host defense function. Insertion of CL-43 RAK sequence or a control Ala-Ala-Ala sequence (AAA) into corresponding position in NCRD increased the efficiency of binding to mannan and changed the inhibitory potencies of competing saccharides to more closely resemble those of CL-43. In addition, RAK resembled CL-43 in its greater capacity to inhibit infectivity of IAV and to increase uptake of IAV by neutrophils (Crouch et al. 2005).

43.15 Lessons from SP-A and SP-D Deficient Mice

SP-D deficient (SP-D$^{-/-}$) mice exhibit an increase in the number and size of airway macrophages, peribronchiolar inflammation, increases in metalloproteinase activity, and development of emphysema. Mice deficient in SP-D$^{-/-}$ develop progressive emphysema with age, associated with loss of parenchymal tissue, subpleural fibrosis, and accumulation of abnormal elastin fibers. The changes in lung structure in SP-D$^{-/-}$ mice are reflected in the mechanical properties of both airway and lung parenchyma measured in vivo (Yoshida and Whitsett 2006).

Gene-targeted mice deficient in SP-D develop abnormalities in surfactant homeostasis, hyperplasia of alveolar epithelial type II cells, and emphysema-like pathology. Alveolar and tissue phosphatidylcholine pool sizes are markedly increased in SP-D$^{-/-}$ mice. The pulmonary lipoidosis in SP-D$^{-/-}$ mice was not associated with accumulation of SP-B or C, or their mRNAs, distinguishing the disorder from alveolar proteinosis syndromes. Surfactant protein A mRNA was reduced and, SP-A protein appeared to be reduced in SP-D$^{-/-}$ compared with wild type mice. Targeting of mouse SP-D gene caused accumulation of surfactant lipid and altered phospholipid structures, demonstrating a unsuspected role for SP-D in surfactant lipid homeostasis in vivo (Botas et al. 1998; Korfhagen et al. 1998; Ikegami et al. 2005). HDL cholesterol was significantly elevated in SP-D$^{-/-}$ mice while treatment of SP-D$^{-/-}$ mice with rhSP-D resulted in decreases of HDL-cholesterol as well as total cholesterol, and LDL cholesterol along with reduced plasma TNF-α in SP-D$^{-/-}$ mice. It shows that SP-D regulates atherogenesis in mouse model (Sorensen et al. 2006). SP-D plays a critical role in the suppression of alveolar macrophage activation, which may contribute to the pathogenesis of chronic inflammation and emphysema (Wert et al. 2000). Oxidant production and reactive oxygen species were increased in lungs of SP-D$^{-/-}$ mice, in turn activate NF-kB and MMP expression. SP-D plays an unexpected inhibitory role in the regulation of NF-kB in AΦs (Yoshida et al. 2001).

Studies indicate that GM-CSF-dependent macrophage activity is not necessary for emphysema development in SP-D-deficient mice, but that type II cell metabolism and proliferation are, either directly or indirectly, regulated by GM-CSF in this model (Hawgood et al. 2001; Ochs et al. 2004). SP-D and GM-CSF play distinct roles in the regulation of surfactant homeostasis and lung structure (Ikegami et al. 2001).

SP-A and SP-D Double Deficient Mice SP-A and SP-D proteins have overlapping as well as distinct functions. Mice singly deficient in SP-A and SP-D have distinct phenotypes and produce altered inflammatory responses to microbial challenges. Adult mice deficient in both SP-A and SP-D ($A^{-}D^{-}$) show fewer and larger alveoli, an increase in the number and size of type II cells, as well as more numerous and larger alveolar macrophages. Chronic deficiency of SP-A and SP-D in mice leads to parenchymal remodeling, type II cell hyperplasia and hypertrophy, and disturbed intracellular surfactant metabolism (Jung et al. 2005) In double deficient mice, there is a progressive increase in bronchoalveolar lavage phospholipid, protein, and macrophage content through 24 week of age. The macrophages from doubly deficient mice express high levels of the MMP-12 and develop intense but patchy lung inflammation. Qualitative changes resemble the lung pathology seen in SP-D-deficient mice (Hawgood et al. 2002).

Treatment of SP-D deficient mice with a truncated recombinant fragment of human SP-D (rfhSP-D) decreased lipidosis and alveolar macrophage accumulation as well as

production of proinflammatory chemokines. The rfhSP-D treatment reduced the structural abnormalities in parenchymal architecture and type II cells characteristic of SP-D deficiency and reduced degree of emphysema and a corrected type II cell hyperplasia and hypertrophy. This suggests that rfhSP-D might become a therapeutic option in diseases that are characterized by decreased SP-D levels in the lung (Knudsen et al. 2007; Zhang et al. 2002).

Treatment with a recombinant fragment of human SP-D consisting of a short collagen-like stalk (but not the entire collagen-like domain of native SP-D), neck, and CRD inhibited development of emphysema-like pathology in SP-D deficient mice. On the other hand, the entire collagen-like domain was necessary for preventing SP-D knockout mice from pulmonary emphysema development. The fragment of SP-D lacking the short collagen-like stalk failed to correct pulmonary emphysematous alterations demonstrating the importance of the short collagen-like stalk for the biological activity of the recombinant fragment of human SP-D (Knudsen et al. 2009; Breij and Batenburg 2008).

NO Production and S-Nitrosylation of SP-D Controls Inflammatory Function SP-D$^{-/-}$ mice exhibit an increase in the number and size of airway macrophages, peribronchiolar inflammation, increases in metalloproteinase activity, and development of emphysema. SP-A inhibited production of NO and inducible nitric oxide synthase (iNOS) in rat AΦ stimulated with smooth LPS. In contrast, SP-A enhanced production of NO and iNOS in cells stimulated with IFN-γ or IFN-γ plus LPS. SP-A contributes to the lung inflammatory response by exerting differential effects on the responses of immune cells, depending on their state and mechanism of activation (Stamme et al. 2000). NO is involved in a variety of signaling processes, and because altered NO metabolism has been observed in inflammation, it is predicted that alterations in its metabolism would underlie the proinflammatory state observed in SP-D deficiency (Atochina et al. 2004a, c). Treatment with the iNOS inhibitor 1,400 W can inhibit inflammatory phenotype and can attenuate inflammatory processes within SP-D deficiency. Mice treated with 1,400 W reduced total lung NO synthase activity (Atochina-Vasserman et al. 2007). Guo et al. (2008) suggest that NO controls the dichotomous nature of SP-D and that posttranslational modification by S-nitrosylation causes quaternary structural alterations in SP-D causing it to switch its inflammatory signaling role. This represents new insight into both the regulation of protein function by S-nitrosylation and NO's role in innate immunity (Guo et al. 2008). Thus, inflammation that occurs in SP-D deficiency is due to an increase in NO production and a shift in the chemistry and targets of NO from a disruption of NO-mediated signaling within the innate immune system. However, purified preparations of SPs often contain endotoxin and the functions of SP-A and SP-D are affected by endotoxin. Therefore, the monitoring of SP preparations for endotoxin contamination is important (Wright et al. 1999).

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Selectins and Associated Adhesion Proteins in Inflammatory disorders

44

G.S. Gupta

44.1 Inflammation

Inflammation is defined as the normal response of living tissue to injury or infection. It is important to emphasize two components of this definition. First, that inflammation is a normal response and, as such, is expected to occur when tissue is damaged. Infact, if injured tissue does not exhibit signs of inflammation this would be considered abnormal and wounds and infections would never heal without inflammation. Secondly, inflammation occurs in living tissue, hence there is need for an adequate blood supply to the tissues in order to exhibit an inflammatory response. The inflammatory response may be triggered by mechanical injury, chemical toxins, and invasion by microorganisms, and hypersensitivity reactions. Three major events occur during the inflammatory response: the blood supply to the affected area is increased substantially, capillary permeability is increased, and leucocytes migrate from the capillary vessels into the surrounding interstitial spaces to the site of inflammation or injury. The inflammatory response represents a complex biological and biochemical process involving cells of the immune system and a plethora of biological mediators. Cell-to-cell communication molecules such as cytokines play an extremely important role in mediating the process of inflammation. Inflammation and platelet activation are critical phenomena in the setting of acute coronary syndromes. An extensive exposition of this complex phenomenon is beyond the scope of this article (Rankin 2004).

Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. However, chronic inflammation can also lead to a host of diseases, such as hay fever, atherosclerosis, rheumatoid arthritis, and even cancer (e.g., gallbladder carcinoma). It is for that reason that inflammation is normally closely regulated by the body. Acute and chronic inflammation differ in matter of causative agent, major cells involved, primary mediators, onset, duration and final outcomes. Generally speaking, acute inflammation is mediated by granulocytes, while chronic inflammation is mediated by mononuclear cells such as monocytes and lymphocytes.

44.2 Cell Adhesion Molecules

Cell adhesion molecules are glycoproteins expressed on the cell surface and play an important role in inflammatory as well as neoplastic diseases. There are four main groups: the integrin family, the immunoglobulin superfamily, selectins, and cadherins. The integrin family has eight subfamilies, designated as β1 through β8. The immunoglobulin superfamily includes leukocyte function antigen-2 (LFA-2 or CD2), leukocyte function antigen-3 (LFA-3 or CD58), intercellular adhesion molecules (ICAMs), vascular adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PE-CAM-1), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). The selectin family includes L-selectin (CD62L), P-selectin (CD62P), and E-selectin (CD62E). Cadherins are major cell-cell adhesion molecules and include epithelial (E), placental (P), and neural (N) subclasses. The binding sites (ligands/receptors) are different for each of these cell adhesion molecules (e.g., ICAM binds to CD11/CD18; VCAM-1 binds to VLA-4). The specific cell adhesion molecules and their ligands that may be involved in pathologic conditions and potential therapeutic strategies by modulating the expression of these molecules have been discussed (Elangbam et al. 1997). Most adhesion molecules

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play fairly broad roles in the generation of immune responses. The three selectins act in concert with other cell adhesion molecules e.g., intracellular adhesion molecule (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and leukocyte integrins to effect adhesive interactions of leukocytes, platelets, and endothelial cells. The structure and functions of selectins, which belong to C-type lectins family, have been reviewed in Chaps. 26, 27, and 28.

44.2.1 Selectins

The selectin family of lectins consists of three closely related cell-surface molecules with differential expression by leukocytes (L-selectin), platelets (P-selectin), and vascular endothelium (E- and P-selectin). Structural identity of a selectins resides in its unique domain composition (Chap. 26). E-, P-, and L-selectin are >60 % identical in their NH2 terminus of 120 amino acids, which represent the lectin domain (Chaps. 26, 27, and 28). The ligands (counter structures) of selectins are sialylated and fucosylated carbohydrate molecules which, in most cases, decorate mucin-like glycoprotein membrane receptors. Their common structure consists of an N-terminal Ca^{2+}-dependent lectin-type domain, an epidermal growth factor (EGF)-like domain, multiple short consensus repeat (SCR) domains similar to those found in complement regulatory proteins, a transmembrane region, and a short cytoplasmic C-terminal domain. Together this arrangement results in an elongated structure which projects from the cell surface, ideal for initiating interactions with circulating leucocytes. The lectin domain forms the main ligand binding site, interacting with a carbohydrate determinant typified by fucosylated, sialylated, and usually sulphated glycans such as sialyl Lewis X (s-Le^X). The EGF domain may also play a role in ligand recognition. The short consensus repeat (SCR) domains (two for L-selectin, six for E-selectin, and nine for P-selectin) probably act as spacer elements, ensuring optimum positioning of the lectin and EGF domains for ligand interaction. The EGF repeats have comparable sequence similarity. Each complement regulatory-like module is 60 amino acids in length and contains six cysteinyl residues capable of disulfide bond formation. This feature distinguishes the selectin modules from those found in complement binding proteins, such as complement receptors 1 and 2, which contain four cysteines (Chap. 26).

The selectins cell-surface receptors play a key role in the initial adhesive interaction between leukocytes and endothelial cells at sites of inflammation. Selectins (P, E and L) and their ligands (mainly P-selectin ligand) are involved in the rolling and tethering of leukocytes on the vascular wall. Activation of endothelial cells (EC) with different stimuli induces the expression of E- and P-selectins, and other adhesion molecules (ICAM-1, VCAM-1), involved in their interaction with circulating cells. Lymphocytes home to peripheral lymph nodes (PLNs) via high endothelial venules (HEVs) in the subcortex and incrementally larger collecting venules in the medulla. HEVs express ligands for L-selectin, which mediates lymphocyte rolling (Horstman et al. 2004). For structure and functions of selectins, the readers are advised to consult Chaps 26–28. In this chapter we will emphasize mainly on the role of selectins in inflammatory disorders including cancer.

44.3 Atherothrombosis

Atherothrombosis, defined as atherosclerotic plaque disruption with superimposed thrombosis, is the leading cause of mortality in the Western world. Atherosclerosis is a diffuse process that starts early in childhood and progresses asymptomatically through adult life. Later in life, it is clinically manifested as coronary artery disease (CAD), stroke, transient ischaemic attack (TIA), and peripheral arterial disease. From the clinical point of view, we should envision this disease as a single pathologic entity that affects different vascular territories. A suggestive analogy is that TIA and intermittent claudication are the unstable angina of the brain and lower limbs, respectively; and stroke and gangrene are the myocardial infarction. Circulating platelets display reversible interactions with atherosclerotic lesions. Atherosclerotic arterial disease is associated with an increased share of platelets unable to express P-selectin and an increased fraction of platelets that microaggregate in citrate anticoagulant. These platelet alterations are not completely explained by either focal arterial injury or abnormal rheology associated with arterial stenosis but appear to be an effect of the atherosclerotic process (McBane et al. 2004). The pathogenesis of arterial thrombotic disease involves multiple genetic and environmental factors related to atherosclerosis and thrombosis.

44.3.1 Venous Thrombosis

Venous Thrombosis is a world wide health problem in the general population. Injury to the endothelium leads to dysfunction. The causes of injury include lipids, immune complexes, microorganisms, smoking, hypertension, aging, diabetes mellitus and trauma. The selectins are thought to be largely responsible for the initial attachment and rolling of leukocytes on stimulated vascular endothelium. Platelet activation is an important process in the pathogenesis of atherothrombosis. Platelet adhesion, activation, and aggregation at the sites of vascular endothelial disruption caused by atherosclerosis are key events in arterial thrombus formation. Platelet tethering and adhesion to the arterial wall,

particularly under high shear forces, are achieved through multiple high-affinity interactions between platelet membrane receptors (integrins) and ligands within the exposed subendothelium, most notably collagen and von Willebrand factor (vWF). Platelet adhesion to collagen occurs both indirectly, via binding of the platelet glycoprotein (GP) Ib-V-IX receptor to circulating vWF, which binds to exposed collagen, and directly, via interaction with platelet receptors GP VI and GP Ia/IIb. Platelet activation, initiated by exposed collagen and locally generated soluble platelet agonists (primarily thrombin, ADP, and thromboxane A2), provides the stimulus for the release of platelet-derived growth factors, adhesion molecules and coagulation factors, activation of adjacent platelets, and conformational changes in the platelet α(IIb)β3 integrin (GP IIb/IIIa receptor). Platelet aggregation, mediated primarily by interaction between the activated platelet GP IIb/IIIa receptor and its ligands, fibrinogen and vWF, results in the formation of a platelet-rich thrombus (Steinhubl and Moliterno 2005).

44.3.2 Arterial Thrombosis

P-selectin expression in platelets is elevated in disorders associated with arterial thrombosis such as coronary artery disease, acute myocardial infarction, stroke, and peripheral artery disease. During thrombosis, P-selectin is expressed on the surface of activated endothelial cells and platelets. P-selectin mediates rolling of platelets and leukocytes on activated endothelial cells as well as interactions of platelets with leukocytes. Platelet P-selectin interacts with PSGL-1 on leukocytes to form platelet-leukocyte aggregates. Furthermore, this interaction of P-selectin with PSGL-1 induces the upregulation of tissue factor, several cytokines in leukocytes and the production of procoagulant microparticles, thereby contributing to a prothrombotic state. P-selectin is also involved in platelet-platelet interactions, i. e. platelet aggregation which is a major factor in arterial thrombosis. P-selectin interacts with platelet sulfatides, thereby stabilizing initial platelet aggregates formed by GPIIb/IIIa-fibrinogen bridges. Inhibtion of the P-selectin-sulfatide interaction leads to a reversal of platelet aggregation. Thus, P-selectin plays a significant role in platelet aggregation and platelet- leukocyte interactions, both important mechanisms in the development of arterial thrombosis. Following activation, P-selectin is rapidly translocated to the cell surface (Merten and Thiagarajan 2004; Wang et al. 2005).

44.3.3 Thrombogenesis in Atrial Fibrillation

Platelet activation occurs in peripheral blood of patients with rheumatic mitral stenosis (MS). The plasma levels of soluble P-selectin are elevated in permanent atrial fibrillation (AF) patients; the plasma levels of soluble P-selectin in the left atrium do not significantly differ from those in the right atrium, femoral vein, or femoral artery. The venous plasma levels of sP-selectin in patients with moderate-to-severe MS are significantly higher than those in healthy volunteers or patients with lone AF. In addition, in patients with MS, there was no difference in the plasma levels of sP-selectin between the left and right atrial blood and between peripheral and atrial blood. Moreover, there was no change in sP-selectin levels as a result of percutaneous transluminal mitral valvuloplasty (PTMV) (Chen et al. 2004). Lip et al. (2005) studied the relations of plasma vWf (an index of endothelial damage and dysfunction) and sP-selectin levels in relation to the presence and onset of clinical congestive heart failure (CHF) and degree of left ventricular dysfunction in patients taking part in SPAF (stroke prevention in AF). While plasma vWf was higher among patients with AF and CHF, plasma P-selectin concentrations were not affected by presence, onset, or severity of heart failure.

44.3.4 Atherosclerosis

Atherosclerosis is a complex chronic inflammatory disease of the arterial wall. Though the inflammatory nature of atherosclerosis has been established, the initial events that trigger this response in the arterial intima remain obscure. Studies reveal a significant rate of genomic alterations in human atheromas. The accumulation of genomic rearrangements in vascular endothelium and smooth muscle cells are important for disease development. It is well accepted that the induction of EC adhesion molecules is a critical component in acute inflammatory responses as well as allogeneic interactions in vascularized allografts and, possibly, atherogenesis. Inflammation and genetics are both prominent mechanisms in the pathogenesis of atherosclerosis and arterial thrombosis. Accordingly, population studies have explored the association of ischaemic heart disease with gene polymorphisms of the inflammatory molecules: tumor necrosis factors (TNF) α and β, transforming growth factors (TGF) β1 and 2, P and E selectins, and platelet endothelial cell adhesion molecule (PECAM) 1. The partly conflicting data provide some evidence that alterations in the genetics of the inflammatory system may modify the risk of ischaemic heart disease.

44.3.4.1 Formation of Reactive Oxygen Species (ROS) as an Initial Event

In recent years, reactive oxygen species (ROS) are considered as initial event in causing atherosclerosis. ROS are a family of molecules including molecular oxygen and its derivatives produced in all aerobic cells. Excessive

production of ROS, outstripping endogenous antioxidant defense mechanisms, has been implicated in processes in which they oxidize biological macromolecules, such as DNA, protein, carbohydrates, and lipids. Many ROS possess unpaired electrons and thus are free radicals. These include molecules such as superoxide anion (O_2^-), hydroxyl radical ($HO^\bullet$), nitric oxide ($NO^\bullet$), and lipid radicals. Other reactive oxygen species, such as hydrogen peroxide (H2O2), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl), are not free radicals per se but have oxidizing effects that contribute to oxidant stress. The cellular production of one ROS may lead to the production of several others via radical chain reactions. For example, reactions between radicals and polyunsaturated fatty acids within cell membrane may result in a fatty acid peroxyl radical ($R\text{-}COO^\bullet$) that can attack adjacent fatty acid side chains and initiate production of other lipid radicals. Lipid radicals produced in this chain reaction accumulate in the cell membrane and may have a myriad of effects on cellular function, including leakage of the plasmolemma and dysfunction of membrane-bound receptors. Of note, end products of lipid peroxidation, including unsaturated aldehydes and other metabolites, have cytotoxic and mutagenic properties. A decline in NO bioavailability may be caused by decreased expression of the endothelial cell NO synthase (eNOS), a lack of substrate or cofactors for eNOS (Fig. 44.1 and 44.2).

In mammalian cells, potential enzymatic sources of ROS include the mitochondrial respiration, arachidonic acid pathway enzymes lipoxygenase and cyclooxygenase, cytochrome p450s, xanthine oxidase, NADH/NADPH oxidases, NO synthase, peroxidases, and other hemoproteins. Although many of these sources could potentially produce ROS that inactivate $NO^\bullet$, 3 sources have been studied extensively in cardiovascular system. These include xanthine oxidase, NADH/NADPH oxidase, and NO synthase (Cai and Harrison 2000; Hamilton et al. 2004; Vijya Lakshmi et al. 2009).

44.3.4.2 CAMs as Predicators of Atherosclerosis

During initial step in atherosclerosis, there is rapid targeting of monocytes to the sites of inflammation and endothelial injury; the adhesion of leukocytes to activated endothelial cells is mediated by ICAM-1. The induction of EC adhesion molecules is a critical component in acute inflammatory responses as well as allogeneic interactions in vascularized allografts and, possibly, atherogenesis. The "inflammatory triad" of IL-1, TNF, and LPS are potent stimulators of the EC activation and adhesion molecules E-selectin or ELAM-1 (or also known as CD62E), ICAM-1 and VCAM-1. PECAM-1 plays also a key role in the transendothelial migration of circulating leukocytes (diapedesis) during vascular inflammation. ICAM-1 and VCAM-1 are inflammatory predicators of adverse prognosis in patients with acute coronary syndromes (ACS) (Postadzhiyan et al. 2008) (Fig. 44.2).

Levels of P-selectin are increased in the blood of patients with familial hypercholesterolemia (FH) in spite of long-term intensive extracorporeal LDL-elimination, documenting the activity of atherosclerosis. Low levels of P-selectin and MCP-1 after hypolidemic procedure can be used as a marker showing the effectivity of the extracorporeal LDL-cholesterol elimination (Blaha et al. 2004). In an extended study, the levels of expression of tissue factor, ICAM-1, P- and E-selectin, and PAI-1 were found low, whereas those of endothelial protein C receptor and VCAM-1 were high (Merlini et al. 2004).

44.3.4.3 Gene Polymorphisms in E-Selectin

Polymorphisms in the E-selectin gene are associated with accelerated atherosclerosis in young (age <40 years) patients, further suggesting a role of inflammation in atherosclerosis. A further change in endothelial physiology is an increase in the surface expression of E-selectin, which regulate adhesive interactions between certain blood cells and endothelium. Intravascular fibrinolysis induced by tissue-type plasminogen activator or urokinase may contribute to the initiation of atherosclerosis by inducing P-selectin and platelet activating factor as well as to plaque rupture, either directly or indirectly, by activating metalloproteinases. As E-selectin is only expressed on activated endothelium, it provides an opportunity to study pathophysiological aspects of this cell in cardiovascular and other disease. However, sE-selectin can be found in the plasma, which has potential role in the pathogenesis of cardiovascular disease as raised levels have been found in hypertension, diabetes and hyperlipidemia, although its association in established atherosclerosis disease and its value as a prognostic factor is more controversial (Holvoet and Collen 1997).

Polymorphisms for three genes, P-selectin, L-selectin, and E-selectin (genes *P-sel, L-sel*, and *E-sel*, respectively) showed that the selectin cluster is linked to markers at chromosome 1q23 (Vora et al. 1994). Significant genomic alterations were found on 1q22-q25 in *Sel-L* gene. The message indicated somatic DNA rearrangements, on loci associated to leukocyte adhesion, vascular smooth muscle cells growth, differentiation and migration, to atherosclerosis development as an inflammatory condition (Arvanitis et al. 2005). Wenzel et al. (1999) and Yoshida et al. (2003) described an adenine to cytosine (A/C) substitution for cDNA position 561 resulting in an amino acid exchange from serine to arginine at position 128 (S/R or $Ser^{128}Arg$) was detected in the epidermal growth factor (EGF) domain. A higher mutation frequency was observed in patients aged 50 years or less with proven severe atherosclerosis as well as in patients aged 40 years or less. If $Ser^{128}Arg$ substitution

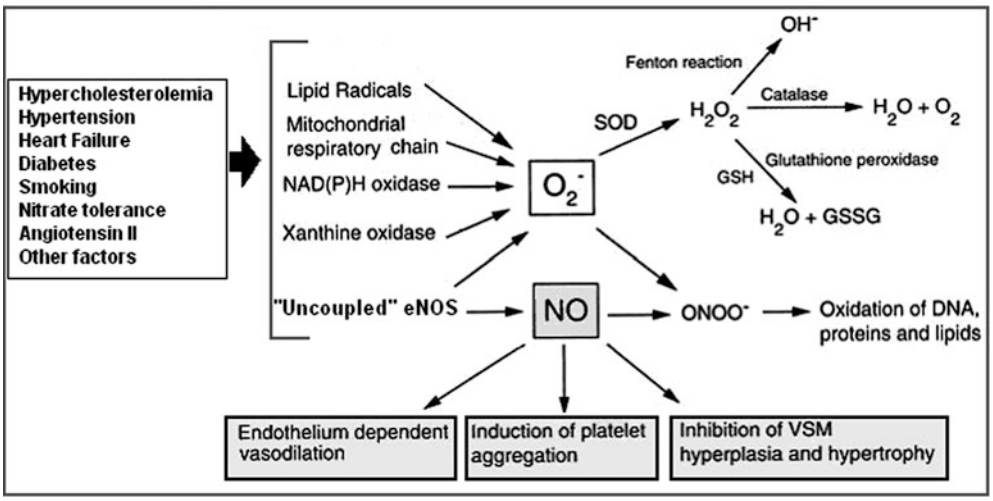

Fig. 44.1 *Mechanisms for oxidant stress-induced endothelial dysfunction in cardiovascular diseases* (Hamilton et al. 2004). Excessive production of ROS has been implicated in processes in which they oxidize biological macromolecules, such as DNA, protein, carbohydrates, and lipids. Many ROS possess unpaired electrons and thus are free radicals. These include molecules such as superoxide anion (O_2^-), hydroxyl racial ($HO^•$), nitric oxide ($NO^•$), and lipid radicals. The cellular production of one ROS may lead to the production of several others via radical chain reactions. A decline in NO bioavailability may be caused by decreased expression of the endothelial cell NO synthase (eNOS), a lack of substrate or cofactors required for eNOS action. Low-density lipoprotein (LDL) is oxidized to oxidized form of LDL (ox-LDL) and initiates the atherosclerotic process in the vessel wall (see Fig. 44.2). Abbreviations: O2−, superoxide; NO, nitric oxide; ONOO−, peroxynitrite; H2O2, hydrogen peroxide; OH−, hydroxyl radical; SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, oxidised glutathione; VSM, vascular smooth muscle

had an effect on the adhesion of blood cells to the endothelium, the polymorphism could be of interest with respect to association studies in a number of pathological conditions, such as cardiovascular diseases. The $Ser^{128}Arg$ polymorphism is associated with a higher risk for early severe atherosclerosis. Yoshida et al. (2003) suggested that the E-selectin $Ser^{128}Arg$ polymorphism could functionally alter leukocyte-endothelial interactions as well as biochemical and biological consequences, which may account for the pathogenesis of myocardial infarction (Li et al. 2005).

Leu^{554}Phe E-selectin mutations in Hypertension and CAD

Wenzel et al. (1996, 1999) detected 17 mutations, five of which resulted in an amino acid substitution. In E-selectin, exchange at $Ser^{128}Arg$ in EGF domain and $Leu^{554}Phe$ in membrane domain, and a DNA mutation from guanine to thymine (position 98) presented different allele frequencies in young patients with severe atherosclerosis, compared with an unselected population. The bi-allelic A/C polymorphism in the E-selectin gene may be implicated in the clinical expression of erythema nodosum (EN) secondary to sarcoidosis (Amoli et al. 2004). However, the E-selectin polymorphism may be associated with severity of atherosclerotic disease, but it is unknown if it is actually a risk factor for atherosclerosis (Ghilardi et al. 2004). **A** strong relationship was confirmed between 561A > C and 98G > T polymorphisms of E-selectin gene and susceptibility to CAD by Zak et al. (2008). A body mass index (BMI)-specific effect of $Leu^{554}Phe$ polymorphism of E-selectin gene on blood pressure has been reported by Marteau et al. (2004) who strengthened the view that E-selectin is implicated in hypertension (Marteau et al. 2004). Serum levels of E- and P-selectin in patients with essential hypertension (EH) are significantly higher than in controls, where as differences in serum levels of soluble L-selectin, VCAM-1, or ICAM-1 between the patients with EH and the controls were not different (Sanada et al. 2005).

44.3.4.4 Genomic Arrangement of P-Selectin Gene

P-Selectin Thr^{715}Pro (A/C) Polymorphism

Genetic analyses of P-selectin in the progression of atherosclerosis have provided conflicting results regarding the role of variation within the P-selectin gene and risk for heart disease. Miller et al. (2004b) suggested that the $Thr^{715}Pro$ C allele was rare in blacks (0.8 %) and intermediate in South Asians (3.0 %) compared to whites (11.2 %). sP-selectin levels were significantly lower in the individuals with the AC or CC compared to the AA genotype in both whites and South Asians. Thus, in whites and South Asians the C allele of the $Thr^{715}Pro$ P-selectin polymorphism is associated with

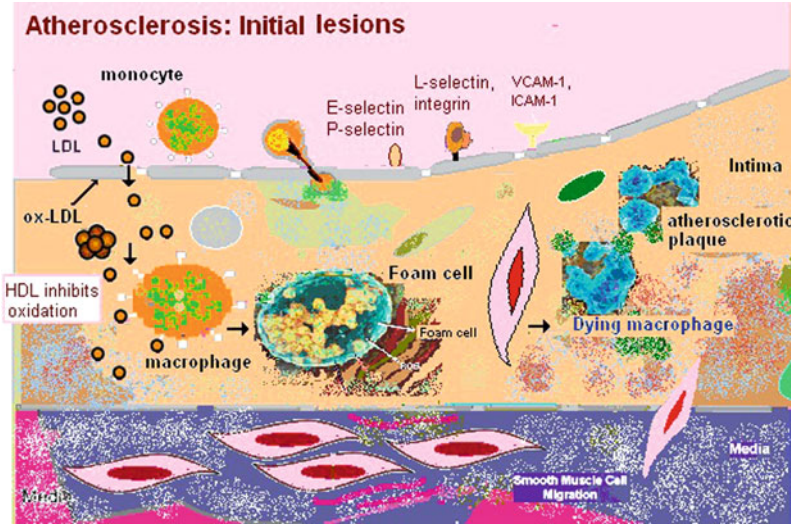

Fig. 44.2 Oxidation of LDL initiates the atherosclerotic process in the vessel wall by acting as a potent stimulus for the induction of inflammatory gene products in vascular endothelial cells. By activating the NF-kB transcription factor, oxidized LDL (ox-LDL) stimulates increased expression of cellular adhesion molecules. There are several different types of adhesion molecules with specific functions in the endothelial leukocyte interaction: The selectins tether and trap monocytes and other leukocytes. Importantly, VCAMs and ICAMs mediate firm attachment of these leukocytes to the endothelial layer. Ox-LDL also augments expression of monocyte chemoattractant protein 1 (MCP-1) and macrophage colony stimulating factor (M-CSF). MCP-1 mediates the attraction of monocytes and leukocytes and their diapedesis through the endothelium into the intima. M-CSF plays an important role in the transformation of monocytes to macrophage foam cells. Macrophages express scavenger receptors, which internalize oxLDL in their transformation into foam cells. Migration of smooth muscle cells (SMCs) from the intima into the media is another early event initiating a sequence that leads to formation of a fibrous atheroma

lower sP-selectin levels (Miller et al. 2004b). The P-selectin Thr^{715}Pro polymorphism is not associated with incident CHD or ischemic stroke in either whites or African-Americans (Volcik et al. 2006).

44.3.4.5 Predictive Value of sE-Selectin in CAD

In CAD, inflammatory biomarkers have been extensively investigated; more evidence exists for C-reactive protein (CRP; Chap. 8). Fatty acid (FA) composition in serum has been associated with CRP and E-selectin but not with other inflammatory markers (Petersson et al. 2009). Studies suggest that, besides CRP, other inflammatory biomarkers such as cytokines, s-CD40 ligand, serum amyloid A (SAA), selectins (E-selectin, P-selectin), ICAM-1, VCAM-1, and several others may have a potential role for the prediction of risk for developing CAD and may correlate with severity of CAD (Eikemo et al. 2004; Fang et al. 2004; Potapov et al. 2005; Zakynthinos and Pappa 2009). The combination of natriuretic peptide (BNP) and E-selectin offers increased predictive value. Plasma levels of adhesive molecules are correlated in patients with stable IHD. Cigarette smoke condensate (CSC)-induced surface expression of ICAM-1, E-selectin, and VCAM-1 in HUVEC. Fang et al. (2005) reported a significant decrease in C allele frequency of PECAM-1 gene and showed that Leu^{125}Val polymorphism of PECAM-1 gene and elevated soluble PECAM-1 were related to severe coronary artery stenosis in CAD patients.

44.3.5 Myocardial Infarction

sP-Selectin is Associated with Myocardial Damage: Platelets are known to be activated during myocardial infarction (MI). Though, the levels of sP-selectin, sE-selectin and sPECAM-1 did not differ significantly in the pathogenesis of atherosclerosis, sP-selectin was substantially increased in patients with acute myocardial infarction (AMI). Yip et al. (2006) tested the hypothesis that platelet activity shown by CD62P is enhanced and predictive of both the extent of myocardial damage and 30-day clinical outcome in patients with ST-se AMI undergoing primary coronary stenting. Xu et al. (2006) suggested that activated-platelets play an important role in the process of myocardial ischemia-reperfusion injury, and platelet-derived P-selectin is a critical mediator. P-selectin expression, along with CD40 ligand and tissue factor is significantly increased in infarcted rabbits with respect to controls. Clopidogrel administration reduced P-selectin expression and CD40 ligand (Molero et al. 2005).

Hyperhomocysteinemia and Selecins in MI: Hyperhomocysteinemia is regarded as an independent risk factor for vascular diseases, and homocysteine is supposed to contribute to oxidative stress and endothelial damage. Hyperhomocysteinemia is significantly associated with MI in comparison with controls with an odd ratio of 6.26 (Khare et al. 2005). Folic acid corrected and reduced hyperhomocysteinemia in a large majority of the cases. Although the levels of sP-selectin, sE-selectin and sPECAM-1 decrease after folic acid therapy, it was only sE-selectin which was significantly reduced. Apart from their lipid-lowering capacity, statins also exert anti-inflammatory and antioxidant effects.

DNA Polymorphism in MI: Some polymorphisms may increase the risk of MI within specific ethnic groups or in certain populations. P-selectin expression is increased in atherosclerotic plaques, and high plasma levels of this molecule have been observed in patients with unstable angina. DNA polymorphisms in P-selectin gene may be a possible candidate for MI. The P-selectin gene is situated on chromosome 1q21-q24, spans >50 kb and contains 17 exons. Four polymorphisms ($Ser^{290}Asn$, $Asn^{562}Asp$, $Leu^{599}Val$ and $Thr^{715}Pro$) predicted a change in the amino acid sequence of P-selectin. In patients with MI from four regions of France and Northern Ireland (the ECTIM study) the P-selectin polymorphisms provided a heterozygosity of 91 %. The polymorphisms were tightly associated with one another and displayed patterns of linkage disequilibrium suggesting the existence of highly conserved ancestral haplotypes. Study illustrates the complexity of the relationship between gene variability and disease and the necessity to explore in detail the polymorphisms of candidate genes (Herrmann et al. 1998; Tregouet et al. 2002).

The E-selectin gene Arg128, 98 T, and Phe554 alleles and PECAM1 $Leu1^{25}Val$ and $Ser^{563}Asn$ polymorphisms may increase the risk of atherosclerosis, but not necessarily the risk of MI. This association seems to be more pronounced in younger patients and may be especially important in patients with a low risk for developing atherosclerosis. Reports indicated that screening for CD14-260 C/T genotypes is unlikely to be a useful tool for risk assessment and it remains unclear whether CD14 polymorphisms significantly increase the risk of MI. The $A^{252}G$ polymorphism of lymphotoxin-α (LTA) gene, a member of the TNF family, is strongly related with the onset of AMI (Auer et al. 2003). Quantitative real-time RT-PCR confirmed that LTA increased the expressions of E-Selectin and VCAM1 both in HUVEC and HCAEC, suggesting the roles of LTA in the development of atherosclerosis. Aminian et al. (2007) determined the possible role of $Gly^{241}Arg$ and $Lys^{469}Glu$ polymorphisms in development of CAD and acute or chronic MI. Although the frequency of Gly-Arg and Arg-Arg genotypes were higher in the control group compared to the CHD patients, no strong corelation was found between $Gly^{241}Arg$ and $Lys^{469}Glu$ polymorphisms and occurrence of CHD and MI in population from Iran.

44.3.6 Atherosclerotic Ischemic Stroke

In ischemic event in patients with atherosclerotic ischemic stroke, though the platelet aggregability was decreased after day 3 compared to that at day 1 of stroke onset, platelet CD63 and P-selectin/CD62P expression remained high even 90 days after the events. This suggested that platelet hyperactivation in atherosclerotic ischemic stroke might be sustained for a considerable period (Cha et al. 2004; Nadar et al. 2004b; Yip et al. 2006). Blood levels of ICAM-1 and CD62P expression in different typing of patients with ischemic stroke are different. Evidences suggest that MPS (Meridian-phlegm stagnancy) group of patients is the key pathogenic factor of ischemic stroke.

Mucosal tolerance to E-selectin after booster tolerization can relieve cerebral ischemia-reperfusion injury and induce ischemia tolerance in rats. The mechanisms may involve decreased frequencies of $CD8^+$ T cells, heightened mRNA expression of IL-10 and lowered mRNA expression of E-selectin in the ischemic hemisphere (Yun et al. 2008). Selakovic et al. (2009) defined changes of soluble CAMS in cerebrospinal fluid and plasma in the patients with the acute brain infarction, in which significant increase in the level of soluble adhesion molecules occurs within the first seven days. Studies show that hypoxia/reoxygenation stimulates ICAM-1 and apoptosis (Antonova et al. 2009).

Cerebral arteriovenous malformations (AVMs) showed significant upregulation of E-selectin, VCAM-1 and ICAM-1 (Storer et al. 2008; Chan and Sukhatme 2008; Tuttolomondo et al. 2009). Li et al. (2008) showed that ICAM-1 $Lys^{469}Glu$ polymorphism was involved in the causation of ischemic stroke, especially in female but not in male (Rodrigues et al. 2008). Two allelic variants were related to ischemic stroke. Multivariable regression analysis after adjustment for vascular risk factors demonstrated that alleles Arg of $Ser^{128}Arg$ and Phe of $Leu^{554}Phe$ polymorphisms are independent risk factors for ischemic stroke. The combination of two minor alleles of E-selectin genes appeared to be the strongest susceptibility factor for ischemic stroke (Haidari et al. 2009). Sarecka-Hujar et al. (2010) could not confirm the relationship between the 98 G > T polymorphism of the E-selectin gene and childhood ischemic stroke. The G allele of the E- selectin 98 G > T polymorphism was more frequently transmitted to the children after stroke compared to the T allele. There is a need for further studies in these areas.

44.3.7 Hypertension

The association between blood pressure and different adhesion molecules appeared to be present in women younger than 50 years, who were likely to be pre-menopausal (Miller et al. 2004a). Serum levels of E- and P-selectin in patients with essential hypertension (EH) are significantly higher than in the controls (Sanada et al. 2005). After adjustment for age, only sE-selectin concentrations were significantly associated with blood pressure. Higher levels of plasma sP-selectin were confirmed in hypertensive patients alone with VEGF (Nadar et al. 2004a). It is stated that decrease in blood pressure may reduce the rate of progression of atherosclerosis by affecting the expression of E- and P-selectin in the endothelium, the platelets, or both.

44.3.8 Reperfusion Injury

In vitro studies indicate that complement activation regulates the expression of P-selectin on endothelial cells. This suggests that in disorders such as ischemia/reperfusion injury, in which both complement and P-selectin have been shown to play a role, complement activation is a primary event and the effects of P-selectin are secondary. In mouse kidney model of I/R injury, results indicated that complement and P-selectin-mediated pathways of renal reperfusion injury are mutually independent (Farrar et al. 2004). Induction of circulating polymorphonuclear neutrophils (PMNs) might contribute to the superior outcome following stenting and early intervention compared to conventional balloon angioplasty (PTCA). A substantial increase in sE-selectin levels early after PTCA and stent implantation may predict development of restenosis (Heider et al. 2006; Kilickap et al. 2004). After reperfusion of myocardial vessels, P-selectin expressed on majority of vessels (77 %) though the expression decreased during subsequent remaining duration of reperfusion (Chukwuemeka et al. 2005). In rats, the mRNA expression for several genes was associated with inflammation after transient middle cerebral artery occlusion (MCAO). Gene expression increased in the injured hemisphere for IL-1β, IL-6 and ICAM-1. TNF-α mRNA was upregulated in the injured versus uninjured hemisphere, while E-selectin mRNA showed a significant increase from 6 to 24 h after MCAO (Berti et al. 2002). Both P-selectin and LFA-1 may be important targets to control pathologic inflammation in I/R-induced tissue injury in the colon (Riaz et al. 2002). The study in intestinal ischemia and reperfusion injury (IR/I) using murine models demonstrated the importance of P-selectin in warm and cold IR/I. The blockade of P-selectin using rPSGL1-lg or the absence of P-selectin KO mice confers a survival advantage and reduction in tissue injury. The mechanism appears to be independent of neutrophil infiltration (Carmody et al. 2004). Enterocyte apoptosis is increased following intestinal I/R injury. Hyperoxia following intestinal I/R in rat increased E-selectin expression in the jejunum and ileum and a concomitant increase in neutrophil recruitment in the ileum, accompanied by increased cell apoptosis (Braun et al. 2004; Sukhotnik et al. 2008). Germ cell-specific apoptosis that occurs after I/R of murine testis is dependent on neutrophil recruitment to the testis and is dependent on E-selectin. Blockage of E-selectin may be a strategy to treat post-ischaemic testis (Celebi and Paul 2008).

44.4 CAMS in Allergic Inflammation

Allergic inflammation is characterized by recruitment of specific leukocyte subpopulations from blood into tissue and requires a series of cell adhesion-molecule-mediated interactions between postcapillary vascular endothelium and the leukocyte cell surface. Three major groups are involved: selectins, integrins, and the immunoglobulin gene superfamily. P- and E-selectin mediate initial leukocyte adhesion, whereas beta 2-integrin/ICAM-1 and VLA-4/VCAM-1 pathways mediate leukocyte arrest and transendothelial migration. Because VLA-4 expression is restricted to eosinophils and lymphocytes, VCAM-1 has been implicated in selective eosinophil recruitment characterizing allergic inflammation. However, additional factors such as profile of cytokine release are likely to operate since tissue eosinophilia has been observed in the absence of VCAM-1 expression (Smith et al. 1993a).

44.4.1 Dermal Disorders

E-selectin is highly expressed on vascular endothelium in atopic dermatitis and psoriasis, and in patients with measles. The cutaneous lymphocyte-associated antigen (CLA), which is expressed on peripheral skin-homing helper memory T cells in healthy persons, is at least partly the sialyl 6-sulfo Le^x determinant (Ohmori et al. 2006) and a ligand for selectins. The differential polyadenylation of E-selectin transcripts may provide the molecular basis for the observed chronic expression of E-selectin in human dermal disorders. In atopic dermatitis, patients express ICAM-1 and ICAM-3, E-selectin and L-selectin (60 %) in the dermis, without expression of E- and L-selectins in the epidermis. A high expression of adhesion molecules in the skin lesions of atopic dermatitis patients may play an important role in the pathogenesis of atopic dermatitis (Lugovic et al. 2006). The blood markers for atopic dermatitis, including soluble forms of E-selectin, VCAM-1 and ICAM-1 were reduced after treatment with cetiridine (Izu and Tokura 2005). The extracts from dust

mites, *Dermatophagoides farinae*, *D. pteronyssinus* and *Euroglyphus maynei* with and without endotoxin (LPS) stimulated endothelial cells to express ICAM-1, VCAM-1, and E-selectin and to secrete IL-6, IL-8, MCP-1, and GM-CSF. Serum levels of sE-selectin are higher in children with measles than in children with atopic dermatitis, atopic asthma and healthy controls. But it was not correlated with measles. There was no correlation between sE-selectin and TNF-α level (Park et al. 2008). Pollinosis from Parietaria judaica is one of the main causes of allergy in the Mediterranean area. The treatment of endothelial cells with pollen extract causes an increase of E-selectin and VCAM-1 protein levels as well as an increase of IL-8 production. The stimulation of cell adhesion molecules was paralleled by an increase of adhesion of polymorphonuclear cells (PMNs) to HMVEC-L monolayer (Taverna et al. 2008).

44.4.2 Rhinitis and Nasal Polyposis

Allergic rhinitis is an inflammatory disease of the nasal mucosa, caused by an IgE-mediated reaction after exposure to the allergen. Persistent inflammation is induced by the presence of an inflammatory cell infiltrate, together with ICAM-1 expression in the epithelial cells of the mucosa exposed to the allergen to which they are sensitized, in the absence of clinical symptoms (Montoro et al. 2007). Nasal polyposis is a chronic non-infectious inflammatory disease of the nasal and paranasal cavity mucosa. Eosinophil migration from blood stream to nasal polyps involves different molecules such as ICAM-1, VCAM-1, and L-, P- and E-selectins. Patients with nasal polyposis exhibit a higher expression of VCAM-1, E-selectin, and L-selectin compared to healthy controls (Corsi et al. 2008). *Staphylococcal enterotoxin A* (SEA) and staphylococcal enterotoxin B (SEB) infection increased ICAM-1 expression and cytokine secretion (Wang et al. 2007).

44.4.3 Lung Injury

Excessive leukocyte accumulation is involved in the pathogenesis of the sepsis-induced acute lung injury. Studies suggest that P-selectin has a substantial role in the pathogenesis of the lung injury induced by LPS (Ohnishi et al. 1999). In bleomycin-induced fibrosis in mice, the L-selectin and/or ICAM-1 deficiency inhibited skin and lung fibrosis with decreased Th2 and Th17 cytokines and increased Th1 cytokines. In contrast, P-selectin deficiency, E-selectin deficiency with or without P-selectin blockade, or PSGL-1 deficiency augmented the fibrosis in parallel with increased Th2 and Th17 cytokines and decreased Th1 cytokines. Yoshizaki et al. (2010) suggest that L-selectin and ICAM-1 regulate Th2 and Th17 cell accumulation in skin and the lung, leading to the development of fibrosis, and that P-selectin, E-selectin, and PSGL-1 regulate Th1 cell infiltration, resulting in the inhibition of fibrosis (Yoshizaki et al. 2010). Adult respiratory distress syndrome (ARDS) appears to develop as the acute lung injury in the course of many severe diseases, as the result of damage of alveolar-capillary barrier. Clinical observations suggest that analysis of E-, P-selectin and ICAM-1 concentrations in the serum of patients with ARDS may be helpful in monitoring the course and treatment of the disease (Skiba-Choińska and Rogowski 1996). In the pathogenesis of paracoccidioidomycosis, Gonzalez et al. (2005) suggest that during early stages, up-regulation of ICAM-1, VCAM-1, CD18 and Mac-1 expression may participate in the inflammatory process.

44.4.4 Bronchial Asthma and Human Rhinovirus

The house dust mite (HDM) is the common indoor allergen associated with bronchial asthma. ICAM-1, VCAM-1, and E-selectin are newly synthesized prior to spontaneous asthma attacks, and their expression may play a key role in eosinophil infiltration into the airway (Ohkawara et al. 1995). Crude extract of *D. farinae* induces ICAM-1 expression in EoL-1 cells through signaling pathways involving both NF-kB and JNK (Kwon et al. 2007). Kirchberger et al. (2006) demonstrated that signaling via ICAM-1 induces adhesiveness of mononuclear phagocytes, which critically involves PECAM-1 and is mediated via LFA-1/ICAM-3. The most common acute infection in humans, Human Rhinovirus (HRV) is a leading cause of exacerbations of asthma and chronic obstruction pulmonary disease. ICAM-1 is a critical target-docking molecule on epithelial cells for 90 % HRV serotypes. ICAM-1 regulates not only viral entry and replication but also signaling pathways that lead to inflammatory mediator production (Lau et al. 2008; Lee et al. 2008). The sICAM-1 but not sE-selectin from patients with asthma is significantly higher than healthy controls. Although serum levels of sICAM-1 are higher in asthmatics, it may be necessary to establish individual baseline values for serial estimation to evaluate their clinical relevance (Bijanzadeh et al. 2009). The serum levels of sICAM-1 were significantly higher in obese nonasthmatic and obese asthmatic children versus control and lean asthmatic children (Huang et al. 2008). P-selectin is an important controller of the inflammation by mediating selective eosinophil cell influx to the lung. It can be used as a sensitive marker in mild asthma (Sjosward et al. 2004).

44.5 Autoimmune Diseases

Adhesion molecule expression and interactions are involved in initiation and propagation of autoimmune diseases including rheumatoid arthritis (RA), systemic lupus erythematosus, Sjögren's syndrome, autoimmune thyroid disease, multiple sclerosis, systemic sclerosis (SSc) and diabetes mellitus. Increased adhesion molecule expression and avidity changes occurring with cellular activation are the principal methods regulating leukocyte adhesion. Although differences between specific autoimmune diseases exist, key interactions facilitating the development of autoimmune inflammation appear to include L-selectin/P-selectin/E-selectin, LFA-1/ICAM-1, very late antigen-4 (VLA-4)/VCAM-1, and α4B7/MadCAM or VCAM-1 adhesion. A vast array of adhesive interactions occurs between immunocompetent cells, endothelium, extracellular matrix, and target tissues during the evolution of an autoimmune disease. Dermatitis herpetiformis (DH) and bullous pemphigoid (BP), the autoimmune diseases, are characterized by destruction of the basement membrane zone (BMZ) and anchoring fibres by autoantibodies and infiltration. Skin biopsies from patients with DH, with BP, and from healthy subjects showed the expression of E and L selectins mainly in the skin leukocytes in all samples where as β1, β3 integrins was detected mainly in basal keratinocytes. Integrins and selectins seem to play an important role in the destruction of BMZ in DH and BP (Erkiert-Polguj et al. 2009). P-selectin levels were significantly higher than normal in RA and SSc, but not in SLE. In contrast, mean L-selectin levels were significantly higher than normal in SLE, but not in RA or SSc. Where as soluble IL-2 receptors in patients with active RA, SSc and SLE were almost double the normal level, showing a strong positive correlation only between L-selectin and sIL-2R, and only in patients with SLE. These findings indicated a distinct pattern of immune cell activation in chronic diseases that share an over-activation of T-lymphocytes (Sfikakis and Mavrikakis 1999).

44.5.1 Endothelial Dysfunction in Diabetes (Type 1 Diabetes)

Adhesion molecules have been implicated in the development and progression of cardiovascular disease, particularly in people with diabetes. Diabetes mellitus type 1 (Type 1 diabetes or T1DM, also called insulin-dependent diabetes mellitus—IDDM, or, formerly, juvenile diabetes) is a form of diabetes mellitus that results from autoimmune destruction of insulin-producing β cells of the pancreas. The chronic hyperglycemic state in T1DM patients produces an aggression to vascular endothelium leading to a premature development of atherosclerosis. In both boys and girls, sE-selectin is an early marker of endothelial dysfunction and a probable risk marker of atherosclerosis in children with T1DM (Carrizo et al. 2008). The levels of C-reactive protein, E-selectin, and cytokines in association with severity index were significantly increased in T1DM and type 1 diabetic patients with microvascular complications (T1DM-MV patients) compared with control subjects (Devaraj et al. 2007). Nerve microvasculitis and ischemic injury appear to be the primary and important pathogenic alterations in lumbosacral radiculoplexus neuropathy (LRPN) of patients with diabetes mellitus (DLRPN) and without diabetes mellitus (LRPN). The up-regulation of inflammatory mediators target different cells at different disease stages and that these mediators may be sequentially involved in an immune-mediated inflammatory process that is shared by both DLRPN and LRPN (Kawamura et al. 2008).

Adhesion molecules are upregulated in endothelial cells of the placental bed in pregnancies complicated by T1DM in association with increased adherence of peripheral blood monocytes. The increase in monocyte adhesion to decidual endothelial cells from diabetic pregnancies was associated with increased endothelial cell expression of ICAM-1, but not VCAM-1. ICAM-1 expression in normal decidual endothelial cells was stimulated by pro-atherogenic and pro-inflammatory stimuli (Xie et al. 2008; Telejko et al. 2009).

Type 2 Diabetes: In contrast to T1DM, type 2 diabetes mellitus (T2DM) results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency (Formerly referred to as non-insulin-dependent diabetes mellitus, NIDDM for short). Endothelial dysfunction in type 2 diabetic patients is associated with inflammation, increased levels of circulating soluble adhesion molecules (VCAM-1 and E-selectin), and inducing production of ROS, and urinary albumin excretion (Potenza et al. 2009). Diabetic patients have increased susceptibility to infection, which may be related to impaired inflammatory response observed in experimental models of diabetes, and restored by insulin treatment (Riad et al. 2008; West et al. 2008).

Serum Levels of CAMs in Diabetic Patients: Abnormal levels some of serum ICAM-1, VICAM-1, E-selectin, P-selectin, L-selectin have been detected in T2DM. High-fat load and glucose alone produce an increase of nitrotyrosine, ICAM-1, VCAM-1, and E-selectin plasma levels in normal and diabetic subjects. A decrease in neutrophil surface CD62L expression and significantly higher concentrations of sICAM-1, sVCAM-1, sE-selectin, vWF, hsCRP, IL-6 and fibrinogen in patients with diabetic microangiopathy in comparison with diabetic group without microangiopathic complications and

healthy controls suggested that: (1) diabetic microangiopathy is accompanied by increase in CD11b expression and decrease in CD62L (L-selectin) expression on peripheral blood neutrophils; (2) neutrophil activation and intensified adhesion; (3) the development of diabetic microangiopathy is accompanied by an increase in soluble adhesion molecules and inflammatory markers concentrations in the blood (Lim et al. 2004; Mastej and Adamiec; 2008). Levels of E-selectin positively correlated with high triglyceride levels in type 2 diabetic subjects with silent ischemia (Adamikova et al. 2008; Okapcova and Gabor 2004; Rubio-Guerra et al. 2008). Though, baseline plasma levels of vascular markers (hsCRP, sICAM-1, sVCAM-1, E-selectin and P-selectin) were significantly elevated, they did not improve after aerobic exercise. The sE-selectin and vWF are elevated in chronic heart failure patients with DM but not in those without DM. High sE-selectin levels may be associated with ischaemic events in patients with DM (Kistorp et al. 2008).

Diabetic Nephropathy (DN) and Diabetic Heart: Diabetic nephropathy (DN) is the leading cause of end stage renal disease (ESRD) (Malatino et al. 2007). Although the pathogenesis of DN is multifactorial, local inflammatory stress may result from both the metabolic and hemodynamic derangements observed in DN. The current evidence supporting the role of inflammation in the early phases of clinical and experimental DN has been reviewed (Fornoni et al. 2008). Inflammatory markers such as IL-18 and TNF-α are increased in the serum of patients with diabetes and DN. This occurs at an early stage of disease, and correlates with the degree of albuminuria. The pharmacologic interventions for DN by angiotensin converting enzyme inhibitors, angiotensin receptor blockers and aldosterone antagonists may have anti-inflammatory effects, which are independent of their hemodynamic effect.

Diabetic Retinopathy: The association between soluble adhesion molecules levels and retinopathy in type 2 diabetic patients has been clarified. sE-selectin levels are elevated in diabetic patients compared to control subjects, with no significant difference in sICAM-1 and sVCAM-1 levels. The progression of retinopathy was not associated with an increase in soluble adhesion molecules. However, Nowak et al. (2008) observed that serum levels of sICAM-1 and sELAM-1 were significantly elevated and the concentration sVCAM-1 was elevated but not significantly in diabetic patients. Increase in sICAM-1 and sVCAM-1 levels, as well as their correlation with high vitreous IL-6 and TNF-α concentrations in patients with diabetic retinopathy seems to confirm the inflammatory-immune nature of this process. Significantly increased TNF-α concentration in the vitreous body was related to the rise of VCAM-1 (Adamiec-Mroczek and Oficjalska-Młyńczak 2008; Leal et al. 2008; Khalfaoui et al. 2008). Intravitreal injection of corticosteroid has been used to treat diabetic macular edema.

44.5.2 Rheumatic Diseases

Plasma levels of vWF and sP-selectin (but not sE-selectin) are significantly higher among Rheumatoid disease (RD) patients compared to controls. Levels of vWF progressively rise with increasing cardiovascular risk (Bhatia et al. 2009). Serum levels of ICAM-1, ICAM-3, VCAM-1, L-selectin, and E-selectin have been detemined in children with a variety of pediatric rheumatic diseases. A trend toward higher levels of sE-selectin was found in vasculitis vs other diagnoses. The sICAM-1 was higher in patients with active vs inactive disease across all diagnoses. Report suggests that (1) elevated E-selectin levels in vasculitis likely reflect the high degree of endothelial activation and possibly overt vascular damage in those conditions. (2) The correlation of sL-selectin with C4 in SLE may indicate that downregulation of shedding of cell surface L-selectin is involved in continued adherence of leukocytes to endothelium, possibly causing further damage and immune complex deposition in this condition. (3) The trend toward inverse correlation between sE-selectin and vWF:Ag in diabetes mellitus is interesting. (4) Levels of sICAM- I may be a useful marker of active vs quiescent disease in general in the pediatric rheumatic diseases, although lack of correlation with disease activity indices indicates that it is too insensitive to smaller differences in disease activity to be recommended for routine clinical use (Bloom et al. 2002).

44.5.3 Rheumatoid Arthritis

Considerable evidence indicates that patients with Rheumatoid arthritis (RA) are at greater risk of developing atherosclerosis and cardiovascular disease. Atherosclerotic cardiovascular mortality is increased in RA patients. The markers proposed for assessing RA activity include rheumatoid factor, anti-citrullinated protein/peptide antibodies, IgM anti-IgG advanced glycation end products, markers of bone/cartilage metabolism, mannose-binding lectin, E-selectin, IL-6, and leptin. Various studies have investigated the correlation between some of these markers and other variables that might indicate disease activity, e.g., inflammatory activity tests and disease activity scores. However, there is as yet insufficient evidence that any of these markers, in isolation or in combination, are useful in the assessment of RA activity. Many numerous endothelial cells become positive for E-selectin and E-selectin mRNA in RA synovial membranes

and the E-selectin expression appeared to correlate with inflammatory activity. P-selectin deficiency in mice resulted in accelerated onset of joint inflammation in the murine collagen-immunized arthritis model. Mice deficient either in E-selectin or in E- selectin and P-selectin (E/P-selectin mutant) also exhibit accelerated development of arthritis compared with wild type mice in CIA model. The strong vascular expression of E-selectin indicates an activation of endothelial cells in the recruitment of cells associated with the chronic inflammation of RA (Foster et al. 2009; da Mota et al. 2009). E-Selectin and ICAM-1 are upregulated on the synovial endothelium, while VCAM-1 plays an important role in synovial lining layer cells and within the synovial stroma. The expression of CAMs may be blocked by mAbs and modified by nonsteroidal anti-inflammatory drugs and disease-modifying antirheumatic drugs. (Cobankara et al. 2004). Serum soluble adhesion molecules concentrations are down-regulated following anti-TNF-α antibody therapy combined with methotrexate (MTX) (Klimiuk et al. 2004, 2007; Levälampi et al. 2007; Bosello et al. 2008).

In comparison with osteoarthritis (OA), patients with early RA are characterized by high serum concentrations of sICAM-1, sVCAM-1, and sE-selectin (Yildirim et al. 2005), while LDL-cholesterol was decreased in all RA patients (Pemberton et al. 2009). P-selectin deficiency in mice results in accelerated onset of joint inflammation in the murine collagen-immunized arthritis model. Mice deficient either in E-selectin or in E-selectin and P-selectin (E/P-selectin mutant) also exhibit accelerated development of arthritis compared with wild type mice, suggesting that these adhesion molecules perform overlapping functions in regulating joint disease. Ruth et al. (2005) suggested that E-selectin and P-selectin expression can significantly influence cytokine and chemokine production in joint tissue, and that these adhesion molecules play important regulatory roles in the development of RA in E/P-selectin mutant mice (Singh et al. 2008).

In RA patients, P-selectin expression, PMC and sCD40L levels were increased when compared with controls. The increase in markers of active platelets, P-selectin and sCD40L, and platelet-monocyte levels might be associated with the increased cardiovascular mortality in RA. Psoriatic arthritis (PsA) is associated with the development of endothelial dysfunction and increased atherosclerotic complications. Endothelial activation might have a role in the pathogenesis of both psoriasis and PsA. Among parameters of platelet activation, only PMC might play a role in the pathogenesis of PsA (Pamuk et al. 2008, 2009). Though, sE-selectin correlated with severity of joint disease, further follow-up studies should evaluate if sE-selectin is useful as prognosis marker for progression of articular damage (Corona-Sanchez et al. 2009).

44.5.4 Other Autoimmune Disorders

Systemic Lupus Erythematosus (SLE): Elevated serum concentrations of ET-1, s-thrombomodulin (TM), and sE-selectin reflect persisting endothelial cell activation in SLE, and point to an important role of ET-1 in the pathogenesis of internal organ involvement (Kuryliszyn-Moskal et al. 2008). The s-E-selectin, TM and s-VCAM-1 are significantly elevated in lupus nephritis (LN) with renal vascular lesions (VLS) than in LN without VLS. A positive correlation was found between TM and serum creatinine in patients with vascular lesions. Therefore, serum TM and s-VCAM-1 can be biomarkers of VLS in LN patients (Yao et al. 2008, Rho et al. 2008).

Autoimmune Thyroiditis: Autoimmune thyroiditis is multifactorial in etiology with genetic and environmental factors contributions. Patients with untreated Graves' disease (GD) show high serum level of sE-selectin, which correlated with the activity of the disease. The expression of ICAM-1 and VCAM-1 was increased in EC from patients from Graves' disease (GD) and Hashimoto's thyroiditis (HT). Results suggest that both the LFA-1/ICAM-1, ICAM-3 and VLA-4/VCAM-1 pathways could play a relevant role in autoimmune thyroid disorders (Marazuela et al. 1994). In patients with GD, the 721 G-A polymorphism was associated with an earlier age of GD onset (before age 40) and that the 1405A-G polymorphism could predispose to Graves ophthalmopathy. It was concluded that G241R and K469E amino acid substitutions in the ICAM1 molecule could influence the intensity/duration of the autoimmunity process and the infiltration of orbital tissues (Kretowski et al. 2003). Chen et al. (2008) suggested that common *SELE* variants may be associated with susceptibility to GD in Chinese population, though the limitation of sample size and multiple test problems exists (Chen et al. 2008).

Sjogren's Syndrome: CAMs are involved in the lymphoid cell infiltration of the salivary and lacrimal glands in Sjogren's syndrome (SS) patients. Biopsies from SS patients showed a marked expression of VCAM-1 and ICAM-1 in the venules surrounded by infiltrated $CD4^+$ $CD45RO^+$ T cells. E-selectin was expressed on vascular endothelium with weak intensity (Saito et al. 1993). Pisella et al. (2000) reported that a significant increase of HLA-DR and ICAM1 expression by epithelial cells was consistently found in patients with keratoconjunctivitis sicca (Sjogren syndrome). These markers were well correlated with each other and correlated inversely with tear break-up time and tear production. Cytokine-mediated up-regulation of VCAM-1 and ICAM-1 that facilitates the recruitment of VLA-4 and LFA-1 expressing T cells might contribute to lymphoid cell infiltration in the salivary and lacrimal glands in SS

44.6 CAMs in System Related Disorders

44.6.1 Gastric Diseases

CAMs mediate the extravasation of leukocytes and their accumulation in inflamed intestinal mucosa. Eosinophilic inflammation is a common feature of numerous eosinophil-associated gastrointestinal (EGID) diseases. Increased intestinal expression of E-selectin has been associated with multiple organ failure and an adverse outcome. VCAM-1 is not altered in in mucosa of patients with inflammatory bowel disease (IBD) regardless of the activity of the inflammatory process. In contrast, E-selectin was not detected in normal colonic mucosa or in colonic mucosa of patients with IBD. However, high levels of E-selectin were consistently found on endothelial surfaces in association with active inflammation in affected areas of colonic mucosa in patients with either ulcerative colitis or Crohn's colitis. In addition, E-selectin appeared to be present within neutrophils which had migrated into crypt abscesses in affected mucosa. Thus E-selectin may play an important role in facilitating leukocyte migration into sites of active IBD involvement (Koizumi et al. 1992)

ICAM-1 was expressed to a greater degree in ulcerative colitis (UC) specimens. Serum ICAM-1 levels in UC patients showed lower levels than those in the control group and were found to vary according to degree of clinical severity (Ogawa et al. 2008). Characterization of integrin expression on colonic eosinophils revealed that colonic CC chemokine receptor 3^+ eosinophils express ICAM-1 counter-receptor integrins αL, αM, and β2. It appears that β2-integrin/ICAM-1-dependent pathways are integral to eosinophil recruitment in colon during GI inflammation associated with colonic injury (Forbes et al. 2006).

McCafferty et al. (1999) examined the role of P-selectin in intestinal inflammation in P-selectin deficient mice alone or in combination with either ICAM-1 or E-selectin and suggested that anti-adhesion therapy might play only a limited, beneficial role and often a detrimental role in intestinal inflammation. The sE-selectin levels of Crohn's disease patients with active disease are higher than those with remission of the disease. L-selectin does not change in patients with active disease compared to those with remission. Thus, determination of sE-selectin in children with Crohn's disease is of significance in estimation of inflammation activity (Adamska et al. 2007).

Khazen et al. (2009) investigated mutations in CAM genes in Tunisian patients, implicated in determining susceptibility to ulcerative colitis (UC) and Crohn's disease (CD). A significant increase in allele frequencies of 206 L of L-selectin and the associated genotype F/L was observed in patients with UC and CD compared with controls; the L206 allele and F/L206 genotype frequencies were significantly increased in UC patients with left-sided type; whereas, the F/L206 genotype was significant in CD patients with ileocolonic location. No significant differences in allele or genotype frequencies were observed for ICAM-1 K469E, E-selectin, and PECAM-1 polymorphisms between UC patients, CD patients, and controls. Khazen et al. (2009) suggest an association of inflammatory bowel disease with allele L206 of L-selectin gene, whereas genotype L/F was associated with a subgroup of UC (left-sided type) and CD patients with more extensive location of disease and stricturing behavior.

However, Vischer et al. (2008) did not reveal any difference in mRNA and protein expression levels for any construct or a major impact of missense variants on ICAM-1 biological function. Pulse-chase experiments showed that two variants, K469E and arg478 to trp (R478W), had a prolonged half-life compared with wildtype ICAM1, whereas two other variants, G241R and pro352 to leu (P352L), had a decreased half-life, implying differences in protein degradation.

Celiac Disease: Celiac disease is a chronic intestinal inflammatory disease that develops in genetically susceptible individuals after gluten ingestion. The *ICAM-1* gene, located in the Celiac disease linkage region 19p13, encodes ICAM-1 involved in inflammatory processes. Increased levels of ICAM-1 were observed in intestinal biopsies and in sera of Celiac disease patients. In addition, an association between the ICAM1 polymorphism G241R and Celiac disease patients has been described in a French population.though, in spanish population results discard the importance of ICAM1 G241R in celiac disease (Dema et al. 2008).

Behçet Syndrome: Behçet's disease/syndrome (BD/BS) is a multisystemic inflammatory disorder of which oral aphthous ulceration is a major feature. CD3 and γδ T-cell expression and other adhesion molecules including VCAM-1 and ICAM-1 were upregulated, whereas CD40 showed little change in BD. The changes in cell-cell and cell-extracellular matrix interactions may affect cell homeostasis and participate in the formation of oral ulcers in BD (Kose et al. 2008). However, Demirkesen et al. (2008) found no significant differences between the BS and control groups in regard to E-selectin, P-selectin, VCAM-1, PNCAM-1 except for ICAM-1.

Systemic Sclerosis or Systemic Scleroderma: Systemic sclerosis or systemic scleroderma is a systemic autoimmune disease or systemic connective tissue disease that is a subtype of scleroderma. Severe fibrosis and increased expression of profibrotic cytokines are important hallmarks in the gastric wall of patients with systemic sclerosis (SSc; scleroderma). The $CD4^+/CD8^+$ T cell ratio is significantly increased in SSc specimens. T cells strongly express the activation markers

VLA-4, LFA-1, and ICAM-1. Endothelial cells showed corresponding surface activation with strong expression of VCAM-1 and ICAM-1. These results provide the evidence that endothelial/lymphocyte activation leading to prominent $CD4^+$ T cell infiltration may play a key pathogenetic role within the gastric wall of patients with SSc (Manetti et al. 2008). In patients with SSc with and without pulmonary arterial hypertension (PAH), serum sICAM-1, sVCAM-1, sP-selectin and sPECAM-1 levels were higher than in healthy donors (HD) at baseline and fell to normal values after 12 months of bosentan therapy. Endothelial activation occurs in SSc, and that changes in the T cell/endothelium interplay take place in SSc-associated PAH. Bosentan seems to be able to hamper these changes and restore T cell functions in these patients (Iannone et al. 2008).

44.6.2 Liver Diseases

Soluble adhesion molecules play a significant role in hepatitis. Biliary atresia (BA) is a congenital or acquired liver disease and one of the principle forms of chronic rejection of a transplanted liver allograft. In the congenital form, the common bile duct between the liver and the small intestine is blocked or absent. The acquired type most often occurs in the setting of autoimmune disease, and is one of the principle forms of chronic rejection of a transplanted liver allograft. The serum sE-selectin of BA patients was higher than that of controls. Subgroup analysis showed that there was an increase in sE-selectin levels of BA patients with jaundice compared to those without jaundice. Also, sE-selectin was positively correlated with serum alanine transferase (ALT), a marker for liver injury, but not with serum gamma glutamyl transpeptidase (GGT) (Vejchapipat et al. 2008).

Cholangitis without a modifier—from Greek *chol-*, bile + *ang-*, vessel + *itis-*, inflammation) is an infection of the bile duct (cholangitis). In secondary cholangitis, ICAM-1 expression is increased along with de novo VCAM-1 and E-selectin appearance on the endothelium of microvessels in chronic exacerbated cholangitis (Gulubova et al. 2008).

Primary biliary cirrhosis (PBC) is an autoimmune disease of the liver marked by the slow progressive destruction of the small bile ducts (bile canaliculi) within the liver. Patients with PBC, primary sclerosing cholangitis and chronic active hepatitis (autoimmune) show significant increase in sICAM-1 compared with normal healthy subjects. Significant elevation in sICAM-1 is also detected in patients with inactive alcoholic cirrhosis, suggesting that impaired liver may, in part, account for the increased serum level in patients with autoimmune liver disease. In contrast, sE-selectin did not differ significantly from healthy controls. Although, peripheral blood mononuclear cells (PBMC) may be a source of sICAM-1, Thomson et al. (1994) suggested that PBMC may not be a significant source of sICAM-1 in this disease. The differential expression of CAMs in the liver is consistent with the suggestion of selectins involvement in neutrophil rolling in the vasculature and ICAM-1 in transendothelial migration and adherence to parenchymal cells (Essani et al. 1995).

Wu et al. (2009) investigated the relationships between the polymorphisms of E-selectin gene and plasma sE-selectin levels in relation to disease progression in a hepatitis B virus (HBV)-infected Chinese Han population. The frequency of C allele (AC or CC) of the $A^{561}C$ polymorphism was significantly increased in patients with liver cirrhosis (LC) compared to normal population. There was no difference in allele distribution of the $G^{98}T$ polymorphism. The $A^{561}C$ polymorphism of E-selectin gene may be associated with disease progression in patients with chronic HBV infection and control the expression of plasma soluble levels, while the $G^{98}T$ polymorphism may be related to fibrotic severity in Chinese population (Wu et al. 2009).

Mice with targeted deletion of the P-selectin gene developed unpolarized type 1/type 2 cytokine responses and severely aggravated liver pathology following infection pathogen *Schistosoma mansoni*. Liver fibrosis increased 6 fold, despite simultaneous induction of IFN-γ and increase in inflammation in absence of P-selectin. This suggested a critical role of P-selectin in the progression of chronic liver disease caused by schistosome parasites (Wynn et al. 2004).

44.6.3 Neuro/Muscular Disorders

Axonal degeneration was confirmed as the major pathological feature of critical illness polyneuropathy (CIP). Expression of E-selectin was significantly increased in endothelium of epineurial and endoneurial vessels, suggesting endothelial cell activation (Fenzi et al. 2003). Increasing evidence indicates that inflammatory responses are implicated in the pathogenesis of cerebral vasospasm after aneurismal subarachnoid hemorrhage (SAH). Murine SAH model provided the evidence of effective prevention of SAH-induced vasospasm by a mAb implied the possible role of E selectin in the pathogenesis of vasospasm after SAH (Lin et al. 2005).

Neuroinflammation is present in the substantia nigra (SN) of patients of Parkinson disease (PD). A large number of ICAM-1-positive reactive astrocytes have been observed in the SN of patients with neuropathologically confirmed PD, including three of familial origin. The ICAM-1-positive reactive astrocytes were mainly concentrated around residual neurons in areas of heavy neuronal loss and extracellular melanin accumulation (Miklossy et al. 2006). The sVCAM-1 plasma levels were higher in late onset Alzheimer's disease (LOAD) and vascular dementia (VD) compared with controls. Among patients (LOAD, VD, and not-dementia

(CDND), sE-selectin levels were higher in individuals with most severe cerebrovascular disease on CT scan. Increased sVCAM-1 plasma levels in LOAD and VD suggest the existence of endothelial dysfunction in both types of dementia. Results support the possible role of E-selectin in the pathogenesis of cerebrovascular disease (Zuliani et al. 2008).

44.6.4 Acute Pancreatitis

Upregulation of ICAM-1, LFA-1, Mac-1 and subsequent leukocyte infiltration appears to be significant events of pancreatic and pulmonary injuries in Acute pancreatitis (AP) (Sun et al. 2006). Proinflammatory cytokines and oxidative stress seem to be involved in the development of local and particularly systemic complications in AP patients. Acute pancreatitis patients show VCAM-1 and P-selectin concentrations significantly lower and L-selectin concentrations significantly higher than the healthy subjects. Only E-selectin was significantly higher in severe than in mild disease (Pezzilli et al. 2008). Kleinhans et al. (2009) showed that the endothelial cell expression of PECAM-1, VCAM, E-selectin, and P-selectin was upregulated in severe porcine pancreatitis. In acute pancreatitis, plasma levels of sE-selectin and soluble thrombomodulin (sTM) serve as endothelial markers; the former is an endothelial activation marker, while the latter is an endothelial injury marker (Chooklin 2009; Ida et al. 2009).

44.6.5 Renal Failure

In patients affected by microscopic polyangiitis (MPA) and associated with myeloperoxidase (MPO)-anti-neutrophil cytoplasmic antibodies (ANCA), higher sICAM-1 and sE-selectin levels during active phase and their slower decline during the treatment period, could be a prognostic risk factor for chronic renal failure development (Di Lorenzo et al. 2004; Musial et al. 2005). An increased level of sE-selectin in patients susceptible to restenosis supports a role for white blood cell/endothelial interaction in restenosis after angioplasty (Sainani and Maru 2005). The impairment of vascular endothelial function was obvious in uremic patients with maintaining hemodialysis (MHD). The changes of ICAM-1 and E-selectin could be accepted as biochemical criterions of vascular endothelial injury (Li et al. 2005). Diuresis, serum creatinine, urea, and enzyme elimination are pathological among patients with acute renal failure (ARF). Higher elimination rates of sICAM-1 and higher values of sE-selectin compared to patients without ARF indicated additional parameters for early signs of kidney damage (Dehne et al. 2008). Both circulating and urinary TNF-α levels are increased in inflammatory chronic renal diseases. TNF-α appeared to play a crucial role in the immunopathogenesis of nephritis by the induction of chemokine, ICAM-1 and VCAM-1 expression via the activation of the intracellular MAPK signaling pathway, which may contribute to macrophage and lymphocyte infiltration (Ho et al. 2008; Li et al. 2008).

SNPs in Selectin genes and IgA Nephropathy: Although intensive efforts have been made to elucidate the genetic basis of Ig A nephropathy (IgAN), genetic factors associated with the pathogenesis of this disease are not well understood. A case–control study, based on linkage disequilibrium among SNPs in selectin gene cluster on chromosome 1q24-25 revealed two SNPs in the E-selectin gene (*SELE8* and *SELE13*) and six SNPs in the L-selectin gene (*SELL1, SELL4, SELL5, SELL6, SELL10,* and *SELL11*), that were significantly associated with IgAN in Japanese patients. *SELE8* and *SELL10* caused amino acid substitutions from His to Tyr and from Pro to Ser for His-to-Tyr substitutions; and *SELL1* could affect promoter activity of the L-selectin gene. The TGT haplotype at these three loci was associated significantly with IgAN. These SNPs in selectin genes may be useful for screening populations susceptible to the IgAN phenotype. (Takei et al. 2002)

Transplant Rejection: Soluble adhesion molecules are not valuable markers for stable kidney graft (STx) rejection reaction. However, patients with chronic renal failure showed increased levels of adhesion molecules, which could reflect an impaired elimination (Alcalde et al. 1995). The expression levels of ICAM-1 and VCAM-1 show positive correlation with the severity of graft rejection and can provide evidence for early diagnosis and prevention of CR. Chronic allograft failure (CAF) is the major cause for late graft loss in renal transplantation. ICAM-1 polymorphisms may represent a predetermined genetic risk factor for CAF. This was substantiated by the polymorphism in exon 4 at the Mac-1 binding site and in exon 6 at fifth Ig-like domain (McLaren et al. 1999). Khazen et al. (2007) found no evidence for an association of any polymorphism with acute rejection in E- and L-selectin. During kidney reperfusion, E-selectin, ICAM-1, and VCAM-1 concentrations correlated positively with hypoxanthine concentrations during reperfusion, whereas concentrations of ICAM-1 correlated negatively with xanthine concentrations, indicating metabolic changes in renal tissue (Domanski et al. 2009).

44.6.6 Other Inflammatory Disorders

Serum CAM levels have been analyzed in many organ diseases, including diseases of nervous system, endocrine disorders and others. Immune dysfunction has been

proposed as a mechanism for pathophysiology of autistic-spectrum disorders. Levels of sP-selectin and sL-selectin were significantly lower in patients than in controls. Furthermore, sP-selectin levels were negatively correlated with impaired social development during early childhood (Iwata et al. 2008). In multiple sclerosis and in its animal model experimental autoimmune encephalomyelitis (EAE), inflammatory cells migrate across the endothelial blood–brain barrier (BBB) and gain access to the CNS. The role of E- and P-selectin in this process has been controversial. Döring et al. (2007) suggest that absence of E- and P-selectin did neither influence the activation of myelin-specific T cells nor the composition of the cellular infiltrates in the CNS during EAE. Thus, E- and P-selectin are not required for leukocyte recruitment across BBB and the development of EAE in C57BL/6 and in SJL mice (Döring et al. 2007). No significant differences in allelic or genotypic frequency in all the SNPs (rs6133, rs4987310 and rs5368 substitutions) tested were found in the Italian population (Fenoglio et al. 2009).

The pathophysiology of cluster headache (CH) is supposed to involve the lower posterior part of the hypothalamus, the trigeminal nerve, autonomic nerves and vessels in the orbital/retro-orbital region. Remahl et al. (2008) compared serum levels of sICAM-1, sVCAM-1 and sE-selectin in patients with episodic CH and in patients with biopsy-positive giant cell arteritis (GCA), a vasculitic disorder of large and medium-sized arteries. Within the CH group, sICAM-1, sVCAM-1 and sE-selectin showed an increasing trend in remission compared with active cluster headache period, but sE-selectin only was significant. Remahl et al. (2008) suggest that cluster headache is not a vasculitic disorder of medium-sized arteries, but CH patients may have an immune response that reacts differently from that of healthy volunteers.

Adhesion molecules have a role in many vasculitic disorders. Compared to controls, Takayasu's arteritis (TA) patients had elevated levels of sE-selectin, sVCAM-1, and sICAM-1. Compared to controls, patients with inactive TA also had elevated levels of sE-selectin, sVCAM-1, and sICAM-1. There was no difference between active TA and controls. The sE-selectin had a trend towards increased levels in inactive versus active TA, but there was no difference in sVCAM-1 and sICAM-1 levels between the groups. Patients with inactive TA had elevated levels of sE-selectin, sVCAM-1, and sICAM-1 that might indicate persistent vasculopathy in clinically inactive disease (Tripathy et al. 2008).

44.6.7 Inflammation in Hereditary Diseases

Serum levels of sVCAM-1, sICAM-1, sTM, P-selectin, E-selectin and CRP levels as inflammation markers are increased in patients of β-thalassemia intermedia and not influenced by treatment (Kanavaki et al. 2009). Pseudoxanthoma elasticum (PXE) is a hereditary disorder predominantly affecting the skin, retina and vascular system. P-selectin concentrations were increased in male and female PXE patients and levels correlated with the ABCC6 gene status of the patients. Patients harboring two mutant ABCC6 alleles had 1.5-fold increased P-selectin concentrations in comparison to patients with at least one wild-type allele. E- and L-selectin levels were within normal range and the allelic frequencies did not differ between from controls. Elevated P-selectin levels in PXE patients are potentially due to oxidative stress and elevated protease activity in PXE (Götting et al. 2008).

Fabry disease, an X-linked systemic vasculopathy, is caused by a deficiency of α-galactosidase A resulting in globotriaosylceramide (Gb_3) storage in cells. Accumulation of Gb_3 in the vascular endothelium of Fabry disease is associated with increased production of reactive oxygen species (ROS) and increased expression of CAMs. Increased Gb_3 induces expression of ICAM-1, VCAM-1, and E-selectin. Reduction of endogenous Gb_3 by treatment of the cells with an inhibitor of glycosphingolipid synthase or α-galactosidase A led to decreased expression of adhesion molecules. This study indicates that excess intracellular Gb_3 induces oxidative stress and up-regulates the expression of CAMs in vascular endothelial cells (Shen et al. 2008).

44.7 Role of CAMs in Cancer

Recent reports have expanded the concept that inflammation is a critical component of tumor progression. Many cancers arise from sites of infection, chronic irritation and inflammation. It is now becoming clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration. In addition, tumor cells have co-opted some of the signaling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis. These insights are fostering new anti-inflammatory therapeutic approaches to cancer development

44.7.1 Selectin Ligands in Cancer cells

Sialosyl Lewis^a in Adhesion of Colon and other Cancers: The complexity of the tumor microenvironment has been revealed in the past decade. The CAMs in the process of inflammation are responsible for recruiting leukocytes onto the vascular endothelium before extravasation to the injured tissues. Some circulating cancer cells have been shown to extravasate to a secondary site using a process similar to inflammatory cells. The most studied ligands for CAMs expressed on cancer cells, s-Lewis^a and s-Lewis^x antigens, are shown to be involved in adhesion to endothelial cells by binding to E-selectin. This process, shared by inflammatory cells and cancer cells, may partially explain the link between inflammation and tumorigenesis. The adhesion of colon cancer cells to E-selectin can be directly affected by changes in the expression level of sialosyl Le^a antigen. The specific lack of expression of sialosyl Le^a carbohydrate structure on the surface of colon cancer cells completely abolished their adhesion to E-selectin. It is proposed that glycoproteins as well as gangliosides carrying sialosyl Le^a structures, when properly exposed and present in high density on surface of cancer cells, can effectively support the adhesion of cancer cells to E-selectin (Klopocki et al. 1998; Kobayashi et al. 2007). In addition to endogenous ligands for L-, P-, and E-selectins (Chap. 26, 27, and 28), several proteins are found in cancer cell lines or solid tumors that act as ligands for E, L, and P selectins. Selectin ligands present in cancers are:

(1) Glycodelin A (GdA) is primarily produced in endometrial and decidual tissue and secreted to amniotic fluid. GdA is expressed in ovarian cancer where it can act as an inhibitor of lymphocyte activation and/or adhesion (Jeschke et al. 2009); (2) The cysteine-rich fibroblast growth factor receptor (FGF-R) represents the main E-selectin ligand (ESL-1) on granulocytes. Hepatic stellate cells (HSC) are pericytes of liver sinusoidal endothelial cells, which are involved in the repair of liver tissue injury and angiogenesis of liver metastases. HSC express FGF-R together with FucT7 and exhibit a functional E-selectin binding activity on their cell surface (Antoine et al. 2009). (3) Although B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cell lines do not express the ligand PSGL-1, a major proportion of carbohydrate selectin ligand was carried by another sialomucin, CD43, in NALL-1 cells. CD43 plays an important role in extravascular infiltration of NALL-1 cells and the degree of tissue engraftment of BCP-ALL cells may be controlled by manipulating CD43 expression (Nonomura et al. 2008). (4) Thomas et al. (2009a) identified podocalyxin-like protein (PCLP) as an alternative selectin ligand. PCLP on LS174T colon carcinoma cells possesses E-/L-, but not P-, selectin binding activity. PCLP functions as an alternative acceptor for selectin-binding glycans. The finding that PCLP is an E-/L-selectin ligand on carcinoma cells offers a unifying perspective on the apparent enhanced metastatic potential associated with tumor cell PCLP overexpression and the role of selectins in metastasis (Thomas et al. 2009b). (5) E-selectin has been shown to play a pivotal role in mediating cell-cell interactions between breast cancer cells and endothelial monolayers during tumor cell metastasis. The counterreceptor for E-selectin was found as CD44v4. However, CD44 variant (CD44v) isoforms was functional P-, but not E-/L- selectin ligands on colon carcinoma cells. Furthermore, a ~180-kDa sialofucosylated glycoprotein(s) mediated selectin binding in CD44-knockdown cells. This glycoprotein was identified as carcinoembryonic antigen (CEA). CEA serves as an auxiliary L-selectin ligand, which stabilizes L-selectin-dependent cell rolling against fluid shear (Thomas et al. 2009b). Zen et al. (2008) identified a ~170 kDa human CD44 variant 4 (CD44v4) as E-selectin ligand, which has a high affinity for E-selectin via sLe^x moieties.

44.7.2 E-Selectin-Induced Angiogenesis

Angiogenesis plays an important role in a variety of pathophysiologic processes, including tumor growth and rheumatoid arthritis. Studies on capillary morphogenesis and angiogenesis in vitro have suggested a role for E-selectin in the process of differentiation into tube-like structures. Soluble E-selectin is a potent mediator of human dermal microvascular endothelial cell (HMVEC) chemotaxis, which is predominantly mediated through the Src and the phosphatidylinositiol 3-kinase (PI3K) pathways (Kumar et al. 2003). Gastrin-17 (G17) has marked proangiogenic effects in vivo on experimental gliomas and in vitro on HUVECs and transiently decreased the expression of E-selectin, but not P-selectin, whereas IL-8 increased the expression of E-selectin. Specific antisense oligonucleotides against E- and P-selectin decreased HUVEC tubulogenesis processes in vitro. This showed that gastrin has marked proangiogenic effects in vivo on experimental gliomas and in vitro on HUVECs. This effect depends in part on the level of E-selectin activation, but not on IL-8 expression/release by HUVECs (Lefranc et al. 2004).

44.7.3 E-Selectin in Cancer Cells

Adhesion molecules are thought to have a role in the host defense against carcinogenesis. Significantly increased P-selectin, s-VCAM-I and s-ICAM-I levels were observed in patients with bladder cancer, and s-VCAM-I levels correlated with tumor stage (Coskun et al. 2006). Selectins mediate attachment of leukocytes to activated endothelium

as well as the adhesion reaction of tumor cells during malignancy (Borsig 2007). In a breast tumor xenograft model, the effect of combined TNF-α and IFN-γ therapy involved the selective destruction of the tumor vasculature and death of tumor cells. Concomitant with these changes RT-PCR analysis revealed the increase of stromal mRNA levels for a series of stromal cytokines, cytokine receptors including TNF-α, sICAM-1, VCAM-1, P-selectin, which could be implicated in the observed events (de Kossodo et al. 1995).

Squamous Cell Carcinomas: In order to evaluate the risk of postoperative haematogenic recurrence of esophageal squamous cell carcinoma (SCC) patients, Shimada et al. (2003) examined the preoperative serum levels of sE-selectin and pathological status of the patients. The patients with a high serum soluble E-selectin concomitant with expression of s-Lewis antigens had a significant risk of postoperative haematogenic recurrence. SCCs of sun-induced skin cancers are particularly numerous in patients on T cell immunosuppression. Blood vessels in SCCs did not express E-selectin, and tumors contained few cutaneous lymphocyte antigen (CLA^+) T cells, the cell type thought to provide cutaneous immunosur-veillance. Clark et al. (2008) found that SCCs evade the immune response at least in part by down-regulating E-selectin and recruiting T_{reg} cells.

Cutaneous T-Cell Lymphoma (CTCL): The CTCL is characterized by accumulation of malignant $CD4^+$ T cells in the skin. In malignant T cells from Sezary syndrome (SS), a leukemic variant of CTCL, in dermal microvessels in mouse skin, Hoeller et al. (2009) found that SS cells rolled along dermal venules in a P-selectin- and E-selectin-dependent manner at ratios similar to $CD4^+$ memory T cells from normal donors. Chemokine CCL17/TARC was sufficient to induce the arrest of SS cells in the microvasculature. Together, experiments suggested molecular adhesion cascade operant in SS cell homing to the skin in vivo. Patients with CTCL showed increased levels of sICAM-1 and sICAM-3 when compared with healthy individuals and patients with inflammatory dermatosis. The sE-selectin and sVCAM-1 levels were not affected (López-Lerma and Estrach 2009).

Hodgkin's Disease: Increased sICAM-1 and sE-selectin have been observed in Hodgkin's Disease/lymphoma (HD/HL) patients at diagnosis and sVCAM-1 at diagnosis correlated with both sICAM-1 and sE-selectin levels. Chemotherapy resulted in a significant decrease of sICAM-1 and sE-selectin (Syrigos et al. 2004). Serum sICAM-1 level increases at advanced stages of untreated multiple myeloma (MM) patients, but did not differ significantly from controls. A positive correlation of IL-6 appeared with sICAM-1 and sE-selectin (Uchihara et al. 2006). Epstein-Barr virus (EBV)-positive NK/T cells showed affinity to vascular components. EBV-positive NK lymphoma cells express ICAM-1 and VCAM-1 at much higher levels than those in EBV-negative T cell lines. Furthermore, NK lymphoma cell lines exhibited increased adhesion to cultured endothelial cells stimulated with TNF-α or IL-1β. The up-regulated expression of VCAM-1 on cytokine-stimulated endothelial cells can be important to initiate the vascular lesions (Kanno et al. 2008).

Non-small Cell Lung Cancer: Serum levels of ICAM-1 increased in advanced stage non-small cell lung cancer (NSCLC) patients, whereas sE-selectin levels were not significantly different from healthy controls Reports suggest that higher serum ICAM-1 can be useful for diagnosis while E-selectin levels have prognostic significance and could be a potential prognostic factor in NSCLC patients (Dowlati et al. 2008; Guney et al. 2008). The Cyfra 21–1 and sE-selectin showed good performance in detecting lung cancer from normal groups. However, Cyfra 21–1 was superior to sE-selectin in discriminating lung cancer from benign lung diseases (Swellam et al. 2008).

44.7.3.1 Thyroid Cancer

Maspin, a serine protease inhibitor belonging to serpin family, is known as a tumor-suppressor protein and also exhibits an inhibitor effect on angiogenesis. Positive correlations were found for maspin positivity and lymph node metastases; E-selectin positivity and lymph node metastases, and P-selectin positivity and lymph node metastases and lymphovascular invasion. Correlations do exist between maspin, E- and P-selectin expressions with each other and with tumor stage. Inactive cytoplasmic maspin cannot act as a tumor suppressor. Expression of E- and P-selectins in tumor cells facilitates the occurrence of metastases, lymphovascular invasion, and perithyroidal soft tissue invasion. Further studies are needed to reveal detailed interactions between maspin, E-selectin, and P-selectin expression (Bal et al. 2008).

Primary Hyperparathyroidism: Patients with primary hyperparathyroidism (PHPT) have impaired vasodilation. Based on small number of patients, a study suggested that classic cardiovascular risk factors seem to be the main determinants for the high plasma levels of sE-selectin and vWF in PHPT. Together with unaltered thrombomodulin and sE-selectin levels, the vWF decrease in plasma after parathyroidectomy reflects a specific mechanism of its endothelial calcium- and/or PTH-stimulated secretion in some PHPT patients without risk factors (Fallo et al. 2006).

Colorectal Cancer: Plasma level of sP-selectin, sE-selectin and ICAM-1 were significantly higher in colorectal cancer (CRC) patients. The highest levels of sE-selectin and ICAM-1 were observed in patients with liver metastasis. There was no correlation between sP-selectin and sE-selectin, but a significant correlation was seen between sE-selectin and ICAM-1 in all patients. Plasma concentration of E-selectin and ICAM-1 may indicate tumor progression and liver metastasis (Dymicka-Piekarska and Kemona 2009; Sato et al. 2010).

The interaction between rE-selectin and CRC cells alters the gene expression profile of cancer cells. A DNA microarry analysis indicated that E-selectin-mediated alterations were significantly more pronounced in metastatic CRC variants SW620 and KM12SM than in the corresponding non-metastatic local SW480 and KM12C variants. The number of genes altered by E-selectin in metastatic variants was 10-fold higher than the number of genes altered in the corresponding local variants. Analysis indicated that E-selectin down regulated (at least by 1.6-folds) the expression of seven genes in a similar fashion, in both metastatic cells. Confocal microscopy indicated that E-selectin down-regulated the cellular expression of HMGB1 protein and enhanced the release of HMGB1 into the culture medium. The released HMGB1 in turn, activated endothelial cells to express E-selectin (Aychek et al. 2008).

The entrapment of malignant cells within the hepatic sinusoids and their interactions with resident non-parenchymal cells are considered very important for the whole metastatic sequence. In the sinusoids, cell connection and signaling is mediated by multiple cell adhesion molecules, such as the selectins. The three members of the selectin family, E-, P- and L-selectin, in conjunction with sialylated Lewis ligands and CD44 variants, regulate colorectal cell communication and adhesion with platelets, leucocytes, sinusoidal endothelial cells and stellate cells. Therefore, trials have already commenced aiming to exploit selectins and their ligands in the treatment of benign and malignant diseases. Multiple pharmacological agents have been developed that are being tested for potential therapeutic applications (Schnaar et al. 2008; Paschos et al. 2010; Zigler et al. 2010).

44.7.4 Metastatic Spreading

The degree of selectin ligand expression by cancer cells is well correlated with metastasis and poor prognosis for cancer patients. Initial adhesion events of cancer cells facilitated by selectins result in activation of integrins, release of chemokines and are possibly associated with the formation of permissive metastatic microenvironment. While E-selectin is one of the initiating adhesion events during metastasis, it is becoming apparent that P-selectin and L-selectin-mediated interactions significantly contribute to this process as well (Gout et al. 2008; Läubli and Borsig 2010).

E-Selectin in Progression of Metastasis of Breast Cancer: Extravasation of cancer cells is a pivotal step in the formation of hematogenous metastasis. Extravasation is initiated by the loose adhesion of cancer cells to endothelial cells via an interaction between endothelial selectins and selectin ligands expressed by the tumor cells. Metastatic spreading is a dreadful complication of neoplastic diseases that is responsible for most deaths due to cancer. It consists in the formation of secondary neoplasms from cancer cells that have detached from the primary site. Leukocytes and tumor cells use E-selectin binding ligands to attach to activated endothelial cells expressing E-selectin during inflammation or metastasis. The formation of these secondary sites is not random and several clinical observations indicate that the metastatic colonization exhibits organ selectivity. This organ tropism relies mostly on the complementary adhesive interactions between cancer cells and their microenvironment. E-selectin and sLewis antigens might play important role in breast tumor, lymph node and liver metastasis. High levels of sE-selectin have been reported in melanoma and some epithelial tumors, especially in colorectal carcinoma. But sE-selectin may not be used as a predictive marker of metastasis in colorectal carcinoma, though high levels of sE-selectin may support diagnosis of liver metastasis (Uner et al. 2004; Eichbaum et al. 2004). It appeared that serum levels of sE-selectin are associated with the clinical course of liver metastases from breast cancer. Eichbaum et al. (2004) observed a possible trend for certain unfavorable prognostic parameters (e.g., young women, low-graded tumors, human epidermal growth factor receptor 2 over-expression) that could be related to higher serum levels of sE-selectin.

Role of E-Selectin in Diapedesis of Cancer Cells: Diapedesis is a vital part of tumor metastasis, whereby tumor cells attach to and cross the endothelium to enter the circulation. E-selectin was found to regulate initial attachment and rolling of colon cancer cells and also the subsequent diapedesis through the endothelium. Evidence indicates that E-selectin-dependent paracellular extravasation is independent of ICAM and VCAM and that it requires the activation of extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase downstream of E-selectin. Studies establish the role of E-selectin in diapedesis of circulating cancer cells (Tremblay et al. 2008; Woodward 2008).

Polymorphisms within E-selectin gene, especially the $S^{128}R$ polymorphism, may increase the risk of metastases by facilitating adhesion of tumor cells to endothelium. Blood DNA from patients treated for stage II or III colorectal

cancer (CRC) and from healthy controls was assessed for three polymorphisms within E-selectin gene ($S^{128}R$, $G^{98}T$ and $L^{554}F$) and one within the P-selectin gene ($V^{640}L$). The $S^{128}R$ polymorphism was detected in 22.3 % patients and was correlated with $G^{98}T$ polymorphism. In multivariate analysis, the $S^{128}R$ polymorphism was associated with shorter event-free survival (EFS) and overall survival (OS) in whole population, in patients with stage II CRC, and in patients with stage III CRC. $L^{554}F$ and $V^{640}L$ polymorphisms had no prognostic value. The $S^{128}R$ polymorphism is a constitutional factor associated with a higher risk of relapse and death in patients treated for CRC and its detection may permit better selection of patients suitable for adjuvant therapy, especially among those with stage II disease (Hebbar et al. 2009).

P-selectin Deficiency Attenuates Tumor Growth and Metastasis: Metastasis is thought to involve the formation of tumor-platelet-leukocyte emboli and their interactions with the endothelium of distant organs. A link between these observations shows that P-selectin, which normally binds leukocyte ligands, can promote tumor growth and facilitate the metastatic seeding of a mucin-producing carcinoma. P-selectin-deficient (P-sele$^{-/-}$) mice showed three potential pathophysiological mechanisms: (1) intravenously injected tumor cells home to the lungs of P-sele$^{-/-}$ mice at a lower rate; (2) P-sele$^{-/-}$ mouse platelets fail to adhere to tumor cell-surface mucins; and (3) tumor cells lodged in lung vasculature after intravenous injection often are decorated with platelet clumps, and these are markedly diminished in P-selectin$^{-/-}$ animals (Kim et al. 1998). However, the surgical procedure did not totally eliminate the factors responsible for platelet activation and did not normalize platelet activation (Dymicka-Piekarska et al. 2005; Hanley et al. 2006).

Role of Sialyl-Lewis Antigens: During inflammation, E- and P-selectins appear on activated endothelial cells to interact with leukocytes through sialyl-Lewis X (sLe^x) and sialyl-Lewis A (sLe^a). These selectins can also interact with tumor cells in a sialyl-Lewis-dependent manner and hence, they are thought to play a key role in metastasis. Diverting the biosynthesis of sialyl-Lewis antigens toward nonadhesive structures is an attractive gene therapy for preventing the hematogenous metastatic spread of cancers. The transduced α1,2-fucosyltransferase-1 (FUT1) efficiently fucosylated the P-selectin ligand PSGL-1 without altering P-selectin binding (Mathieu et al. 2004).

The metastasis of cancer cells and leukocyte extravasation into inflamed tissues share common features. Carbohydrate antigen sLe^a (CA19-9) is the most frequently applied serum tumor marker for diagnosis of cancers in the digestive organs. The normal counterpart of the determinant, namely disialyl-Le^a is predominantly expressed in non-malignant epithelial cells of the digestive organs. The disialyl-Le^a determinant carries one extra sialic residue attached through a 2 → 6 linkage to GlcNAc moiety compared to cancer-associated sLe^a, which carries only one 2 → 3 linked sialic acid residue (monosialyl Lewis A) (Fig. 44.3). Disialyl-Le^a in normal epithelial cells serves as a ligand for immunosuppressive receptors such as sialic acid binding Ig-like lectins (siglec-7 and -9) expressed on resident monocytes/macrophages and maintains immunological homeostasis of mucosal membranes in digestive organs. Epigenetic silencing of a gene for a 2 → 6 sialyl-transferase in the early stages of carcinogenesis results in impairment of 2 → 6 sialylation, leading to incomplete synthesis and accumulation of sLe^a, which lacks the 2 → 6 linked sialic acid residue, in cancer cells. Simultaneous determination of serum levels of sLe^a and disialyl-Le^a, and calculation of the sLe^a/disialyl-Le^a ratio provides information useful for excluding a false-positive serum diagnosis. During cancer progression in locally advanced cancers, tumor hypoxia induces transcription of several glyco genes involved in sLe^a synthesis. Expression of the determinant, consequently, is further accelerated in more malignant hypoxia-resistant cancer cell clones, which become predominant clones in advanced stage cancers and frequently develop hematogenous metastasis. sLe^a, as well as its positional isomer sLe^a, serves as a ligand for vascular E-selectin and facilitates hematogenous metastasis through mediating adhesion of circulating cancer cells to vascular endothelium. Patients having both strong sLe^a expression on cancer cells and enhanced E-selectin expression on vascular beds are at a greater risk of developing distant hematogenous metastasis (Kannagi 2007). In a human-mouse model, the selectin ligand s-Le^a is involved in in vivo extravasation of colorectal carcinoma (CRC) cells. Highly metastatic CRC cells expressing high levels of s-Le^a extravasate more efficiently than non-metastatic CRC cells expressing low levels of s-Le^a. Down-regulating the expression of s-Le^a in CRC cells by genetic manipulations, significantly reduced CRC extravasation. The arrest and adhesion of CRC cells, and possibly of other types of cancer cells as well, to endothelium depend on the expression of the selectin ligand sLe^a by the tumor cells (Ben-David et al. 2008). 3'-Sulfo-Le^a is known to be the potent ligand of E-selectin which is important in cell adhesion and migration. The serum 3'-sulfo-Le^a can provide important information in patients with primary gastric cancer, which might be useful as a predictive marker especially for the detection of tumor metastasis (Zheng et al. 2009).

Specialized carbohydrates modified with sLe^x antigens on leukocyte membranes are ligands for selectin adhesion molecules on activated vascular endothelial cells at

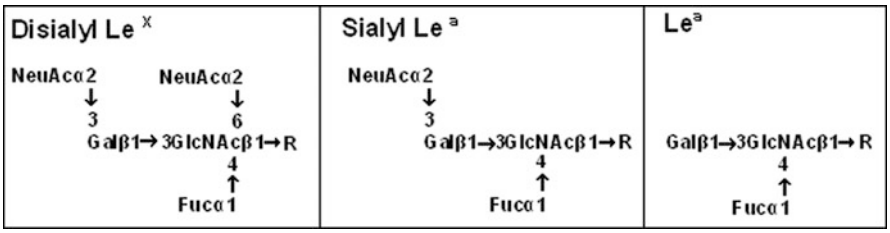

Fig. 44.3 Structures of three carbohydrate determinants, disialyl Lewis x (Le^x), sialyl Lewis a and Lewis a (Le^a). In panel, note that the only difference between the three determinants is the linkage of sialic acid residues. The $\alpha(2 \rightarrow 6)$ sialic acid residue in disialyl Le^a is synthesized by a sialyltransferase ST6GalNAcVI, which shows a significant decrease in its mRNA level upon malignant transformation

inflammatory sites. The sLe^x expression of invasive micropapillary carcinoma was higher than that of invasive ductal carcinoma, which was also associated with lymph node metastasis. E-selectin combined with sLe^x might play an important role in lymph node metastasis in invasive micropapillary carcinoma. The expression pattern of sLe^x in invasive micropapillary carcinoma suggested that the reversal of cell polarity of invasive micropapillary carcinoma might be as an important factor for the morphogenesis and possibly the pathogenesis, especially their higher rates of lymph node metastasis (Wei et al. 2010). The activity of core 2 β1,6 N-acetylglucosaminyltransferase (C2GnT1) in leukocytes greatly increases their ability to bind to endothelial selectins. C2GnT1 is essential for the synthesis of core 2-branched O-linked carbohydrates terminated with sLe^x (C2-O-sLe^x). E-selectin and its ligand-sLe^x are closely correlated with the metastasis of hepatocellular carcinoma. C2-O-sLe^x is a potentially useful early predictor of metastasis (Zhang et al. 2002). The expression profiles of C2-O-sLe^x in the malignant progression and metastasis of colorectal adenocarcinomas is upregulated in colorectal adenocarcinomas and metastatic liver tumors (St Hill et al. 2009).

44.7.5 Survival Benefits of Heparin

Endothelial P-Selectin as a Target of Heparin Action: Metastasis can be effectively inhibited by the anticoagulant heparin in different tumor models. At the cellular level, many of the antimetastatic effects of heparin in vivo are due to its action on P-selectin-mediated binding. Ludwig et al. (2007) addressed the potential contribution of endothelial P-selectin expression to adhesive events between the microvasculature and melanoma cells in vivo. Heparin not only inhibits P-selectin-mediated melanoma cell rolling but also attenuates melanoma metastasis formation in vivo, supporting the concept that endothelial P-selectin expression may represent an additional target of heparin in experimental melanoma lung metastasis (Ludwig et al. 2007). The low molecular weight heparin (LMWH) significantly improved colonic inflammation in rats with trinitrobenzene sulphonic acid (TNBS) induced colitis. The effect is possibly related to inhibition of proinflammatory cytokine IL-8, but not involved platelet surface P-selectin expression (Xia et al. 2004). The survival benefits in patients with cancer treated with LMWH may result from a LMWH-mediated effect on the immune system or on the cross-talk between platelets and tumor cells. However, survival observed with LMWH in patients with cancer apparently cannot be explained by a LMWH effect on these circulating markers (Di Nisio et al. 2005). Nonetheless, in vivo antimetastatic effects of heparins reflect their action on P-selectin-mediated binding. Therefore, these commonly used anticoagulants widely differ in their potential to interfere with P-selectin mediated cell binding. Importantly, the superior inhibitory capacity on P-selectin function of unfractionated heparin and LMWH nadroparin as opposed to LMWH enoxaparin and synthetic heparin pentasaccharide fondaparinux strongly correlated to the inhibitory potency of each in inhibiting experimental lung metastasis in vivo. Hence, P-selectin inhibition constitutes a valuable feature to identify anticoagulants that are suitable for anticancer therapy (Ludwig et al. 2006). Stevenson et al. (2005) studied metastasis inhibition by clinically relevant levels of various heparins and investigated the structural basis for selectin inhibition differences. Five clinically approved heparins were evaluated for inhibition of P-selectin and L-selectin binding to carcinoma cells and showed differing abilities to inhibit selectins, likely explained by size distribution. It should be possible to size fractionate heparins and inhibit selectins at concentrations that do not have a large effect on coagulation. Gao et al. (2005) prepared periodate-oxidized, borohydride-reduced heparin (RO-heparin) and tested its anticoagulant and anti-inflammatory activities. Compared with heparin, RO-heparin had greatly reduced anticoagulant activity. Intravenous administration of this compound led to reduction in the peritoneal infiltration of neutrophils in a

mouse acute inflammation model. In vitro studies showed that the effect of RO-heparin on inflammatory responses was mainly due to inhibiting the interaction of P-selectin with its ligands. These results indicate that RO-heparin may be a safer treatment for inflammation than heparin, especially when selectin is targeted.

To clarify the mechanism of heparin antimetastatic activity, several biological effects are being investigated. Cancer progression and metastasis are associated with enhanced expression of heparanase, which is inhibited efficiently by heparin. Heparin is also a potent inhibitor of selectin-mediated interactions. P- and L-selectin were shown to contribute to the early stages of metastasis, which is associated with platelet-tumor cell thrombi formation. Low anticoagulant heparin preparations still inhibited metastasis efficiently indicating that anticoagulation is not a necessary component for heparin attenuation of metastasis. Modified heparins characterized for heparanase inhibitory activity are also potential inhibitors of selectins. Selectin inhibition is a clear component of heparin inhibition of metastasis. The contribution of selectin or heparanase inhibition by heparin can provide evidence about its antimetastatic activity (Borsig 2007). One of the mechanisms by which heparin inhibits metastasis is by blocking the P-selectin-based interaction of platelets with tumor cell. The sulfate groups at C6/N and especially C6, but not C2 and C3, of heparin play a critical role in P-selectin recognition and that 2-O,3-O-desulfated heparin can block P-selectin-mediated A375 human melanoma cell adhesion. Thus chemical modification of heparin, especially 2-O,3-O-desulfation, may result in a therapeutic agent that is anti-metastatic because it blocks unwanted P-selectin-dependent adhesion but that lacks dose-limiting anticoagulant effects (Wei et al. 2005).

Heparin-Induced Thrombocytopenia: The pathophysiology of heparin-induced thrombocytopenia (HIT) is a complex process which involves platelets, vascular endothelium, and leukocytes. The activation products from these sites also contribute to the activation of coagulation and to the fibrinolytic deficit. Many of the markers of hemostatic activation processes have been found to be at increased levels during acute phases of the HIT syndromes. Since the pathophysiology of HIT involves the activation of platelets, endothelium, and leukocytes, it is expected that activation products related to these hemostatic systems, including soluble selectins, will also be increased in circulating blood. These alterations may provide an index of the pathophysiologic process. Fareed et al. (1999) reviewed on the circulating levels of P-, E-, and L-selectins in HIT patients and their modulation after therapeutic intervention. With the availability of recombinant hirudin, it is now possible to provide alternate anticoagulants to HIT patients. However, Fareed et al. (1999) suggest that the immunoactivation of platelets and other cells may require additional adjunct therapeutic approaches.

44.8 Adhesion Proteins in Transplantation

Activated protein C (APC) is the major physiological anticoagulant with concomitant anti-inflammatory properties. Turunen et al. (2005) suggest that APC has an anti-inflammatory role in I/R injury in clinical renal transplantation (Turunen et al. 2005). Bimosiamose prolongs survival of kidney allografts. Binding of the P-, L-, and E-selectins to sLe X retards circulating leukocytes, thereby facilitating their attachment to the blood vessels of allografts. Selectin inhibitor bimosiamose (BIMO) inhibits the rejection process of kidney allografts in a rat model in association with reduced intragraft expression of P-selectin glycoprotein ligand-1, CX(3)CL1, CCL19, CCL20, and CCL2. Thus, BIMO blocks allograft rejection by reduction of intragraft expression of cytokines and chemokines (Langer et al. 2004).

Brain death (BD), a significant antigen-independent process, the donor-related injury up-regulates variety of inflammatory mediators in peripheral organs. One of the immediate responses is the expression of selectins by endothelial cells of the transplanted tissues, which in turn trigger a cascade of nonspecific events that may enhance host alloresponses. Using a rat model in which donor BD accentuates subsequent renal allograft injury, Gasser et al. (2005) tested the effects of therapy with rPSGL-Ig alone, or in combination with sirolimus (SRL) and cyclosporin A. It was found that in contrast to the effects of standard doses of SRL or cyclosporine, rPSGL-Ig decreased inflammation in the early posttransplant period such that lower doses of maintenance immunosuppression were sufficient to maintain long-term graft function.

Intestinal transplantation (ITx) is severely limited by ischemia-reperfusion (I/R) injury. T lymphocyte is an important regulatory cell in this inflammatory process (Farmer et al. 2005a). rPSGL-Ig treatment leads to marked improvement in the outcome. The mechanism of action seems to involve the blockade of neutrophil and lymphocyte infiltration that leads to a decreased inflammatory response possibly driven by Th2 cytokines (Farmer et al. 2005b).

It was suggested that liver transplantation and liver resection, together with portal clamping time, might be a potential stimulus for platelet activation. Becker et al. (2004) indicated that neither liver transplantation nor liver resection influences GPIIb/IIIa and P-selectin expression on circulating platelets (Becker et al. 2004).

44.9 Inflammation During Infection

44.9.1 Microbial Pathogens

Endothelial activation contributes significantly to the systemic inflammatory response to bacteraemia. Release of soluble endothelial markers into the circulation has been demonstrated together with elevated plasma levels of CAMs and has been reported in bacteraemic patients. It has been proposed that the infection of endothelial cells with *Staphylococcus aureus, Streptococcus sanguis, or Staphylococcus epidermidis* induces surface expression of ICAM-1 and VCAM-1 and monocyte adhesion. In general, leukocyte/endothelial cell interactions such as capture, rolling, and firm adhesion should be viewed as a series of overlapping synergistic interactions among adhesion molecules resulting in an adhesion cascade. These cascades thereby direct leukocyte migration, which is essential for the generation of effective inflammatory responses and the development of rapid immune responses (Golias et al. 2007). *Helicobacter pylori* is a common bacterial pathogen that infects world's population up to 50 %. Carbohydrate components on *H. pylori* (sequences related to Le^x or Le^a antigens) are responsible for the persistent inflammation through interactions with leukocyte-endothelial adhesion molecules of the host. *H. pylori* isolates from patients with chronic gastritis, duodenal ulcer and gastric cancer interact with E- and L-selectins (Galustian et al. 2003). Expression of E-selectin was specifically upregulated in *H. pylori*-induced gastritis but not in gastritis induced by acetylsalicylic acid or pouchitis. The upregulated E-selectin expression was localized to the gastric mucosa rather than being a systemic response to the infection (Svensson et al. 2009).

Although mice with mutations in individual selectins showed no spontaneous disease and had a mild or negligible deficiencies of inflammatory responses, Bullard et al. (1996), in contrast, found that mice with null mutations in both endothelial selectins (P and E) develop a phenotype of leukocyte adhesion deficiency characterized by mucocutaneous infections in response to intraperitoneal *S. pneumoniae* peritonitis. These mice provide strong evidence for the functional importance of selectins in vivo (Bullard et al. 1996). Anthrax lethal toxin (LT), a key virulence factor of *Bacillus anthracis*, enhanced VCAM-1 expression on primary human endothelial cells suggesting a causative link between dysregulated adhesion molecule expression and the poor immune response and vasculitis associated with anthrax. Results suggest that LT can differentially modulate NF-kB target genes and highlight the importance of VCAM-1 enhancement (Warfel and D'Agnillo 2008). Vascular endothelium stimulation in vitro that lead to the upregulation of CAMs is known for the pathogenic spirochaetes, including rLIC10365 of *Leptospira interrogans*. The recombinant proteins of *L. interrogans* in *E. coli* as a host were capable to promote the upregulation of ICAM-1 and E-selectin on monolayers of HUVECS. In addition, pathogenic and non-pathogenic Leptospira are both capable to stimulate endothelium E-selectin and ICAM-1, but the pathogenic *L. interrogans serovar Copenhageni* strain promoted a higher activation than the non-pathogenic *L. biflexa serovar Patoc* (Atzingen et al. 2009; Gómez et al. 2008). *Chlamydia pneumoniae* has been associated with cardiovascular disease and atherosclerosis. To determine the ability of *C. pneumoniae* to elicit inflammation, Högdahl et al. (2008) infected human coronary artery endothelial cells (HCAEC) with *C. pneumoniae*. Secretion of IL-8, MCP-1, and ICAM-1 was significantly increased after *C. pneumoniae* infection of HCAEC in comparison with uninfected controls, where as release of E-selectin or MMP-1did not change. This suggested that *C. pneumoniae* initiates and propagates vascular inflammation in ways that contribute to coronary artery disease (Högdahl et al. 2008).

CAMs in Gingival Crevicular Fluid: The sICAM-1, sVCAM-1, and sE-Selectin are present in gingival crevicular fluid (GCF) and changes in their levels may be a sensitive indicator to differentiate healthy sites from those with periodontitis (Hannigan et al. 2004; Tamai et al. 2007). *Porphyromonas gingivalis* is a Gram-negative bacterium that is an important etiologic agent of human adult periodontitis. *E. coli* LPS and isoforms of *P. gingivalis* LPS were potent in stimulating the expression of inflammatory markers, with *E. coli* LPS being more potent (Liu et al. 2008). DNA samples from blood of periodontitis patients genotyped for E-selectin $Ser^{128}Arg$ and L-selectin $Phe^{206}Leu$ revealed a significant difference in the $Ser^{128}Arg$ polymorphism of E-selectin, but not in L-selectin, between periodontal patients and controls; the 128Arg allele was present more frequently in patients. Houshmand et al. (2009) suggested that $Ser^{128}Arg$ polymorphism of E-selectin might contribute to the susceptibility of Iranian individuals to periodontitis.

CAMs in Subjects with HIV Disease: Swingler et al. (2003) suggested that while both soluble CD23 and ICAM1 promote resting cell HIV1 infection, productive infection of cycling cells requires soluble ICAM1. Swingler et al. (2003) noted that these results may explain in part the existence of a resting T-cell reservoir infected with HIV-1. Subjects with HIV disease have multiple risk factors for cardiovascular disease, including elevated levels of ICAM-1 and VCAM-1. Many of the variables associated with

ICAM-1 and VCAM-1 levels can be related to their impact on inflammation (Melendez et al. 2008). The LFA-1, ICAM-1, and ICAM-3 are enriched at virological synapse (VS). The cognate adhesion molecule interactions at VS are important for HIV-1 spread between T cells (Jolly et al. 2007).

44.9.2 Yeasts and Fungi

Zuccarello et al. (2002) described a distinct form of familial chronic mucocutaneous candidiasis characterized by early-onset infections by different species of Candida, restricted to the nails of the hands and feet and associated with low serum concentration of ICAM-1. Phan and Filler (2009) measured the effects of *C. albicans* on the endothelial cell production of E-selectin and TNF-α in vitro. During invasive pulmonary aspergillosis, *A. fumigatus* hyphae invade the abluminal endothelial cell surface, whereas they invade the luminal endothelial cell surface during haematogenous dissemination. Infection with hyphae stimulates endothelial cells to synthesize E-selectin, VCAM-1, IL-8, and TNF-α in vitro. In neutropenic mice infected with wild-type *A. fumigatus*, increased pulmonary expression of E-selectin and TNF-α occurred only when neutropenia had resolved. In nonneutropenic mice immunosuppressed with corticosteroids, *A. fumigatus* stimulated earlier pulmonary expression of E-selectin and VCAM-1, while expression of ICAM-1 and TNF-α was suppressed. In both mouse models, expression of E-selectin was associated with high pulmonary fungal burden, angioinvasion, and neutrophil adherence to endothelial cells (Chiang et al. 2008; Kamai et al. 2009).

44.9.3 Parasites and Amoeba

44.9.3.1 Falciparum Malaria

Significant differences are observed between falciparum malaria patients and the healthy people in term of levels of both sE-selectin and thrombomodulin (TM). The levels of both sE-selectin and TM correlated positively with temperature, levels of IFN-γ and levels of TNF-α; and negatively with hemoglobin levels. Trends of positive correlations were observed between sP-selectin or vWF and temperature (Matondo et al. 2008). Evidence from autopsy and in vitro binding studies suggests that adhesion of erythrocytes infected with *Plasmodium falciparum* to the human host ICAM-1 receptor is important in the pathogenesis of severe malaria. Fernandez-Reyes et al. (1997) identified a mutation (K29M) in the ICAM1 gene, which they designated 'ICAM1 Kilifi,' that was associated with susceptibility to cerebral malaria with relative risks of 2.23 and 1.39 for homozygotes and heterozygotes, respectively. The available epidemiological, population genetic and functional evidence link ICAM-1(Kilifi) to severe malaria susceptibility (Fry et al. 2008; Cojean et al. 2008).

Increased serum concentrations of soluble sICAM-1, CD54 and of soluble E-, but not soluble P- and L-selectins were detected in Malagasy patients living in hyperendemic focus of Schistosoma mansoni. Serum levels of ICAM-1 were significantly correlated with the disease severity (Esterre et al. 1998). Studies in several models of inflammation have underscored the importance of P- and E-selectins in the migration of T cells to inflamed tissues. $CD4^+$ T cells recruited to the cutaneous compartment during infection with Leishmania major express P- and E-selectin ligands. Results suggest that by blocking P- and E-selectins, the immune pathology associated with cutaneous leishmaniasis might be ameliorated without compromising immunity to infection (Zaph and Scott 2003). Invasive amebiasis offers a new model that poses an inadequate immune response leading to a continuous and prolonged activation of endothelial cells (ECs) by amebas, amebic molecules and cytokines, leading to necrosis. Hyperactivated endothelial cells continuously express ICAM-1 and E-selectin, pro-coagulant molecules (tissue factor, vWF, and the plasminogen activator inhibitor), resulting in ever greater inflammation and thrombosis (Campos-Rodríguez et al. 2009)

44.9.3.2 Sepsis

Sepsis is a multifactorial, and often fatal, disorder typically characterized by widespread inflammation and immune activation with resultant endothelial activation. Though bacterial sepsis is most common, sepsis occurs with fungal, parastic and mycobacterial organisms. During bacterial sepsis in vivo, in wild-type mice and mice with E-, P-, or E-/P-selectin deficiencies, a phenotypic abnormality in E-selectin-deficient mice suggested that E- and P-selectin are important in the host defense against *S. pneumoniae* infection (Munoz et al. 1997). P-selectin is an important mediator of eosinophil recruitment to the cornea from limbal vessels to the corneal stroma, suggesting that P-selectin interactions may be potential targets for immunotherapy in eosinophil-mediated ocular inflammation (Kaifi et al. 2000).

Staphylococcus aureus is one of the most significant pathogens in human sepsis and endocarditis. Peptidoglycan induced surface expression of EC inflammation markers ICAM-1 and VCAM-1, which supported the adhesion of monocytes to these ECs (Mattsson et al. 2008). Teoh et al. (2008) assigned adiponectin as a modulator of survival and endothelial inflammation in experimental sepsis and a potential mechanistic link between adiposity and increased sepsis. Newborn infants with clinical diagnosis of sepsis demonstrated significantly higher plasma sE-selectin levels

in infected infants. Infants with gram-negative sepsis had higher sE-selectin levels than did those with gram-positive sepsis. C-reactive protein was the best test for diagnosis of neonatal sepsis (Zaki and el-Sayed 2009).

Hofer et al. (2008) compared two different models of sepsis LPS-induced endotoxemia and cecal ligation perforation (CLP) bacteremia in rats with respect to changes in endothelial expression of CAMS as a marker for capillary breakdown of the blood brain barrier. Increased ICAM-1 expression might be an early factor involved in these pathogenic events. Although the role of PECAM-1 could not be determined, it was possible to show its expression on cerebral endothelium in all groups (Hofer et al. 2008). In mouse models of sepsis, Shapiro et al. (2009) demonstrated increased circulating levels of sE-selectin, sICAM-1, sVCAM-1 and sP-selectin at 24 h, while CLP was associated with increased levels of sE-selectin alone. In real-time PCR, mRNA levels for P-selectin, ICAM-1 and PAI-1 were increased in skin from endotoxemic mice. In CLP, mRNA levels for P-selectin, ICAM-1, E-selectin and PAI-1 were elevated, while VCAM-1 expression was reduced in skin. Most, but not all of these changes correlated with alterations in immunohistochemical staining (Shapiro et al. 2009).

44.10 Action of Drugs and Physical Factors on CAMS

The field of selectin inhibition has matured significantly in recent years in the ability to inhibit selectin/ligand interactions with drug-like molecules and to demonstrate disease modification in human trials. A comprehensive review of new developments in the field of selectin inhibition through discussion of patents/patent applications from 2003 to August 2009 has been reported by Bedard and Kaila (2010)

44.10.1 Inhibitors of Gene Transcription

Treatment of human endothelial cells with cytokines such as IL-1, TNF-α or IFN-γ induces the expression of specific leukocyte adhesion molecules on the endothelial cell surface. Interfering with either leukocyte adhesion or upregulation of adhesion protein is an important therapeutic target as evidenced by the potent anti-inflammatory actions of neutralizing antibodies to these ligands in various animal models and in patients. The induction of E-selectin, VCAM-1, and ICAM-1 genes requires the transcription factor NF-κB. Pharmaceutical agents, which prevent the induced expression of one or more of cell adhesion molecules on endothelium, might be expected to provide a novel mechanism to attenuate the inflammatory responses associated with chronic inflammatory diseases. E-selectin expression is induced on the endothelial cell surface of vessels in response to inflammatory stimuli but is absent in the normal vessels. Thus, E-selectin is an attractive molecular target, and high affinity ligands for E-selectin could be powerful tools for the delivery of therapeutics and/or imaging agents to inflamed vessels. Zimmerman and Blanco (2008) reviewed the structure and regulation of LFA-1 and different classes of inhibitors that interfere LFA-1/ICAM-1 interactions. Alicaforsen (ISIS 2302), an antisense to ICAM-1, designed to inhibit ICAM-1 expression did not reveal significant effect in Crohn's disease. However, topical enemas for ulcerative colitis demonstrated some effect in secondary outcomes, and initial studies in pouchitis are promising (Philpott and Miner 2008). ICAM-1 antibody (UV3) was highly effective at slowing the growth of tumors and/or prolonging survival in SCID mice xenografted with human multiple myeloma, lymphoma, melanoma and other cell lines (Brooks et al. 2008). A structurally diverse collection of small molecule inhibitors has been characterized and developed either to bind the IDAS site of α_L I-domain or to the MIDAS of the β_2 I-like domain.

44.10.2 Anti-NF-kB Reagents

CAMs play important roles in a critical step of tumor metastasis and arrest of tumor cells onto the venous or capillary bed of the target organ. In this process, IL-1β induces nuclear translocation of NF-kB in HUVE cells, followed by induction of cell surface expression of E-selectin, ICAM-1, and VAM-1, and subsequent adhesion of those cancer cells expressing sialyl Le^X antigen, which is a ligand to E-selectin. The adhesion of tumor cells to IL-1β-treated HUVE cells can be inhibited by anti-NF-kB reagents such as N-acetyl L-cysteine, aspirin, or pentoxifylline. These observations indicate the involvement of NF-kB in cancer metastasis and the feasibility of using anti-NF-kB reagents in preventing metastasis (Tozawa et al. 1995). Incubation of HUVEC with N,N,N-trimethylsphingosine (TMS) resulted in a dose-dependent inhibition of IL-1β-induced E-selectin expression. Sphingosine or N,N-dimethylsphingosine had no effects on the expression. This inhibitory effect of TMS on IL-1β-dependent endothelial cell activation may partly explain the known anti-inflammatory or anti-metastatic effect of TMS in vivo (Masamune et al. 1995). Cimetidine inhibits the expression of E-selectin on vascular endothelial cells in gastric- and colorectal cancer patients, treated for chemotherapy (Kawase et al. 2005). Since the expression of E-selectin and Mac-1 is regulated either directly or indirectly by NF-kB, studies provide in vivo evidence that

tepoxalin is a potent inhibitor of NF-kB mediated events in animal models and this novel molecular mechanism clearly defines it as a new class of anti-inflammatory compounds. E-selectin transcription requires binding of transcription factors, NF-kB, ATF-2, and HMG-I(Y). HUVE cells treated with TNF-α showed E-selectin surface expression, which peaked at 4 h and then declined. However, ATF-2 binding was unchanged after stimulation with TNF-α. The termination of E-selectin expression is controlled at the level of transcription, with loss of protein-DNA interactions at only one of three NF-kB-binding sites in the E-selectin promoter (Boyle et al. 1999).

E-selectin is synthesized following X-ray exposure to doses as low as 0.5 Gy. X-ray-induced expression of E-selectin and ICAM-1 has been proposed to contribute to radiation injury in normal tissues. E-selectin expression does not require cytokine synthesis, but involves NF-kB activation (Hallahan et al. 1995). NFκB inhibition using NFκB inhibitors abrogates X-ray induced inflammatory mediators (Hallahan et al. 1998). Andrographolide, the principal component of medicinal plant Andrographis paniculata, has been shown to inhibit NF-kB activity and may attenuate allergic asthma via inhibition of the NF-kB signaling pathway. Andrographolide attenuated OVA-induced lung tissue eosinophilia and airway mucus production, mRNA expression of E-selectin, and inducible NOS in lung tissues. Findings implicate a potential therapeutic value of andrographolide in the treatment of asthma (Bao et al. 2009; Jiang et al. 2007).

Effects of TGF-β and IFN-γ on E-Selectin Expression: Transforming growth factor (TGF-β) has been shown to decrease the adhesiveness of endothelial cells for neutrophils, lymphocytes, and tumor cells. TGF-β inhibits the basal E-selectin expression and TNF-stimulated expression. While TGF-β had no effect on the expression of VCAM-1 and ICAM-1, the effect was additive with IL-4 in inhibiting the expression of E-selectin. Thus, perivascular TGF-β appears to act as an inhibitor of inflammatory responses involving neutrophils and a subset of lymphocytes (Gamble et al. 1993). IFN-γ down-regulates the induction by a viral mimetic, polyinosinic-polycytidylic acid [poly-(I:C)], of E-selectin. The inhibitory effect of IFN-γ on poly(I:C)-induced E-selectin was specific for dsRNA. Results indicated the role for IFN-γ in the regulation of E-selectin gene expression in response to dsRNA by a transcriptional mechanism independent of NF-kB, as well as by a minor decrease in message stability (Faruqi and DiCorleto 1997).

Retinoic Acid Inhibits the Expression of VCAM-1 but not E-Selectin: Several genes are regulated by tocopherols which can be categorized, based on their function. Genes that are related to inflammation, cell adhesion and platelet aggregation include E-selectin, ICAM-1, and others (Azzi et al. 2004). Retinoic acid and synthetic derivatives are known to exert anti-inflammatory effects in cutaneous diseases. Pretreatment with all-trans-retinoic acid (t-RA) specifically prevented TNFα-induced VCAM-1 expression, but not ICAM-1 and E-selectin induction (Gille et al. 1997). The TNFα-mediated activation of the human VCAM-1 promoter was also inhibited after t-RA treatment, while the ICAM-1 promoter activation was unaffected, indicating that the selective inhibition of CAM expression is regulated in part at the level of gene transcription. Furthermore, the transcriptional inhibition by t-RA appears to be mediated by its effects upon the activation of NF-kB-dependent complex formation. The specific inhibition of cytokine-mediated VCAM-1 gene expression in vitro provides a potential basis by which retinoids exert their biological effects at sites of inflammation in vivo (Gille et al. 1997). Radiation-induced expression of E-selectin was also blocked by t-RA, whereas 9-cis retinoic acid was ineffective. Application of statins and t-RA might have clinical impact in protecting against E-selectin-promoted metastasis, which might arise as an unwanted side effect from radiation treatment (Holler et al. 2009; Nubel et al. 2004).

Methylation of E-Selectin Promoter Gene Represses NF-kB Transactivation: The E-selectin promoter in cultured endothelial cells is under-methylated in comparison with non-expressing HeLa cells. Thus, methylation is likely to play a role in blocking E-selectin expression in non-endothelial cells (Smith et al. 1993). In intestine, MUC2 is the main mucin carrying s-Le^x, which interacts with E-selectin. This interaction may contribute to the extravasation of tumor cells and thus to the metastases. In several colorectal carcinoma cell lines the methylation of the 5'-flanking region of MUC2 correlated with the suppression of the MUC2 gene. The increase in MUC2 expression after the inhibition of the methylation with 5-aza-2' deoxycytidine strongly supports the notion that the suppression of MUC2 gene is related to the methylation of the promoter (Riede et al. 1998).

44.10.3 Strategies to Combat Atherogenesis and Venous Thrombosis

The advances in the development of adhesion molecule blocking agents, as well as an insight into the potential of these molecules in cardiovascular therapy have been reviewed from time to time (Lutters et al. 2004). Prophylactic dosing of a recombinant P-selectin ligand decreases venous thrombosis in a dose-dependent fashion in both feline and nonhuman primate animal models. Additionally,

treatment of 2-day iliac thrombi with a recombinant protein, P-selectin inhibitor, significantly improves vein reopening in nonhuman primates (Register 2009). It is interesting to note that P-selectin inhibition decreases thrombosis without adverse anticoagulation. Myers et al. (2005) evaluated an orally bioavailable inhibitor of P-selectin (PSI-697), which decreased thrombosis. Since, P-selectin is expressed on the surface of activated endothelial cells and platelets during thrombosis, targeting the plasminogen activator (PA) to P-selectin would enhance local thrombolysis and reduce bleeding risk. A urokinase (uPA)/anti-P-selectin antibody (HuSZ51) fusion protein is known to increase fibrinolysis in a hamster pulmonary embolism (Dong et al. 2004)

Aspirin reduces risks of myocardial infarction, stroke and cardiovascular death (Serebruany et al. 2004). The impact of cyclooxygenase (COX)-2 antagonist treatment on acute coronary risk is controversial. Prolonged COX-2 inhibition attenuates CRP and IL-6, does not modify P-selectin and MMP-9, and has no deleterious effect on endothelial function in stable patients with a history of recurrent acute coronary events and raised C-reactive protein (CRP) (Bogaty et al. 2004). Statins used in the control of hypercholesterolemia exert a protective effect on the endothelium reflected by a reduced level of circulating adhesion molecules. Statins exert a beneficial effects on endothelial function and atherosclerotic plaque, modulating oxidative stress and inflammation, with subsequent, well documented, primary and secondary prevention of CAD. Following statin treatment, sP-selectin, and ICAM-1 and highly sensitive CRP decreased compared to baseline levels. Other proteins (sVCAM-1, sE-selectin and platelet ECAM-1) did not show significant changes. In contrast to CRP, the reduction of sP-selectin concentrations correlated directly with the lowering of total cholesterol and inversely with the progression of CAD (Marschang et al. 2006)

44.10.4 Anti-inflammatory Drugs

While diclofenac is capable of inhibiting the expression of E-selectin, ICAM-1 and VCAM-1, the SJC13 is selective in inhibiting the expression of E-selectin and VCAM-1, but not ICAM-1 in endothelial cells. Nonsteroidal anti-inflammatory agents, such as sodium salicylate and aspirin, inhibit NF-κB-dependent gene activation. Salicylate blocked the TNF-α-induced increase in mRNA levels of adhesion molecules and gave a dose-dependent inhibition of TNF-α-induced surface expression of VCAM-1 and ICAM-1 with higher doses required to inhibit E-selectin expression. Ibuprofen appeared a potent inhibitor of IL-1α and TNF-α-induced surface expression of VCAM-1 and a less potent inhibitor of ICAM-1. Indomethacin, a nonsalicylate cyclooxygenase inhibitor, had no effect on surface expression of adhesion molecules, suggesting that the effects were not due to inhibition of cyclooxygenase (Pierce et al. 1996). Methimazole, used in treating autoimmune diseases, may also diminish pathological inflammation by suppressing E-selectin expression. The phenyl methimazole can also reduce cytokine-induced E-selectin expression and consequent leukocyte adhesion. Compound 10, which dramatically inhibits TNF-α-induced VCAM-1 mRNA and protein expression in human aortic endothelial cells, has a modest inhibitory effect on TNF-α induced E-selectin expression and has no effect on ICAM-1 expression (Dagia et al. 2004).

A thieno(2,3-d)pyrimidine, A-155918 inhibits the TNFα-induced expression of E-selectin, ICAM-1, or VCAM-1 on HEVCs (Stewart et al. 2001). Co-treatment of human endothelial cells with certain hydroxyflavones and flavanols blocks cytokine-induced ICAM-1, VCAM-1, and E-selectin expression on human endothelial cells. One of the potent flavones, apigenin, exhibited a dose- and time-dependent, reversible effect on adhesion protein expression as well as inhibiting adhesion protein upregulation at the transcriptional level (Gerritsen et al. 1996). Enalapril and losartan but not placebo induced a small but stable decrease of cardiovascular ICAM-1 and VCAM-1, while E-selectin and leukocyte expression of ICAM-1 remained unchanged. The lowering of plasma adhesion molecules may indicate an antiatherogenic effect of angiotensin II blockade in hypercholesterolemia (Graninger et al. 2004).

Carbohydrates, Synthetic Oligopeptides and Steroids

Targeting interaction of selectins and appropriate carbohydrate ligand is a promising approach to treat chronic inflammation. β-1,3-glucan sulfate (PS3) has inhibitory activity toward L and P-selectins under static conditions (Alban et al. 2009). Access to synthetic carbohydrates is an urgent need for the development of carbohydrate-based drugs, vaccines, adjuvants as well as novel drug delivery systems. Besides traditional synthesis in solution, synthetic carbohydrates have been generated by chemoenzymatic methods as well as automated solid-phase synthesis. Synthetic oligosaccharides have proven to be useful for identifying ligands of carbohydrate-binding proteins such as C-type lectins and siglecs using glycan arrays. Furthermore, glyconanoparticles and glycodendrimers have been used for specific targeting of lectins of the immune system such as selectins, DC-SIGN, and CD22 (Lepenies et al. 2010).

Compounds that target both heparanase and selectins offer a promising approach for cancer therapy. Borsig et al.

(2011) reported semisynthetic sulfated tri mannose C-C-linked dimers (STMCs) which are endowed with heparanase and selectin inhibitory activity. STMC hexasaccharide is an effective inhibitor of P-selectin in vivo. P-selectin-specific STMC attenuated metastasis in animal models, indicating that inhibition of tumor cell interaction with the vascular endothelium is critical for cancer dissemination. The small size, the stability of the C-C bond, and the chemically defined structure of STMCs make them superior to heparin derivatives and signify STMCs as valuable candidates for further evaluation.

Steroids down-regulate the expression of CAMs in endothelial cells stimulated by LPS in vitro. Low-dose hydrocortisone is a new treatment of patients with septic shock, a state that is characterized by an endothelial injury. Treatment with glucocorticoids differently affected the pattern of evolution of sCAMs, with sE-selectin being decreased and sICAM-1 being increased. Expression of sP-selectin and sVCAM-1 was not affected (Leone et al. 2004). Methotrexate (MTX) markedly reduces the expression of vascular E-selectin. A positive correlation between disease severity and the frequency of cutaneous lymphocyte-associated antigen (CLA)-positive T cells in the blood of untreated patients with psoriasis has been observed. It is suggested that MTX decreases the expression of CLA and E-selectin and that this may be a major mechanism for the therapeutic effect of MTX on psoriatic skin lesions (Sigmundsdottir et al. 2004).

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Polycystins and Autosomal Polycystic Kidney Disease

45

G.S. Gupta

45.1 Polycystic Kidney Disease Genes

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common monogenic disorders, and globally is the third most common cause of end-stage kidney disease. Although cystic renal disease is the major cause of morbidity, the occurrence of nonrenal cysts, most notably in the liver (occasionally resulting in clinically significant polycystic liver disease) and the increased prevalence of other abnormalities including intracranial aneurysms, indicate that ADPKD is a systemic disorder. Approximately 85% of ADPKD cases are attributable to mutations in polycystic kidney disease (PKD) gene 1 (*PKD1*) on chromosome 16, while mutations in *PKD2* gene on chromosome 4 account for almost all of the remaining cases. These two diseases are phenotypically almost identical, differing only by the higher age of diagnosis with *PKD2*, and its slower progression to end-stage renal disease (Rapoport 2007). The product of *PKD1*, polycystin-1 (PC-1), is a very large protein (4,303 amino acids), and is a membrane glycoprotein widely expressed in epithelial cells. It is also expressed in tight junctions, adherens junctions, desmosomes, apical junctions and primary cilia. Polycystin-2 (PC-2), the product of *PKD2*, is a smaller protein (968 amino acids) mainly present in the endoplasmic reticulum, but also in the cell plasma membrane. PC-1 and -2 are joined via a domain in the carboxy-tail of PC-1, and appear to act in concert (Ong and Harris 2005). PC-2 acts as a Ca^{2+} channel. It appears that PKD1 and PKD2 proteins associate physically in vivo and may be partners of a common signaling cascade involved in tubular morphogenesis (Qian et al. 1997). Qian et al. (1997) defined naturally occurring pathogenic mutations of *PKD1* and *PKD2* that disrupt their associations. Portions of the cellular populations of PC-1 and PC-2 localize to the primary cilium. The ADPKD is the founding member of the "ciliopathies," a recently defined class of genetic disorders that result from mutations in genes encoding cilia-associated proteins.

The human *PKD1* gene encodes an ~14-kb transcript, but full characterization was complicated, because most of the gene lies in a genomic region that is duplicated elsewhere on chromosome 16; the duplicate area encodes three genes with substantial homology to *PKD*. The *PKD1* has been identified in the chromosome region 16p13.3. Other *PKD1*-like loci on chromosome 16 are approximately 97% identical to *PKD1*. The 14.5 kb *PKD1* transcript encodes a 4303/4 amino acid protein with a calculated mass of ~460 kDa with a novel domain architecture. The PKD1 gene covers ~52 kb of genomic DNA and is divided into 46 exons. The amino-terminal half of the protein consists of a mosaic of domains, including leucine-rich repeats flanked by characteristic cysteine-rich structures, LDL-A and C-type lectin domains, and 16 units of a novel 80–85 amino acid domain. The presence of these domains suggests that the PKD1 protein is involved in adhesive protein-protein and protein-carbohydrate interactions in the extracellular compartment. The C-terminal third of the protein has multiple hydrophobic regions, and modeling of this region suggests the presence of many transmembrane domains and a cytoplasmic C terminus. The ADPKD phenotype suggests that polycystin may play a role in cell-matrix communication, which is important for normal basement membrane production and for controlling cellular differentiation (Harris et al. 1995; International Polycystic Kidney Disease Consortium 1995). Mutations in PKD1 gene are the most common cause of ADPKD.

Mouse gene transcript homologous to human PC-1 predicted 79% protein identity to human PC-1 and showed the presence of most of the domains identified in the human sequence. The mouse homolog is transcribed from a unique gene and there are no transcribed, closely related copies as observed in human *PKD1*. In mouse at the junction of exons 12 and 13, several different splicing variants lead to a predicted protein that would be secreted. These forms are predominantly found in newborn brain, while in kidney the

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transcript homologous to human RNA predominates (Löhning et al 1997). The murine homolog of human *PKD2* gene, *Pkd2* is localized on mouse Chromosome 5 proximal to anchor marker D5Mit175, spans at least 35 kb of the mouse genome, and consists of 15 exons. Its translation product consists of 966 amino acids, and the peptide shows a 95% homology to human PC-2. Functional domains are particularly well conserved in the mouse homolog. The expression of mouse PC-2 in the developing embryo at day 12.5 post conception is localized in mesenchymally derived structures. In adult mouse, the protein is mostly expressed in kidney, which suggests its functional relevance for this organ (Pennekamp et al. 1998).

45.1.1 Regulatory Elements in Promoter Regions

The *PKD1* and *PKD2* genes are developmentally regulated and their aberrant expression leads to cystogenesis. The 5′-flanking regions of the murine and canine *PKD1* genes have been characterized and compared with sequences from human and Fugu rubripes orthologues as well as the *PKD2* promoters from mouse and human (Lantinga-van Leeuwen et al. 2005). Sequences revealed a variety of conserved putative binding sites for transcription factors and no TATA-box element. Nine elements were conserved in the mammalian *PKD1* promoters: AP2, E2F, E-Box, EGRF, ETS, MINI, MZF1, SP1, and ZBP-89; six of these elements were also found in the mammalian *PKD2* promoters. Deletion studies with the mouse *PKD1* promoter defined a functional promoter region for *PKD1* and implied that E2F, EGRF, Ets, MZF1, Sp1, and ZBP-89 are potential key regulators of *PKD1* and *PKD2* gene products in mammals. The proximal *PKD1* promoter region is a potential target of Ets family transcription factors, which regulate the polycystic kidney disease-1 promoter (Puri et al. 2006).

45.2 Polycystins: The Products of PKD Genes

45.2.1 Polycystins

Polycystins are transmembrane proteins that form a distinct subgroup of the transient receptor potential (TRP) superfamily of channels (Montell 2005; Clapham 2003). The polycystin family is divided structurally and functionally into two subfamilies, the polycystin 1 (PKD1)-like or TRPP1 and polycystin 2 (PKD2)-like or TRPP2 proteins, both of which have a modest degree of sequence similarity between subfamilies (Delmas 2004; Igarashi and Somlo 2002; Nauli and Zhou 2004). In humans, the PKD2-like subgroup contains three homologous proteins—PKD2, PKD2L1 and PKD2L2—which are now referred to as TRPP2, TRPP3 and TRPP5. The PKD1-like subgroup contains five homologous proteins—all with an 11-transmembrane topology and by virtue of their structure are not considered as members of the TRP superfamily. Although channel activity has not been demonstrated for PKD1, it has 11 transmembrane segments of which the last 6 are similar to both PKD2 and other voltage-gated calcium channels (Hughes et al. 1995; Mochizuki et al. 1996). PKD2 assembles with PKD1 to form a functional Ca^{2+}-permeable cation channel complex in the plasma membrane (Delmas et al. 2004; Hanaoka et al. 2000) and the primary cilium (Nauli et al. 2003). In addition to currents observed at the plasma membrane in conjunction with PKD1, PKD2-mediated channel activity has also been shown in other sub-cellular membranes where it acts as an intracellular Ca^{2+} release channel (Koulen et al. 2002). Interactions between PKD1 and PKD2 are driven by their cytoplasmic carboxyl-terminal regions (Hanaoka et al. 2000; Newby et al. 2002). PKD1–PKD2 interactions are critical to kidney architecture and function because complex-disrupting mutations in either partner lead to development of autosomal dominant polycystic kidney disease (ADPKD), which affects 1 in 1,000 individuals (Wilson 2004; Igarashi and Somlo 2002; Molland et al. 2010).

45.2.2 Polycystin-1 (TRPP1) with a C-type Lectin Domain

Polycystin-1 (PC-1) is a large transmembrane protein with an estimated molecular weight of ~500 kDa having 4303 amino acids (Hughes et al. 1995; Burn et al. 1995). The large extracellular N-terminal region contains several specific motifs including leucine-rich repeats (LRRs), C-type lectin domain, LDL-A region, multiple Ig-like domains (or PKD domains), REJ domain and GPS domain. It has 11 transmembrane domains, with a PLAT domain located in the first cytoplasmic loop and a small cytoplasmic tail with a G-protein-binding motif and coiled-coil region (Figs. 45.1a and 45.2). The 16 Ig-like domains are segmented such that the first Ig-like domain is localized between the LRRs and the C-type lectin domain, while the remaining 15 Ig-like domains are clustered together between LDL-A and REJ domains. This Ig-like domain cluster forms strong homophilic interactions that are important for cell-cell adhesion (Bycroft et al. 1999; Ibraghimov-Beskrovnaya et al. 2000; Streets et al. 2003). Polycystin is likely a multifunctional protein with important roles in cell-cell/matrix adhesion and ciliary functions (Hildebrandt and Otto 2005; Wilson 2004). Polycystin-1 undergoes partial cleavage at the GPS domain such that N-terminal and C-terminal polypeptides remain non-covalently linked (Qian et al.

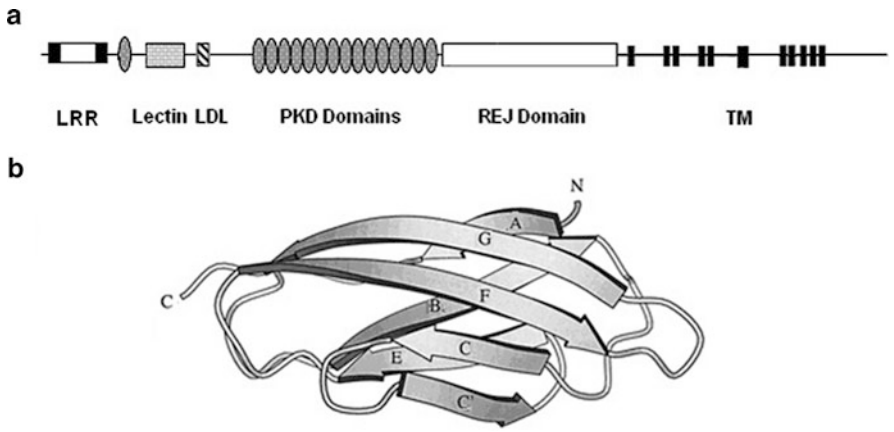

Fig. 45.1 (**a**) A schematic representation of the domains in polycystin-1. *LRR* leucine-rich repeat domain with flanking cysteine-rich regions, *Lectin* C-type lectin domain, *LDL* LDL-A module, *PKD* PKD (Ig-like) domains, *REJ domain* similar to sea urchin REJ protein, *TM* transmembrane domains. (**b**) PKD (Ig-like)-1 showing the elements of secondary structure. The β-strands are labeled (Reprinted with permission from Macmillan Publishers Ltd: EMBO J, Bycroft et al. ©1999)

2002; Wei et al. 2007). It is subsequently cleaved at the second site, which releases its C-terminal tail. The cytoplasmic tail of PC-1 enters the nucleus and regulates cell signaling events. This signaling function of PC-1 is regulated by PC-2 and may possibly be initiated by mechanical stimuli (Chauvet et al. 2004).

Analysis of the C-terminal cytosolic domain of human and mouse PC-1 has identified a number of conserved protein motifs, including a 20-amino-acid heterotrimeric G-protein activation sequence, suggesting that PC-1 may function as a heterotrimeric G-protein coupled receptor (Parnell et al. 1998). The *Xenopus* homologue of human *PKD1* (x*PKD1*) gene predicts the sequence of the putative protein, homologous to human PKD1 with a high level of expression in the kidney. A similar analysis in developing embryos and in an in vitro nephrogenic system suggests that *xPKD1* is associated with development of the amphibian pronephros (Burtey et al. 2005). The C-terminal intracellular segments of PC-1 have been extensively studied, mainly with respect to their putative involvement in cell signaling. Mutations in PC-1 result in ADPKD which is characterised by perturbation of transport resulting in fluid accumulation, cell proliferation and modification of the extracellular matrix (Weston et al. 2003). Cloned and expressed *PKD1* C-type lectin domain demonstrated that PC-1 may be involved in protein–carbohydrate interactions in vivo, and that Ca^{2+} is required for this interaction (Weston et al. 2001). Mutations in *PKD1* are implicated in human autosomal dominant polycystic disease.

Pletnev et al. (2007) made a model on a 3D structure and derived function of individual domains of PC-1. A three dimensional model of CTLD of PC-1 (sequence region 405–534) complexed with galactose and a Ca^{2+} was developed. The model suggests a αβ structural organization, which is composed of eight β strands and three α helices, and includes three disulfide bridges. It is consistent with the observed Ca^{2+} dependence of sugar binding to CLD and identifies the amino acid side chains (E^{499}, H^{501}, E^{506}, N^{518}, T^{519} and D^{520}) that are likely to bind the ligand (Pletnev et al. 2007). A coiled-coil domain within the C terminus of polycystin has been described to bind specifically to the C terminus of PKD2.

45.2.2.1 PKD (or Ig-like) Domains

The extracellular portion of PC-1 is modular in nature, and has a β sandwich-Ig-like protein module called PKD domain and comprises approximately 40% of the structure (Hughes et al. 1995). PC-1 consists of 16 copies of the PKD domain. Although the β-sandwich fold is common to a number of cell-surface modules, the PKD domain represents a distinct protein family. The tenth PKD domain of human and Fugu polycystin-1 showed extensive conservation of surface residues suggesting that this region could be a ligand-binding site. This structure will allow the likely effects of missense mutations in a large part of PKD1 gene to be determined (Bycroft et al. 1999) (Fig. 45.1b).The mechanical properties of PC-1 PKD domains (Forman et al. 2005; Qian et al. 2005) were first investigated for their importance in PKD. Atomic force microscopy experiments, applying force at the N and C termini of PKD domains, showed that PKD domains resist unfolding under significant force, a requirement for their function as mechanosensors (Forman et al. 2005; Qian et al. 2005). Hence, PC-1 was proposed to act as a mechanosensor, transducing fluid flow detected by the cilia of kidney epithelial cells into changes in intracellular calcium levels (Nauli et al. 2003). On the basis of predicted domain structure PC-1 seems to be involved in protein-protein and protein-carbohydrate interactions.

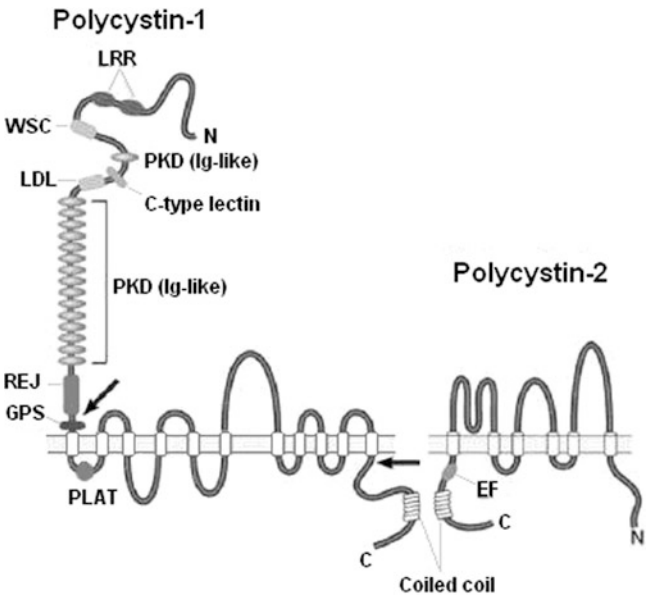

Fig. 45.2 The structure of polycystin-1 and polycystin-2 (*LRR* leucine-rich repeats, *WSC* cell wall integrity and stress response component 1, *PKD* (Ig-like), Ig-like domains, *LDL* low density lipoprotein domain, *REJ* receptor for egg jelly, *GPS* proteolytic G protein-coupled receptor proteolytic site, *PLAT* lipoxygenase domain, *EF* EF hand (helix-loop-helix) domain). Polycystin-1 undergoes cleavage at site shown by the *arrow* (Adapted with permission from Ibraghimov-Beskrovnaya and Bukanov 2008 © Springer)

45.2.2.2 Amino Acid Polymorphism

Many disease-causing mutations have been characterized in *PKD1* gene, most of them resulting in premature protein termination. A few intragenic polymorphisms have been described that are also useful for linkage studies. A new diallelic polymorphism is described for amino acid residue 4058, Ala/Val4058, with allelic frequencies of 0.88 and 0.12, respectively, and a heterozygosity of 0.23, in the Greek and Greek-Cypriot populations. Interestingly, this polymorphism and Ala4091-A/G, which were described in Caucasians, were not detected in DNA from 44 Japanese. This is particularly important when allelic frequencies in a particular population are used for linkage analysis of families of different ethnic origin. Also, observation of the two polymorphisms together as haplotypes suggests that the Ala/Val4058 polymorphism occurred more recently than the establishment of the Ala4091-A/G polymorphism, and specifically on the G allele (Constantinides et al. 1997).

45.2.3 Polycystin-2 (TRPP2)

Polycystin-2 (or TRPP2) is a 968-amino acid containing protein of ~110 kDa with six transmembrane domains and cytoplasmic N- and C-terminal domains (Fig. 45.2) (Mochizuki et al. 1996). Polycystin-2 is thought to be a new member of the transient receptor potential (TRP) family of ion channels. It was shown to be a cation channel with some selectivity for Ca^{2+} (Gonzalez-Perrett et al. 2001) and functions in multiple subcellular locations including plasma membrane (Hanaoka et al. 2000; Babich et al. 2004), endoplasmic reticulum (Koulen et al. 2002) and the primary cilia (Nauli et al. 2003). Several domains present in N and C termini of PC-2 are responsible for PC-2's protein–protein interactions and Ca^{2+} sensitivity. At least two domains, one in each cytoplasmic tail, contribute to PC-2 oligomerization. Immediately distal to PC-2's last transmembrane domain is a functionally complex region of the C terminus that includes coiled-coil, EF-hand (helix-loop-helix), and ER retention domains. A calcium-binding EF hand domain begins upstream of and extends into the PC-1-interacting coiled-coil region (Mochizuki et al. 1996; Qian et al. 1997; Celić et al. 2008). The helix-loop-helix structure of the EF-hand binds Ca^{2+}, permitting the protein to sense or to buffer changes in Ca^{2+} (Gifford et al. 2007). The PC-2 EF-hand has a single Ca^{2+}-binding site with micromolar affinity (Celić et al. 2008). Slightly overlapping with both the coiled-coil and the EF-hand is the sequence that is required for maintaining PC-2's ER and Golgi localization (Cai et al. 1999). A naturally occurring truncation mutation that removes this C-terminal domain, and thus presumably abrogates all of its interactions and regulatory potential, is sufficient to cause ADPKD (Mochizuki et al. 1996).

Polycystin-2 (or TRPP2) has been implicated in various biological functions including cell proliferation, sperm fertilization, mating behaviour, mechanosensation and asymmetric gene expression. Polycystin-1 and -2 can function together as a complex as well as independently in a variety of subcellular compartments. Direct interaction between the cytoplasmic tails of the polycystins has been shown using yeast two-hybrid assay (Qian et al. 1997; Tsiokas et al. 1997). Being a calcium-activated channel PC-2 releases calcium from intracellular stores in response to local increases in calcium concentrations. The calcium-conducting pore of PC-2 is likely formed by the loop between the fifth and sixth transmembrane domains, with some involvement of the third transmembrane domain (Koulen et al. 2002). A missense mutation that perturbs this putative conducting pore (D511V) is causative of ADPKD (Koulen et al. 2002). PC2 also indirectly regulates cytoplasmic calcium levels through interactions with two major intracellular Ca^{2+} channels: the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor (IP3R). The ryanodine receptor mediates calcium-induced calcium release, and PC-2 inhibits its function by binding the channel in its open state and decreasing its

conductance (Anyatonwu et al. 2007). PC-2 also modifies IP3-induced Ca^{2+} flux through direct binding between the PC-2C terminus and the IP3R (Li et al. 2005b, 2009).

45.2.3.1 Polycystin-L

Polycystin-L (PC-L), the third member of the polycystin family of proteins, functions as a Ca^{2+}-modulated nonselective cation channel when expressed in Xenopus oocytes. PC-L is predominantly expressed in adult mouse tissues and has a more restricted pattern of expression than either PC-1 or -2. In kidney, PC-L expression was first detected at E16, and levels increased into adulthood. Localization of PC-L was predominantly found in the apical region of the principal cells of inner medullary collecting ducts. PC-L was also found in discrete cell types of the retina, testis, liver, pancreas, heart, and spleen, but not in the lung. The expression pattern of PC-L suggests that it is unlikely to be a candidate gene for ADPKD (Basora et al. 2002). Results raise the possibility that PKD2L1 represents the third genetic locus that is responsible for PKD. However, its CTLD status remains to be investigated.

45.2.4 Interactions of Polycystins

PC-1 and PC-2 interact through their C-terminal cytoplasmic tails. This interaction results in an up-regulation of PC-1 but not PC-2. Furthermore, the cytoplasmic tail of PC-2 but not PC-1 formed homodimers through a coiled-coil domain distinct from the region required for interaction with PC-1. These interactions indicate that PC-1 and PC-2 may function through a common signaling pathway that is necessary for normal tubulogenesis and that PC-1 may require the presence of PC-2 for stable expression (Tsiokas et al. 1997). The intermediate filament (IF) protein vimentin is a strong PC-1-interacting partner. Cytokeratins K8 and K18 and desmin were also found to interact with PC-1. These interactions were mediated by coiled-coil motifs in PC-1 and IF proteins. Polycystin-1 may utilize this association for structural, storage, or signaling functions (Xu et al. 2001). Fibrocystin, a ~450 kDa protein, may form a complex with PC-2 to regulate calcium responses in kidney epithelia, but its exact role in normal and cystic epithelia is unknown (Wang et al. 2007).

A potential target for PC-1 signal transduction is the β catenin signal transduction pathway. This cytoplasmic protein is a key player in the regulation of cell polarity, proliferation, and morphogenesis, processes that are all affected in ADPKD. It was found that polycystin-1 interacts with the E-cadherin–catenin complex containing β catenin and suggested that polycystin-1 modulates signaling by β catenin. Several lines of evidence implicate β catenin in the pathogenesis of polycystic kidney disease. It appeared that aberrant β catenin signaling is a common feature of polycystic kidney diseases. Cytoplasmic β catenin interacts with members of the LEF/TCF family of transcription factors (van Adelsberg 2000).

45.2.5 Tissue and Sub-Cellular Distribution of Polycystins

Both proteins localize to primary cilia of renal epithelial cells, where they are implicated in mechanosensitive transduction signals (Yoder et al. 2002; Nauli et al. 2003). Polycystin 2/TRPP2 has been also documented at the plasma membrane (PM) and the ER—but it is still disputed whether it functions as an intracellular or a plasmalemmal channel. The finding that TRPP2 is retained in the ER of most cell systems has supported the view that TRPP2 might function as a reticular Ca^{2+}-release channel (Koulen et al. 2002). Conversely, TRPP2 has been shown to reside and act at the PM, notably in Madine–Darby canine kidney (MDCK) cells derived from cortical collecting ducts (Luo et al. 2003; Scheffers et al. 2002). These apparently incongruent views might be reconciled by the demonstration that subcellular transport and localization of TRPP2 are controlled by many interactions with adaptor proteins and enzymes (Köttgen et al. 2005; Streets et al. 2006; Geng et al. 2006). Such varied transport behaviour provides a mechanism for the dynamic regulation of TRPP2 channel density at the ER, PM and cilial localizations, and for different subcellular TRPP2 signaling functions. Polycystin is localized in membranes of renal and endothelial cells. When cultured cells made cell-cell contact, polycystin was localized to the lateral membranes of cells in contact (Ibraghimov-Beskrovnaya et al. 1997). Subcellular localization studies found PKD-1 to be a component of various cell junctional complexes and to be associated with the cytoskeleton (Xu et al. 2001). Scheffers et al. (2002) suggest that polycystin-2 can move freely in certain regions of the membrane where it probably functions as a channel, activated by, or in complex with, polycystin-1. PC-1 and PC-2 are functionally expressed in B-lymphoblastoid cells (LCLs), easily obtainable from ADPKD patients, to study PKD gene expression and function (Aguiari et al. 2004).

In fetal kidney, polycystin was localized to the plasma membranes of ureteric bud and comma and S-shaped bodies.

However, in more mature tubules in fetal kidney, in adult kidney, and in polycystic kidney, the majority of polycystin staining was intracellular. The temporal and spatial regulation of polycystin during renal development indicates that polycystin may play a role in nephrogenesis (van Adelsberg et al. 1997; van Adelsberg 1999a). Thus, the knowledge of the compartment-specific regulations of TRPP2 is of crucial importance for the understanding of its roles in health and disease (Giamarchi et al. 2006). The pattern of polycystin expression changed with gestational age in kidney. The widespread distribution of polycystin is consistent with the systemic nature of ADPKD and the role of epithelial cells in the disease (Weston et al. 2001).

45.3 Functions of Polycystin-1 and Polycystin-2

45.3.1 Polycystin-1 and Polycystin-2 Function Together

Polycystin-1 and -2 are thought to function together as part of a multiprotein membrane-spanning complex involved in cell-cell or cell-matrix interactions. Polycystin-1 and -2 interact to produce new calcium-permeable non-selective cation currents. Neither polycystin-1 nor -2 alone is capable of producing currents. Moreover, disease-associated mutant forms of either polycystin protein that are incapable of heterodimerization do not result in new channel activity. Thus, polycystin-1 and -2 co-assemble at the plasma membrane to produce a new channel and to regulate renal tubular morphology and function (Hanaoka et al. 2000). Polycystin-1 and -2 co-assembly can initiate signal transduction, leading to the activation of a number of downstream effectors, including heterotrimeric G-proteins, protein kinase C, mitogen-activated protein kinases, β-catenin, and the AP-1 transcription factor. In addition, PC-2 may function in mediating calcium flux.

The overlapping expression and localization patterns of polycystin-1 and -2 support their role as a complex in regulating multiple processes in tubular epithelia (Ong 2000). Both proteins are found in basolateral membranes and the primary cilium, where they may act together to regulate cellular adhesion and Ca^{2+} signaling. On the other hand, PC-2 is mainly expressed in ER, where it functions as a Ca^{2+} release channel (Koulen et al. 2002). In addition, PC-1 is highly expressed during development, with significant down-regulation of its expression in adult tissues. In contrast, expression of PC-2 seems to persist into adult life (Ong 2000). Experimental evidence from several groups has established an important role for polycystins in epithelial cell morphogenesis, including differentiation and maturation in vivo (Kim et al. 2000; Lu et al. 1997). Studies using MDCK cells demonstrated that expression of polycystin-1 at cell-cell junctions at controlled levels is critical for proper tubular differentiation (Bukanov et al. 2002). It has been shown that PC-1 is directly involved in intercellular adhesion *via* formation of strong homophilic interactions of its PKD (Ig-like) domains (Ibraghimov-Beskrovnaya et al. 2000). A direct role for Ig-like domains in cell-cell adhesion was demonstrated by specific perturbation of intercellular adhesion using antibodies against Ig-like domains in cell cultures (Ibraghimov-Beskrovnaya et al. 2000; Streets et al. 2003). Polycystin-1 was localized to the cell-cell adhesion complexes with adherens junctions and desmosomal junctions in epithelial cells of different origin (Bukanov et al. 2002; Huan and van Adelsberg 1999; Scheffers et al. 2000). Because alterations in PC-1-mediated adhesion may cause the abnormal epithelial cell phenotype observed in ADPKD cells, including dedifferentiation and loss of epithelial polarity, several studies examined cell-cell adhesion junctions in primary cells derived from ADPKD kidneys (Streets et al. 2003; Roitbak et al. 2004; Russo et al. 2005). Abnormal adherens and desmosomal junctions are found in ADPKD: intracellular junctions are devoid of desmosomal cadherins and associated proteins, which were sequestered to the cytoplasmic pools, and adherens junctions appeared disrupted, accompanied by a great reduction of E-cadherin expression and partial compensatory expression of N-cadherin (Roitbak et al. 2004) Streets et al. (2003) demonstrated that one of the primary functions of polycystin-1 is to mediate cell-cell adhesion in renal epithelial cells, probably via homophilic or heterophilic interactions of the PKD domains. Polycystin-1 localizes with desmosomal junctions (DJs) and adherens junctions (AJs). AJs and DJs are disrupted in ADPKD cells, while tight junctions (TJ) remain intact. In normal epithelial cells, PC-1 is found in a complex with talin (TAL), paxillin (PAX), vinculin (VINC), focal adhesion kinase (FAK), c-src (SRC), p130-cas (CAS), nephrocystin (NPH1) and tensin (TEN). In ADPKD cells, expression of FAK is lost from the focal adhesion complex (Ibraghimov-Beskrovnaya and Bukanov 2008; Chapin and Caplan 2010).

45.3.2 Cell-Cell and Cell-Matrix Adhesion

The *PKD* genes are required for normal fetal development. Consistent with its manifestations, PC-1 is widely expressed in both epithelial and nonepithelial tissues during embryological development. Mice with targeted mutations of either the *PKD1* or the *PKD2* genes die during embryogenesis. The observation, that loss of polycystin-1 or -2 function causes death during embryogenesis, suggests that *PKD1* and *PKD2*

might be part of a morphoregulatory pathway (van Adelsberg 1999b). The cystein-flanked leucine-rich repeats (LRR) of PC-1 act as mediators of the PC-1 interaction with the ECM. The observed suppression effect of the LRR on cell proliferation suggests a functional role of the LRR-mediated PC-1 involvement in cell-matrix and cell-cell interactions. These interactions may result in the enhanced cell proliferation that is a characteristic feature of ADPKD (Malhas et al. 2002; Chapin and Caplan 2010).

45.3.3 Role in Ciliary Signaling

The study of the polycystins has revealed some entirely novel insights into fundamental cell biology but these have not yet been satisfactorily integrated into a verified pathogenetic pathway for the development of ADPKD (Sutters 2006). The majority of epithelial cells along the nephron, except intercalated cells, possess a primary cilium, an organelle projecting from the cell's apical surface into the luminal space. Recent studies indicate that renal cilia have a sensory function. Many of the molecular players, which should help solve the mystery of how the renal cilium senses fluid flow, have been studied. Several proteins implicated in the pathogenesis of polycystic kidney disease localize to cilia. The role(s) of the polycystin signaling complex in mediating mechanosensory function by the primary cilium of renal epithelium as well as of the embryonic node have been reviewed (Nauli and Zhou 2004; Chapin and Caplan 2010). It is likely that the central pathogenetic pathway for cystogenesis stems from de-differentiation of tubular epithelial cells. Available evidence indicates that loss of polycystin activity leads to subtle derangements of cell calcium regulation through several possible pathways. Abnormal cell calcium homeostasis might then lead to altered differentiation in affected cells. The precise mechanism by which PC-1 functions, however, remains unclear. Polycystin-1 undergoes a proteolytic cleavage that releases its C-terminal tail (CTT), which enters the nucleus and initiates signaling processes. The cleavage occurs in vivo in association with alterations in mechanical stimuli. Polycystin-2 modulates the signaling properties of the PC-1 CTT.

Results show also a novel pathway by which PC-1 transmits messages directly to the nucleus (Chauvet et al. 2004). Low et al. (2006) showed that PC-1 tail interacts with the transcription factor STAT6 and the coactivator P100, and it stimulates STAT6-dependent gene expression. Under normal conditions, STAT6 localizes to primary cilia of renal epithelial cells. Cessation of apical fluid flow results in nuclear translocation of STAT6. Cyst-lining cells in ADPKD exhibit elevated levels of nuclear STAT6, P100, and the PC-1 tail. Exogenous expression of human PC-1 tail results in renal cyst formation in zebrafish embryos. This is a novel mechanism of cilia function in transduction of a mechanical signal to changes of gene expression involving PC-1 and shows that this pathway is inappropriately activated in ADPKD.

Primary Cilia of inv/inv Mouse Renal Epithelial Cells Sense Physiological Fluid Flow: Anomalous structure of primary cilia and/or impairment of increases in intracellular Ca^{2+} in response to fluid flow are thought to result in renal cyst formation in conditional kif3a knockout, Tg737 and pkd1/pkd2 mutant mice. The mutant inv/inv mouse develops multiple renal cysts like kif3a, Tg737 and pkd1/pkd2 mutants. Inv proteins have been shown to be localized in the renal primary cilia (Shiba et al. 2005).

Polycystins have been suggested to form mechanosensory transduction complexes involved in a variety of biological functions including sperm fertilization, mating behavior, and asymmetric gene expression in different species. Furthermore, their dysfunction is the cause of cyst formation in human kidney disease. The extracellular region of polycystin-1, which has a number of putative binding domains, may act as a mechanosensor. New evidence shows that a mechanosensitive signal, cilia bending, activates the PC-1-PC-2 channel complex. When working properly, this functional complex elicits a transient Ca^{2+} influx, which is coupled to the release of Ca^{2+} from intracellular stores (Cantiello 2003). Delmas (2004) focused on the pros and cons of their candidacy as mechanically gated channels and on recent findings that have significantly advanced our physiological insight. Nauli et al. (2006) proposed that calcium response to fluid-flow shear stress can be used as a read out of polycystin function and that loss of mechanosensation in the renal tubular epithelia is a feature of PKD cysts. Report supports a two-hit hypothesis as a mechanism of cystogenesis.

A large proportion of the extracellular region of polycystin-1 consists of β-sandwich PKD domains in tandem array. Using atomic force microscopy, it was shown that these domains, despite having a low thermodynamic stability, exhibit a remarkable mechanical strength, similar to that of Ig domains in the giant muscle protein titin. The simulations suggest that the basis for this mechanical stability is the formation of aforce-stabilised intermediate. Results suggest that these domains will remain folded under external force supporting the hypothesis that polycystin-1 could act as a mechanosensor, detecting changes in fluid flow in the kidney tubule (Forman et al. 2005). Both polycystins are localized to motile oviduct cilia and this localization is greatly increased upon ovulatory gonadotropic stimulation. It was suggested that PC-1 and -2 play an important role in granulosa cell differentiation and in development and maturation of ovarian follicles. In the oviduct both TRPV4 and polycystins could be important in relaying physiochemical

changes in the oviduct upon ovulation (Nauli et al. 2003; Teilmann et al. 2005).

45.3.4 Cilia and Cell Cycle

There is an intimate link between cilia and the cell cycle. The basal bodies/centrosomes of the cilia act as organizers of the mitotic spindle poles during cell division, directly connecting ciliogenesis with cell cycle regulation, and cilia are resorbed when cells enter the cell cycle. Because a number of cystoproteins causing PKD in humans and animals are expressed, at least partially, in cilia or the basal body of the cilia, polycystins and other cystoproteins may play an important role in connecting the mechanosensory function of the cilia to the centrosome and thus influence cell cycle control. Disruption of cystoproteins associated with cilia or basal bodies could, therefore, lead to dysregulation of the cell cycle and proliferation, resulting in cystic disease. Several lines of evidence support this hypothesis. Li et al. (2005b) showed that PC-2 regulates the cell cycle through direct interaction with Id2, a member of the helix-loop-helix (HLH) protein family that is known to regulate cell proliferation and differentiation. Id2 expression suppresses the induction of a cyclin-dependent kinase inhibitor, p21, by either PC-1 or PC-2. It was proposed that Id2 has a crucial role in cell-cycle regulation that is mediated by PC-1 and PC-2 (Chapin and Caplan 2010).

45.3.5 Polycystins and Sperm Physiology

Polycystins also play a significant role in sperm development and function. Drosophila PC-2 is associated with the head and tail of mature sperm. Targeted disruption of the PKD2 homologue results in nearly complete male sterility without disrupting spermatogenesis. Mutant sperm are motile but are unable to reach the female storage organs (seminal receptacles and spermathecae). The sea urchin PC-1-equivalent suPC-2 colocalizes with the PC-1 homolog REJ3 to plasma membrane over the acrosomal vesicle (Galindo et al. 2004; Kierszenbaum 2004). Like other PC-2 family members, suPC-2 is a six-pass transmembrane protein containing C-terminal cytoplasmic EF hand and coiled-coil domains. The location of suPC-2 suggests that it may function as a cation channel mediating the sperm acrosome reaction. The low cation selectivity of PC-2 channels would explain data indicating that Na^+ and Ca^{2+} may enter sea urchin sperm through same channel during the acrosome reaction (Neill et al. 2004). This localization also suggests that the suPC-2-REJ3 complex may function as a cation channel mediating acrosome reaction when sperm contact the jelly layer surrounding the egg at fertilization. Future studies leading to the identification of specific ligands for polycystins, including the signaling pathways, might define the puzzling relationship between renal tubular morphogenesis and sperm development and function.

45.4 Autosomal Dominant Polycystic Kidney Disease

45.4.1 Mutations in *PKD1* and *PKD2* and Association of Polycystic Kidney Disease

Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited nephropathy affecting over 1:1000 of worldwide population. It is a systemic condition with frequent hepatic and cardiovascular manifestations in addition to the progressive development of fluid-filled renal cysts that eventually result in loss of renal function in the majority of affected individuals. Mutations in either PC-1 or PC-2 account for the majority of autosomal dominant PKD. The cysts that grow in the kidney are the result of any number of mutations within the genes *PKD1* and *PKD2* that code for PC-1 and PC-2, respectively. Over 90 mutations have been discovered in *PKD1* alone, which lead to the disruption of proper function. These mutations have been found to be missense, nonsense, and frame-shift (Eo et al. 2002). The diagnosis of ADPKD is typically made using renal imaging despite the identification of mutations in *PKD1* and *PKD2* that account for virtually all cases. Most *PKD* gene mutations are loss of function and a 'two-hit' mechanism has been demonstrated underlying focal cyst formation. The protein products of the *PKD* genes, the polycystins, form a calcium-permeable ion channel complex that regulates the cell cycle and the function of the renal primary cilium. Abnormal cilial function is now thought to be the primary defect in several types of PKD including autosomal recessive polycystic kidney disease and represents a novel and exciting mechanism underlying a range of human diseases (Boucher and Sandford 2004; Chapin and Caplan 2010).

Russo et al. (2005) indicated that polycystin-1 is involved in cell proliferation and morphogenesis. Mutated PC-1 affects intercellular adhesion as shown by desmosomal junctions in primary cells derived from ADPKD cysts. While, primary epithelial cells from normal kidney showed co-localization of PC-1 and desmosomal proteins at cell-cell contacts, a striking difference was seen in ADPKD cells, where PC-1 and desmosomal proteins were lost from the intercellular junction membrane, despite unchanged protein expression levels. Results demonstrated that, in the absence of functional PC-1, desmosomal junctions cannot be properly assembled and remain sequestered in cytoplasmic compartments. Thus, PC-1 is crucial for formation of intercellular contacts. The abnormal expression of PC-1 causes disregulation of cellular

adhesion complexes leading to increased proliferation, loss of polarity and, ultimately cystogenesis.

45.4.2 Proliferation and Branching Morphogenesis in Kidney Epithelial Cells

Grimm et al. (2006) showed that the basal and EGF-stimulated rate of cell proliferation is higher in cells that do not express PC-2 versus those that do, indicating that PC-2 acts as a negative regulator of cell growth. In addition, cells not expressing PC-2 exhibited significantly more branching morphogenesis and multicellular tubule formation under basal and Hepatocyte GF-stimulated conditions than their PC-2-expressing counterparts, suggesting that PC-2 may also play an important role in the regulation of tubulogenesis. Cells expressing a channel mutant of PC-2 proliferated faster than those expressing the wild-type protein, but exhibited blunted tubule formation. Thus, the channel activity of PC-2 may be an important component of its regulatory machinery. Finally, PC-2 regulation of cell proliferation appears to be dependent on its ability to prevent phosphorylated extracellular-related kinase from entering the nucleus. These results indicate that PC-2 is necessary for the proper growth and differentiation of kidney epithelial cells (Grimm et al. 2006). Polycystin-2 is thought to function with PC-1, as part of a multiprotein complex involved in transducing Ca^{2+}-dependent information. Although its function as a Ca^{2+}-permeable cation channel is well established, its precise role in the plasma membrane, the endoplasmic reticulum and the cilium is controversial. Studies suggest that PC-2 (TRPP2) function is highly dependent on the subcellular compartment of expression, and is regulated by many interactions with adaptor proteins (Giamarchi et al. 2006).

Although the PKD genes were identified a decade ago, the pathway(s) leading from mutation to disease remain the subject of intense investigation. The pathogenesis of cyst formation is thought to involve increased cell proliferation, fluid accumulation, and basement membrane remodeling. It appears that cAMP metabolism is a central component of cyst formation, stimulating apical chloride secretion and driving the accumulation of cyst fluid. Evidence has shown that ADPKD cells also have an altered responsiveness to cAMP. In contrast to normal kidney cells whose cell proliferation is inhibited by cAMP, ADPKD cells are stimulated to proliferate. Thus, it is likely that an alteration in polycystin function transforms the normal cellular phenotype to one that responds to elevated cAMP by an increased rate of cell proliferation and that the enlarging cyst expands by an increased rate of cAMP-driven fluid secretion. Cyclic AMP and growth factors, including EGF, have complementary effects to accelerate the enlargement of ADPKD cysts, and thereby to contribute to the progression of the disease. This knowledge should facilitate the discovery of inhibitors of signal transduction cascades that can be used in the treatment of ADPKD (Calvet and Grantham 2001). Li et al. (2005) suggested that PC-2 and IP3R functionally interact and modulate intracellular Ca^{2+} signaling. Therefore, mutations in either PC-1 or PC-2 could result in the misregulation of intracellular Ca^{2+} signaling, which in turn could contribute to the pathology of ADPKD (Li et al. 2005).

Involvement of G-proteins was demonstrated by Delmas et al. (2004) who found that full-length PC-1 functions as a constitutive activator of $G_{i/o}$-type but not G_q-type G-proteins and modulates the activity of Ca^{2+} and K^+ channels via release of $G_{\beta\gamma}$ subunits. PC-1 lacking N-terminal 1,811 residues replicated the effects of full-length PC-1. Evidence indicates that full-length PC-1 acts as an untraditional G-protein-coupled receptor, activity of which is physically regulated by PC-2. Thus, it suggests that mutations in PC-1 or PC-2 that distort the polycystin complex would initiate abnormal G-protein signaling in ADPKD (Delmas et al. 2002). Regulation of intracellular Ca^{2+} mobilization has been associated with the functions of PC-1 and PC-2. PC-1 can activate the calcineurin/NFAT (nuclear factor of activated T-cells) signaling pathway (Puri et al. 2004). Puri et al. (2006) suggested a model in which PC-1 signaling leads to a sustained elevation of intracellular Ca^{2+} mediated by PC-1 activation of $G_{\alpha q}$ followed by PLC activation, release of Ca^{2+} from intracellular stores, and activation of store-operated Ca^{2+} entry, thus activating calcineurin and NFAT.

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Endogenous Lectins as Drug Targets

46

Rajesh K. Gupta and Anita Gupta

46.1 Targeting of Mannose-6-Phosphate Receptors and Applications in Human Diseases

46.1.1 Lysosomal Storage Diseases

The lysosome is an intracytoplasmic acidic vacuole containing more than 60 hydrolytic enzymes for digestion of macromolecules, such as nucleic acids, proteins, lipids and complex carbohydrates. Expression of lysosomal enzyme activities is regulated by various intracellular environmental factors. Mutation of a gene coding for a lysosomal enzyme results in a specific genetic disease, often involving the central nervous system in children. Three groups of functional proteins are known at present for regulation of the expressed enzyme activity in lysosomes. Targeting of a newly synthesized protein is achieved by the mannose 6-phosphate receptor (M6PR) system (Chaps. 3–5), which was revealed in the course of I-cell disease research. Many lysosomal enzymes are excessively secreted in the extracellular compartment in the absence of this regulatory system in patients with this disease (Kornfeld and Sly 2001). Lysosomal enzymes are also components of cell type-specific compartments referred to as lysosome-related organelles which include melanosomes, lytic granules, MHC class II compartments, platelet-dense granules, and synaptic-like microvesicles. Lysosomal storage diseases (LSDs) are inherited metabolic disorders caused by deficient activity of a single lysosomal enzyme or other defects resulting in deficient catabolism of large substrates in lysosomes. There are more than 40 forms of inherited LSDs known to occur in humans, with an aggregate incidence estimated at 1 in 7,000 live births. Clinical signs result from the inability of lysosomes to degrade large substrates; because most lysosomal enzymes are ubiquitously expressed, a deficiency in a single enzyme can affect multiple organ systems. Thus LSDs are associated with high morbidity and mortality and represent a significant burden on patients and society. Because lysosomal enzymes are trafficked by M6PR mechanism, normal enzyme provided to deficient cells can be localized to the lysosome to reduce and prevent storage. However, many LSDs remain untreatable, and gene therapy holds the promise for effective therapy. Other therapies for some LSDs do exist, or are under evaluation, including heterologous bone marrow or cord blood transplantation, enzyme replacement therapy (ERT), and substrate reduction therapy, but these treatments are associated with significant concerns, including high morbidity and mortality, limited positive outcomes, incomplete response to therapy the cost (Haskins 2009), life-long therapy, and the cost (Haskins 2009). Moreover, proteins, vesicles, nano-particles or other polymers could be functionalized with mannose 6-phosphate or its analogues in order to be used as carriers of bioactive molecules for the treatments of different diseases (Gary-Bobo et al. 2007).

Transport of lysosomal enzymes is mediated by two M6PRs: a cation dependent (CD-MPR) and a cation independent receptor (CI-MPR) (see Chaps. 3–5 for structures and functions). In hepatocytes of MPR-deficient neonatal mice lysosomal storage occurs when both MPRs are lacking, whereas deficiency of CI-MPR only has no effect on the ultrastructure of the lysosomal system (Schellens et al. 2003). Some structural features have been shown to be crucial for the binding of M6P to CI-MPR. The hydroxyl group at 2-position of pyranose ring must be axial to allow a strong binding to the CI-MPR. The analogues synthesized to target CI-MPR must be isosteric to M6P to efficiently bind to the receptor. Moreover, a single negative charge is sufficient to allow the binding to the receptor while the phosphorus atom is not necessary to ensure recognition. However, the

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best ligands for CI-MPR contain two negative charges like in the malonate or phosphonate isosteric analogues of M6P.

46.1.2 Enzyme Replacement Therapy (ERT)

Enzyme replacement therapy (ERT) represents a major breakthrough in the treatment of LSDs, initially used for the treatment of Gaucher disease and now available for several other LSDs. For example ERT with recombinant human acid α-glucosidase (GAA) (rhGAA) is presently the only approach for the treatment of Pompe disease patients. To date, several studies have pointed to the role of a variety of structural and biochemical responses triggered by intracellular storage, which are considered to be responsible for the pathogenetic manifestation of LSDs. Abnormal intracellular trafficking of lipids and proteins may affect the function of membrane-bound proteins, such as receptors, and ligands. The CI-MPR is of particular interest as it is a key player in the internalization of exogenous enzymes, and for the possible consequences of deranged CI-MPR function on ERT efficacy. At the steady state, a fraction (~10%) of CI-MPR is located on cell surface, where it mediates the uptake of lysosomal enzymes. CI-MPR being an integral membrane glycoprotein follows a complex and finely regulated itinerary from the trans-Golgi network (TGN), where it binds newly synthesized lysosomal hydrolases, travels through the early endosomes towards the late endosomal compartments, and recycles back to the TGN. CI-MPR at the cell surface mediates the uptake of lysosomal enzymes, internalize in the early endocytic compartment and then recycles again to the plasma membrane through the endocytic recycling compartment (ERC). Some of these advances in LSDs therapies have been enumerated in sections to follow. However many of these studies are at experimental stages.

Pompe Disease (Glycogen Storage Disease Type II)

Pompe disease also called Glycogen storage disease type II (or acid maltase deficiency) is an autosomal recessive metabolic disorder which damages muscle and nerve cells throughout the body. It is caused by an accumulation of glycogen in the lysosome due to deficiency of the lysosomal acid α-glucosidase (GAA). The GAA enzyme is responsible for breaking down glycogen in the lysosome. In individuals afflicted with Pompe Disease, the GAA enzyme is either absent or present in a very low quantity and glycogen builds in the lysosome, resulting in symptoms associated with this disorder. The glycogen storage disease with a defect in lysosomal metabolism is the first glycogen storage disease to be identified, in 1932. The build-up of glycogen causes progressive muscle weakness (myopathy) throughout the body and affects various body tissues, particularly in the heart, skeletal muscles, liver and nervous system. There are four primary forms of Pompe Disease: The classic infantile form, the non-classic infantile, the juvenile form, and the adult form. The classic infantile form is the most severe. Pompe Disease is estimated to occur in about one in 40,000 births worldwide.

One of the major therapeutic applications of M6P derivatives could be the enzyme replacement therapy (ERT) for lysosomal diseases. To address the consequences of abnormalities of cellular morphology and function on CI-MPR subcellular localization, fibroblasts from Pompe disease patients with different genotypes and phenotypes have been studied. In these cells, which showed abnormalities of cellular morphology, CI-MPR is mislocalized and its availability at the plasma membrane is reduced. These abnormalities in CI-MPR distribution result in a less efficient uptake of rhGAA by Pompe disease fibroblasts. CI-MPR-mediated endocytosis of rhGAA is an important pathway by which the enzyme is delivered to the affected lysosomes of Pompe muscle cells. Hence, the generation of rhGAA containing high affinity ligands for the CI-MPR represents a strategy by which the potency of rhGAA and the clinical efficacy of enzyme replacement therapy for Pompe disease may be improved. To enhance the delivery of rhGAA to the affected muscles in Pompe disease, the carbohydrate moieties on the enzyme were remodelled to exhibit a high affinity ligand for CI-MPR. This was achieved by chemically conjugating on to rhGAA, a synthetic oligosaccharide ligand bearing M6P residues in the optimal configuration for binding the receptor (Zhu et al. 2004, 2005). This approach allowed to decrease the effective dose of enzyme and to treat either less accessible tissues, such as skeletal muscles, or tissues with a relatively low abundance of CI-MPR (Gary-Bobo et al. 2007).

The ERT for Pompe disease was recently approved in Europe, the U.S., Canada, and Japan using a recombinant human GAA (Myozyme, alglucosidase alfa) produced in CHO cells (CHO-GAA). The ERT with rhGAA is at present the only approved treatment for Pompe disease, in addition to supportive and physical therapies. However, ERT shows limited efficacy in some patients and does not completely correct the disease phenotype. Recently, an improved knowledge of Pompe disease pathophysiology has provided clues to explain the limitations of ERT. A mechanical effect of lysosomal inclusions on muscle contractility has been proposed as a key factor of disease resulting in a severe loss of contractility. In addition, it has been shown that secondary abnormalities of housekeeping cellular functions, such as autophagy, have an important role in the pathogenesis of cell damage in Pompe disease. Abnormalities of intracellular trafficking of vesicles and membrane-bound proteins, such as the CI-MPR, may be deleterious for the efficacy of ERT. Other approaches, also in a pre-clinical stage, include substrate reduction and gene therapy (Parenti and Andria 2011; McVie-Wylie et al. 2008).

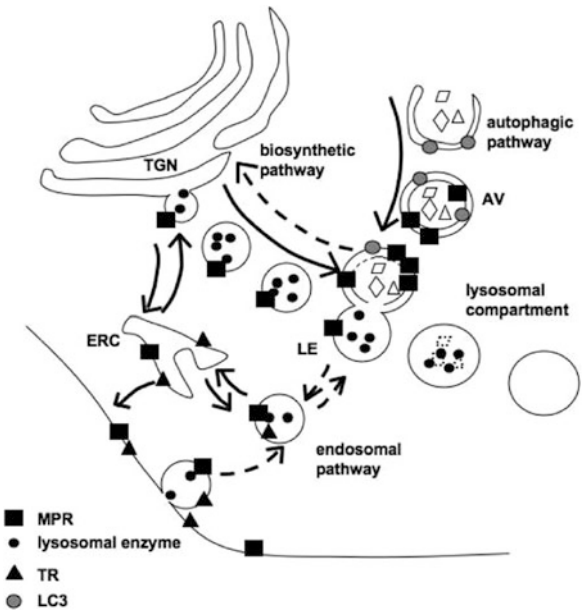

Fig. 46.1 Schematic representation of CI-MPR trafficking in the cells. CI-MPR follows different routes, including the biosynthetic pathway from the trans-Golgi network (*TGN*), where it binds and delivers newly synthesized lysosomal hydrolases to the late endosomal compartment. A fraction (~ 10%) of CI-MPR is located on the cell surface, where it mediates the uptake of lysosomal enzymes, is internalized in the endocytic pathway and recycles through the endocytic recycling compartment (*ERC*), again to the plasma membrane. Increased co-localization of CI-MPR with microtubule-associated protein 1 light chain 3 (*LC3*) in Pompe disease fibroblasts indicates that the CI-MPR in these cells is diverted from its normal routes and is sequestered in autophagosomes and autophagolysosomes. Since transferrin and transferring receptor trafficking is not affected in Pompe disease fibroblasts, it is likely that the disruption of CI-MPR trafficking occurs at the late endosomal compartment and in the retrograde route to the TGN (Cardone et al. 2008)

To investigate the mechanisms underlying the variable response to ERT, Cardone et al. (2008) studied cell morphology of Pompe disease fibroblasts, the distribution and trafficking of the CI-MPR that mediates rhGAA uptake, and rhGAA uptake itself. Immunofluorescence analysis showed abnormal intracellular distribution of CI-MPR in Pompe disease fibroblasts, increased co-localization with microtubule-associated protein 1 light chain 3 (LC3) and reduced availability of the receptor at the plasma membrane. The recycling of CI-MPR from the plasma membrane to the trans-Golgi network was also impaired. All these abnormalities were more prominent in severe and intermediate Pompe disease fibroblasts, correlating with disease severity. In severe and intermediate Pompe disease cells rhGAA uptake and processing were less efficient and correction of GAA activity was reduced. Results indicate a role for disrupted CI-MPR trafficking in the variable response to ERT in Pompe disease with implications for ERT efficacy and optimization of treatment protocols (Fig. 46.1).

Although ERT with acid α-glucosidase has become available for Pompe disease, the response of skeletal muscle, as opposed to the heart, has been attenuated. The poor response of skeletal muscle was attributed to the low abundance of the CI-MPR in skeletal muscle compared to heart. In CI-MPR-KO/GAA-KO (double KO) mice, the role of CI-MPR was emphasized by the lack of efficacy of ERT as demonstrated by markedly reduced biochemical correction of GAA deficiency and of glycogen accumulations in double KO mice, in comparison with the administration of the same doses in GAA-KO mice. Clenbuterol, a selective β_2-agonist, enhanced the CI-MPR expression in skeletal tissue and also increased efficacy from GAA therapy, thereby confirming the key role of CI-MPR with regard to ERT in Pompe disease. Biochemical correction improved in both muscle and non-muscle tissues, indicating that therapy could be similarly enhanced in other lysosomal storage disorders. In summary, enhanced CI-MPR expression might improve the efficacy of ERT in Pompe disease through enhancing receptor-mediated uptake of GAA (Koeberl et al. 2011).

Aspartylglycosaminuria

Aspartylglucosaminuria also called aspartylglycosaminuria (AGU), a severe lysosomal storage disease, is caused by the deficiency of the lysosomal glycosylasparaginase (GA), and accumulation of aspartylglucosamine (GlcNAc-Asn) in tissues. Aspartylglucosaminuria is caused by mutations in the gene encoding for a soluble aspartylglucosaminidase (AGA). Kyttälä et al. (1998) indicated that endocytotic capability of cultured telencephalic neurons for recombinant AGA was mediated by M6PRs. Human leukocyte glycosylasparaginase can correct the metabolic defect in Epstein-Barr virus (EBV)-transformed AGU lymphocytes rapidly and effectively by M6PR-mediated endocytosis or by contact-mediated cell-to-cell transfer from normal EBV-transformed lymphocytes, and that 2–7% of normal activity is sufficient to correct the GlcNAc-Asn metabolism in the cells. The combined evidence indicates that cell-to-cell transfer of GA plays a main role in enzyme replacement therapy of AGU by normal lymphocytes (Dunder and Mononen 2001).

Niemann-Pick Disease (NPD) Type-A, B and C

Acid sphingomyelinase (ASM), a member of the saposin-like protein family, is a lysosomal hydrolase that converts sphingomyelin to ceramide. Deficiency of ASM causes a variant form of Niemann-Pick disease. Progressive accumulation of lipid-laden macrophages is the hallmark of the ASM-deficient forms of Niemann-Pick disease (i.e. Types A and B NPD). Studies using receptor-specific ligands to inhibit enzyme uptake revealed that in normal cells rhASM was taken up by a combination of mannose receptor (MR) and M6PR,

respectively. Whereas in the ASM deficient (ASM-KO) cells the M6PR had a minimal role in rhASM uptake. Expression of M6PR mRNA was normal in the ASM-KO cells, although it was hypothesized that lipid accumulation in ASM-KO macrophages led to abnormalities in M6PR trafficking and/or degradation, resulting in reduced enzyme uptake. Consistent with this hypothesis, it was also found that, when rhASM was modified to expose terminal mannose residues and target mannose receptors, the uptake of this modified enzyme by ASM-KO cells was tenfold greater when compared with the "complex" type rhASM. These findings have important implications for NPD enzyme replacement therapy, particularly in the lung (Dhami and Schuchman 2004). Further studies suggest that ASM uses in part the M6P-R and Sortilin, a type I transmembrane glycoprotein that belongs to a family of receptor proteins, involved in lysosomal targeting (Ni and Morales 2006).

Niemann-Pick disease type C (NPC) is an autosomal recessive disorder that leads to massive accumulation of cholesterol and glycosphingolipids in late endosomes and lysosomes. NPC, caused by mutations in the *NPC1* gene or the *NPC2* gene, is characterized by the accumulation of unesterified cholesterol and other lipids in lysosomal compartment. NPC2 is a small lysosomal protein that is targeted to this compartment via a M6P-inhibitable pathway. Either of M6PRs alone is sufficient to transport NPC2 to the endo/lysosomal compartment, although M6PR-300 seems to be more efficient than M6PR-47. In the absence of both MPRs, NPC2 is secreted into the culture medium, and only a small amount of intracellular NPC2 can be detected, mainly in the ER. This leads to massive accumulation of unesterified cholesterol in the endo/lysosomal compartment of the MPR46/300-deficient fibroblasts, a phenotype similar to that of the NPC patient fibroblasts. The lysosomal targeting of NPC2 is strictly dependent on M6PRs in fibroblasts. In another concept, Rab9 is likely sequestered in an inactive form on Niemann-Pick type C membranes, as cation-dependent M6PRs were missorted to the lysosome for degradation, a process that was reversed by over-expression of Rab9. It seems that cholesterol contributes directly to the sequestration of Rab9 on Niemann-Pick type C cell membranes, which in turn, disrupts M6PR trafficking (Naureckiene et al. 2000; Ganley and Pfeffer 2006).

Mucolipidosis Type II and III

Mucolipidosis (ML) II and III are rare autosomal recessive inherited diseases characterized by deficiency of multiple lysosomal enzymes resulting in a generalized storage of macromolecules in lysosomes of cells of mesenchymal origin. The M6P lysosomal targeting signal on acid hydrolases is synthesized by the sequential action of uridine 5′-diphosphate-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) and GlcNAc-1-phosphodiester α-N-acetylglucosaminidase ("uncovering enzyme" or UCE). Mutations in the two genes that encode GlcNAc-1-phosphotransferase give rise to lysosomal storage diseases (ML type II and III). In ML II and ML III fibroblasts, most of the newly synthesized lysosomal enzymes are secreted into the medium instead of being targeted correctly to lysosomes. Studies indicate that phospholipases, like most other lysosomal enzymes in these diseases, are secreted into the blood instead of being targeted specifically to lysosomes. The M6PR pathway is needed for proper delivery of lysosomal phospholipases to lysosomes (Jansen et al. 1999). Boonen et al. (2009) demonstrated that UCE accounts for all the uncovering activity in the Golgi. In the absence of UCE, the weak binding of the acid hydrolases to the CI-MPR allows sufficient sorting to lysosomes to prevent the tissue abnormalities seen with GlcNAc-1-phosphotranferase deficiency (Boonen et al. 2009).

46.1.3 M6PR-Mediated Transport Across Blood-Brain Barrier

Enzyme replacement therapy has been used successfully in many lysosomal storage diseases. However, correction of brain storage has been limited by the inability of infused enzyme to cross the blood-brain barrier (BBB). Delivering therapeutic levels of lysosomal enzymes across the BBB is pivotal issue in treating CNS diseases, including the mucopolysaccharidoses. The newborn mouse is an exception because recombinant enzyme is delivered to neonatal brain after M6PR-mediated transcytosis. Access to this route is limited for 2 weeks of age, after which this transporter is lost with maturation.

Glial and Neuronal Cells Express CI-MPR

Enzyme replacement therapy for lysosomal storage disorders depends on efficient uptake of recombinant enzyme into the tissues of patients. This uptake is mediated by oligosaccharide receptors including the CI-MPR and the mannose receptor (MR). Studies on the uptake of recombinant α-(L)-iduronidase into glial and neuronal cells, produced by retrovirally transduced NIH3T3 fibroblasts indicate that: (1) neuronal and glial cells take up α-(L)-iduronidase released into the medium by retrovirally transduced fibroblasts expressing high levels of α-(L)-iduronidase; (2) both glial and neuronal cells express the CI-MPR responsible for lysosomal enzyme uptake; and (3) uptake of the lysosomal enzyme could be blocked by excess free M6P, but not glucose-6-phosphate. Thus, various brain cells take up α-(L)-iduronidase, possibly through a CI-MPR mediated pathway, and this uptake is higher in actively dividing or immature brain cells (Stewart et al. 1997).

GM2 Gangliosidosis

Intralysosomal stability of β-galactosidase is regulated by a multifunctional protein that interacts with two lysosomal enzymes, β-galactosidase and sialidase, and also exerts catalytic activities as carboxypeptidase, esterase and deamidase under various pH conditions. It is encoded by a gene on chromosome 20, and its mutation results in a neurodegenerative disease in children and adults (galactosialidosis). For digestion of lipid substrates, lysosomal enzymes need specific activator proteins as natural detergents for molecular interaction with these nonpolar compounds. Two different groups of proteins have been revealed. A protein encoded by a gene on chromosome 5 interacts with ganglioside GM2 and its asialo derivative, for their catalytic hydrolysis by β-hexosaminidase A. Another protein encoded by a gene on chromosome 10 is expressed as a precursor (prosaposin) which is then processed to four small proteins (saposins) with heterogeneous functions. They are essential for hydrolysis of sphingolipid substrates, and genetic deficiency of each protein results in various lipid storage diseases.

Sandhoff disease is an autosomal recessive lysosomal storage disease caused by a defect of the β-subunit gene (*HEXB*) associated with simultaneous deficiencies of β-hexosaminidase A (HexA; αβ) and B (HexB; ββ), and excessive accumulation of GM2 ganglioside (GM2) and oligosaccharides with N-acetylglucosamine (GlcNAc) residues at their non-reducing termini. From the neonatal brains of Sandhoff disease model mice (SD mice) produced by disruption of the murine Hex β-subunit gene allele ($Hexb^{-/-}$) recombinant Hex isozyme sub-units were found to be incorporated into the SD microglia via cell surface CI-MPR and mannose receptor to degrade the intracellularly accumulated GM2 and GlcNAc-oligosaccharides (Tsuji et al. 2005).

Tay-Sachs disease is a severe neurodegenerative disorder due to mutations in the *HEXA* gene coding for the α-chain of the αβ heterodimeric lysosomal enzyme β-hexosaminidase A (HexA). Guidotti et al. (1998) corrected HexA-deficient cells by *HEXA* gene transfer. Murine HexA-deficient fibroblasts derived from HexA-/- mice were transduced with the G.HEXA vector. Transduced cells overexpressed the α-chain, resulting in the synthesis of interspecific HexA (human α-chain/murine β-chain) and in a total correction of HexA deficiency. The α-chain was secreted in the culture medium and taken up by HexA-deficient cells via M6PR binding, allowing for the restoration of intracellular HexA activity in non-transduced cells.

To develop a novel enzyme replacement therapy for Tay-Sachs disease (TSD) and Sandhoff disease (SD), which are caused by deficiency of Hex A, Matsuoka et al. (2011) designed a genetically engineered *HEXB* encoding the chimeric human β-subunit containing partial amino acid sequence of the α-subunit and succeeded in producing the modified HexB by a CHO cell line stably expressing the chimeric HexB, which can degrade artificial anionic substrates and GM2 ganglioside in vitro, and also retain the wild-type (WT) HexB-like thermostability in presence of plasma. The modified HexB was efficiently incorporated via CI-MPR into fibroblasts derived from Tay-Sachs patients, and reduced the GM2 ganglioside accumulated in the cultured cells. The intracerebroventricular enzyme replacement therapy involving the modified HexB should be more effective for Tay-Sachs and Sandhoff than that utilizing the HexA, especially as a low-antigenic enzyme replacement therapy for Tay-Sachs patients who have endogenous WT HexB.

Mucopolysaccharidosis (MPS)

Recently, several studies showed that multiple infusions of high doses of enzyme partially cleared storage in adult brain. For example, the neonate uses the M6PR to transport phosphorylated β-glucuronidase (P- GUS) across the BBB. These results raised the question if correction of brain storage by repeated high doses of enzyme depends on M6P-mediated uptake or whether enzyme gains access to brain storage by another route when brain capillaries are exposed to prolonged, high levels of circulating enzyme (Grubb et al. 2008). Pharmacological manipulation with epinephrine restores functional transport of P-GUS across the adult BBB. The effect of epinephrine on the transport of P-GUS was ligand specific (Urayama et al. 2007).

The rat amniotic epithelial cells (AEC) over-express and secrete human β-glucuronidase (GUS) following transduction with an adenoviral vector encoding human GUS. The AEC were used as donor cells for cell-mediated gene therapy of CNS lesions in mice with mucopolysaccharidosis type VII (MPSVII), a lysosomal storage disorder caused by an inherited deficiency of GUS activity. After confirmation that the secreted GUS was taken up mainly via M6PRs in primary cultured neurons, the AECs were transplanted into the brains of adult MPSVII mice. Results suggest that intracerebral transplantation of genetically engineered AEC has therapeutic potential for the treatment of CNS lesions in lysosomal storage disorders (Kosuga et al. 2001).

The availability of both $MR^{+/+}$ and $MR^{-/-}$ mice led to study the effects of eliminating the MR on MR- and MPR-mediated plasma clearance and tissue distribution of infused phosphorylated (P) and nonphosphorylated (NP) forms of human β-glucuronidase. In $MR^{+/+}$ MPS VII mice, the MR clearance system predominated at doses up to 6.4 mg/kg P-GUS. Genetically eliminating the MR slowed plasma clearance of both P- and NP-GUS and enhanced the effectiveness of P-GUS in clearing storage in kidney, bone, and retina. Saturating the MR clearance system by high doses of enzyme also improved targeting to MPR-containing tissues

such as muscle, kidney, heart, and hepatocytes. Although ablating the MR clearance system genetically is not practical clinically, blocking the MR-mediated clearance system with high doses of enzyme is feasible. This approach delivers a larger fraction of enzyme to MPR-expressing tissues, thus enhancing the effectiveness of MPR-targeted ERT (Sly et al. 2006).

Mucopolysaccharidosis type VI (MPS VI) is an autosomal recessive disease caused by the deficiency of N-acetylgalactosamine 4-sulfatase (4S) leading to the lysosomal accumulation and urinary excretion of dermatan sulfate. MPS VI has also been described in the Siamese cat. Endocytosis of recombinant feline 4S (rf4S) by cultured feline MPS VI myoblasts was predominantly mediated by a M6PR and resulted in the correction of dermatan sulfate storage. The mutation causing feline MPS VI was identified as a base substitution at codon 476, altering a leucine codon to a proline (L476P) (Yogalingam et al. 1996). Differences in glycosylation of lysosomal enzymes can be an important factor in altering enzyme uptake by different cell types. In alternative approaches, carbohydrate modification variants may be useful for altering the distribution of exogenous enzyme in vivo (Fuller et al. 1998). Reports also suggest that muscle-mediated gene replacement therapy may be a viable method for achieving circulating levels of recombinant f4S (rf4S) in the MPS VI cat (Yogalingam et al. 1997).

Mucopolysaccharidosis Type IIIA (MPS IIIA) is a lysosomal storage disorder caused by a deficiency in the lysosomal enzyme sulfamidase, which is required for the degradation of heparan sulfate. The disease is characterized by neurological dysfunction but relatively mild somatic manifestations. In a naturally occurring mouse model to MPS IIIA, recombinant murine sulfamidase was able to correct the storage phenotype of MPS IIIA fibroblasts after endocytosis via M6PR (Bielicki et al. 1998; Gliddon et al. 2004; Urayama et al. 2008).

Mucopolysaccharidosis type IIIB (MPS-IIIB, Sanfilippo type B Syndrome) is a heterosomal, recessive lysosomal storage disorder resulting from a deficiency of α-N-acetylglucosaminidase (NAGLU). The use of secreted NAGLU in future enzyme and gene replacement therapy protocols will be with limited success due to its small degree of mannose-6-phosphorylation (Weber et al. 2001). Mucopolysaccharidosis type IIID or Sanfilippo D syndrome, a lysosomal storage disorder, is caused by the deficiency of N-acetylglucosamine-6-sulphatase (Glc6S). In addition to human patients, a Nubian goat with this disorder has been described. The r-caprine Glc6S was endocytosed by fibroblasts from patients with mucopolysaccharidosis type IIID via the M6PR-mediated pathway resulting in correction of the storage phenotype of these cells (Litjens et al. 1997). Mucopolysaccharidosis IVA (MPS IVA; Morquio A syndrome) is a lysosomal storage disorder caused by deficiency of N-acetylgalactosamine-6-sulfatase (GALNS), an enzyme that degrades keratan sulfate. The recombinant phosphorylated enzyme was dose-dependently taken up by M6PR thereby restoring enzyme activity in MPS IVA fibroblasts. Penetration of the therapeutic enzyme throughout poorly vascularized, but clinically relevant tissues, as well as macrophages and hepatocytes in wild-type mouse, supports development of rhGALNS as enzyme replacement therapy for MPS IVA (Dvorak-Ewell et al. 2010).

Palmitoyl-Protein Thioesterase (PPT) is a lysosomal long-chain fatty acyl hydrolase that removes fatty acyl groups from modified cysteine residues in proteins. Mutations in palmitoyl-protein thioesterase were found to cause the neurodegenerative disorder infantile neuronal ceroid lipofuscinosis, a disease characterized by accumulation of amorphous granular deposits in cortical neurons, leading to blindness, seizures, and brain death by the age of 3. The accumulation in cultured cells is reversed by the addition of recombinant palmitoyl-protein thioesterase that is competent for lysosomal uptake through M6PR (Lu et al. 1996). PPT expressed in COS-1 cells is recognized by M6PR and is routed to lysosome, but a substantial fraction of PPT is secreted. The PPT has a role outside the lysosomes in the brain and may be associated with synaptic functioning (Lehtovirta et al. 2001).

MPR as Target in MPS VII in Mice: Although ERT is an established method for treating lysosomal storage diseases, an alternative strategy to rectify lysosomal storage diseases depends on the interaction of a fragment of IGF2, with the IGF2 binding site on the IGF2/CI-MPR as tested in a murine mucopolysaccharidosis type VII (MPS VII) model. A chimeric protein containing a portion of mature human IGF2 fused to the C terminus of human β-glucuronidase was taken up by MPS VII fibroblasts in a M6P-independent manner. The tagged enzyme was delivered effectively to clinically significant tissues in MPS VII mice and effective in reversing the storage pathology. The peptide-based, glycosylation-independent lysosomal targeting system may enhance enzyme-replacement therapy for certain human lysosomal storage diseases (LeBowitz et al. 2004).

Lysosomal storage diseases may be treated by the transplantation of cells that secrete the enzyme which is deficient in patients. Secretion of lysosomal enzymes can be enhanced by reducing the MPR involved in the lysosomal sorting of newly synthesized lysosomal enzymes. Hammerhead ribozymes targeting the mRNA of murine CI-MPR cleave

RNA fragments efficiently and reduce the levels of murine MPR mRNA in transient transfection experiments. Thus, the reduction in MPR is sufficient to increase a lysosomal enzyme secretion (Yaghootfam and Gieselmann 2003).

46.1.4 Other Approaches using CI-MPR as Target

CI-MPR Binds and Internalizes Leukemia Inhibitory Factor: Leukemia inhibitory factor (LIF) is a multifunctional, highly glycosylated soluble protein belonging to the interleukin-6 (IL-6) subfamily of helical cytokines. LIF exerts an important role in neuronal, platelet and bone formation. Blanchard and co-workers have reported that glycosylated LIF and the macrophage-colony-stimulating factor were able to bind to CI-MPR in a M6P-sensitive manner (Blanchard et al. 1999). The M6P-containing cytokine LIF is rapidly internalized and degraded by cells expressing CI-MPR (Blanchard et al. 1999). Thus, CI-MPR is a candidate natural molecule, like other inhibitors of the IGF2 pathway, for the development of novel therapeutic strategies in treatment of haematopoietic disorders in which IL-6-type cytokines play a role, particularly multiple myeloma. CI-MPR can also bind and internalize renin and pro-renin (Admiraal et al. 1999). After internalization, pro-renin is swiftly activated by proteolytic cleavage (Saris et al. 2002). CI-MPR in myocytes may participate in the cardiac (pro)renin uptake by binding to the fraction of human (pro) renin, that is characterized by the presence of the M6P recognition marker (Saris et al. 2002).

Lysosomal Targeting for Cancer Therapy: CI-MPR has been reported to be a potential tumor suppressor in 70% of hepatocarcinomas and 15–30% of breast cancers (Hankins et al. 1996; Chappell et al. 1997; Oates et al. 1998). Moreover, over-expression of CI-MPR induced regression of tumors in mice and growth inhibition in cancer cells. These effects are probably due to the different functions of this receptor that indirectly controls cell growth: (1) internalization and degradation of various growth-promoting factors, such as IGF2 (Kiess et al. 1988), glycosylated LIF, and other M6P-containing cytokines, such as the macrophage colony-stimulating factor (Blanchard et al. 1999); (2) binding and uptake of granzyme B, an essential factor for T cell-mediated apoptosis (Motyka et al. 2000), and (3) regulation of secreted lysosomal enzymes that are responsible for extracellular matrix degradation and tumor dissemination. Attempts to target the acidic extracellular compartment of solid tumors have been performed by loading doxorubicin into pH-sensitive poly/PEG/folate micelles (Lee et al. 2005) or liposomes (Storm et al. 1987).

Inhibitors of PI3-Kinase Activity in Late Endocytic Pathway: Addition of wortmannin to normal rat kidney cells causes redistribution of the lysosomal type I integral membrane proteins Igp110 and Igp120 to a swollen vacuolar compartment. Wortmannin does not show gross morphological effect on TGN or lysosomes, or any effect on the delivery to the TGN of endocytosed antibodies against the type I membrane protein TGN38. The observed effects of wortmannin were due to inhibition of membrane traffic between CI-MPR-positive late endosomes and the TGN and to inhibition of membrane traffic between a novel Igp120-positive, CI-MPR-negative late endosomal compartment and lysosomes. The effects of wortmannin suggest a function for a PI3-kinase(s) in regulating membrane traffic in the late endocytic pathway (Reaves et al. 1996). 3-methyladenine (3-MA), a well-known inhibitor of autophagic sequestration, can also inhibit PI3-kinase activity, which is required for many processes in endosomal membrane trafficking. The treatment with 3-MA results in a specific redistribution of CI-MPR from the TGN to early/recycling endosomal compartments containing internalized transferrin. However, in contrast to wortmannin and LY294002, 3-MA did not cause the enlargement of late endosomal/lysosomal compartments. Hence, the effect of 3-MA is restricted to the retrieval of CI-MPR from early/recycling endosomes (Hirosako et al. 2004).

46.2 Cell Targeting Based on Mannan-Lectin Interactions

Carbohydrates exhibit properties of potential interest when developing drug-delivery mechanisms, such as specificity in their interaction with their receptors and the varied nature of potentially targetable receptors available. Macromolecular glycoconjugates, in particular, have shown some promise. Synthetic glycopolymers and glycoproteins have been used as carriers of covalently conjugated drugs, bearing carbohydrate ligands that provide delivery specificity. However, these systems commonly rely on endogenous mechanisms, such as lysosomal degradation, for release of the active drug, and so the unwanted release of the drug at sites other than the desired site of action is possible. Glycotargeting is being followed through two approaches: (1) relying on use of oligosaccharide moiety or (2) using the lectin as a component of the drug delivery system (Gao et al. 2007; Gupta et al. 2009; Minko 2004; Smart 2004). In the first approach, oligosaccharides or neoglycoconjugates form part of the drug delivery system, whereas, in the second approach using lectins towards glycotargeting, the principle is reversed of the first approach.

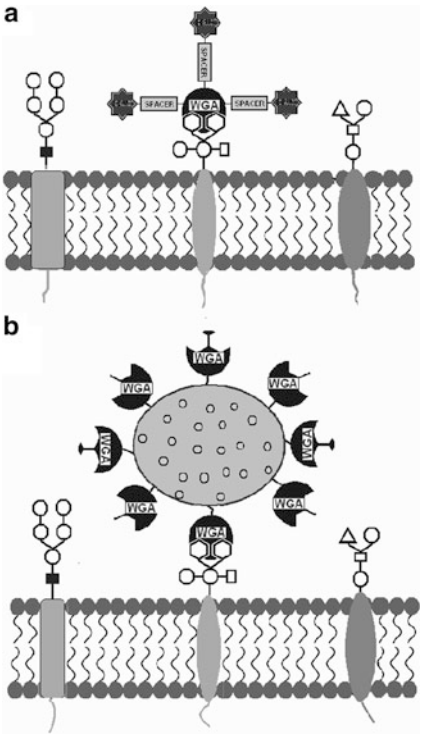

Fig. 46.2 Lectin-grafted formulations. (**a**) Lectin-grafted prodrug, (**b**) Lectin-grafted carrier system. Galactose, N-acetylgalactosamine, N-acetylglucosamine, Spacer, Fructose, Drug, Mannose

The delivery of therapeutic agents to, or via, the oral cavity is limited by the efficient removal mechanisms that exist in this area. The bio-recognition between lectinised drug delivery systems and glycosylated structures in the intestine can be exploited for improved peroral therapy. Lectins as proteins bind to specific sugar residues, and can interact with the glycoconjugates present on cell surfaces or salivary mucins. Endogenous lectins could also be used as points of attachment for carbohydrate-containing delivery systems. As lectins are multifunctional molecules, the possibility of using them is considered as a potential innovation for targeted and prolonged therapy within the oral cavity but considerations such as toxicity needs to be addressed before their routine use becomes a reality (Gao et al. 2007; Gupta et al. 2009; Minko 2004; Smart 2004).

The lectin-carbohydrate interaction can be made use of by the development of nanoparticles containing carbohydrate moieties that are directed to certain lectins (direct lectin targeting) as well as incorporating lectins into nanoparticles that are directed to cell surface carbohydrates (reverse lectin targeting) (Gabor et al. 2004) (Fig. 46.2). Treatment of glioblastoma multiforme (GBM), a primary malignant tumor of the brain, is one of the most challenging problems as no currently available treatment is curative. Surgery remains the basic treatment in which the bulk of the tumor is removed and the peripheral infiltrating part is the target of supplementary treatments. Many of the technologies based on nanotechnology can be applied in the improvement of drug delivery to GBM (Jain 2007).

46.2.1 Receptor-Mediated Uptake of Mannan-Coated Particles (Direct Targeting)

Cell surface-bound receptors represent suitable entry sites for delivery of macromolecules or supramolecular structures into cells by receptor-mediated endocytosis. Carrying the carbohydrate-tag, the drug delivery system can be recognized by the cells/tissues and internalised by endogenous lectins at the cell surface. Mammalian mannose/fucose/galactose specific cell surface receptors are expressed on macrophages and other antigen presenting cells such as dendritic cells (DCs) in skin and M-cells in intestine. Macrophages and DCs play an important role in host immune functions such as antigen presentation. Attempts have been made to modulate the function of macrophages for the treatment of genetic metabolic diseases. The mannose receptor (ManR) on macrophages mediates the internalization of a wide range of molecules or microorganisms in a pattern recognition manner (see Chaps. 15 and 35 for structure and functions of ManR). Therefore, it represents an attractive entry for specific drug, gene, or antigen delivery to macrophages and antigen-presenting DCs. Particles coated with carbohydrate ligands offer potential future in the site directed delivery of macromolecules and therapeutic drugs. Based on this principle delivery systems containing asialofetuin, galactose, mannose, or N-acetyl-galactosamine were developed and tested for endocytois by macrophages, DCs, and liver cells (Chiu et al. 1994). The use of carbohydrate-modified HPMA or liposomes gave improved results (Andre et al. 2000; Dasi et al. 2001). Liver or the colon, macrophages and mouse brain have been shown to be targeted by mannosylated liposomes (Man-liposome) (Umezawa and Eto 1988).

46.2.2 Polymeric Glyco-Conjugates as Carriers

Mannosylated Conjugates as Cell-Specific Carrier: Hepatic uptake characteristics of mannosylated bovine serum albumin (Man-BSA) were assessed as a liver-specific carrier system (Ogawara et al. 1999). The ^{111}In-Man18-BSA accumulated in the liver up to 70%; the endothelial cells and Kupffer cells contributed major fraction. These results provide useful information in designing drug targeting systems to the liver nonparenchymal cells via mannose receptors (Ogawara et al. 1999).

Mannosylpolyethylenimine (ManPEI) Conjugates: Presence of mannose receptors on DCs can be exploited for targeted gene transfer by employing mannosylpolyethylenimine (ManPEI) conjugates. Several ManPEI conjugates have been used for formation of ManPEI/DNA transfection complexes. Results demonstrated that DCs transfected with ManPEI/DNA complexes containing adenovirus particles are effective in activating T cells of T cell receptor transgenic mice in an antigen-specific fashion (Diebold et al. 1999).

Poly-(L-Lysine Citramide Imide): Commercially available quinic and shikimic acids appear as stable mannose bioisosteres, which should prove valuable tools for specific cell delivery (Grandjean et al. 2001). With the aim of promoting the targeting of macrophage mannose receptors and the internalization of the norfloxacin antibiotic, which is active against some intracellular bacteria, was coupled to a polymeric carrier, namely poly-(L-lysine citramide imide). This carrier, derived from two metabolites, citric acid and L-lysine, is known to be biocompatible and slowly degradable under slight acidic conditions. Prodrug macromolecules compete effectively with glucose oxidase and thus should be able to bring the drug up to the mannosyl receptor-bearing membranes of macrophages infected by intracellular bacteria (Gac et al. 2000).

Poly[N-p-Vinylbenzyl-O-β-Mannopyranosyl-(1-4)-D-Gluconamide (PV-Man): PV-Man is a polystyrene derivative that contains mannose moieties and interacts with ManR-carrying cell line. The PV-Man strongly binds to macrophage cells, probably due to a specific interaction mediated by ManRs on the cell membrane. Using a PV-Man glycopolymer, receptor-mediated gene transfer via ManR is another method for targeted gene delivery into macrophages (Park et al. 2005). Polymeric nanospheres (NS) with a polystyrene core and a glucosyloxyethyl methacrylate (GEMA) oligomer corona nanosphere proved to be a useful material for studying sugar-biomolecule recognition and offered a potential for using a multi-lectin nanoparticle array in glycoprotein mapping (Fromell et al. 2005; Serizawa et al. 2001).

Cyclodextrin Conjugates: A structure based saccharide-directed molecular delivery system through biological receptors was studied by Benito et al. (2004). The dendritic β-cyclodextrin (βCD) derivatives bearing multivalent mannosyl ligands were assessed for their binding efficiency towards the ConA and mammalian mannose/fucose specific cell surface receptor from macrophages. This new type of βCD-dendrimer construct showed high drug solubilization capability. A subtle change in the structure of the conjugate may have important consequences on receptor affinity (Benito et al. 2004).

McNicholas et al. (2007) synthesized amphiphilic β-cyclodextrins bearing hexylthio, dodecylthio, and hexadecylthio chains at the 6-positions and glycosylthiocarbamoyl-oligo(ethylene glycol) units at the 2-positions. The glycosyl residues (α-D-mannosyl and β-L-fucosyl) were intended for cell-targeting. These amphiphilic glycosylated cyclodextrins form vesicles in water. Hexylthio assemblies exhibited selective binding to Lens culinaris lectin. A bioeliminable amphiphilic poly(ethylene oxide)-b-poly(ε-caprolactone) (PEO-b-PCL) diblock copolymer end-capped by a mannose residue, synthesized by sequential controlled polymerization of ethylene oxide and epsilon-caprolactone, followed by the coupling of a reactive mannose derivative to the PEO chain end showed that these colloidal systems have great potential for drug targeting and vaccine delivery systems (Rieger et al. 2007).

Delivery of Antisense Oligonucleotides: Antisense oligonucleotides (ONs) are useful for selective inhibition of gene expression. However, their effective use is limited by inefficient cellular uptake and lack of cellular targeting. A drug targeting system which utilizes ManR-mediated endocytosis to enhance cellular uptake of ONs in alveolar macrophages (AMs), employs a molecular complex consisting of partially substituted mannosylated poly(L-lysine) (MPL), linked to ON. Upon recognition by the macrophage ManRs, the MPL was internalized by the receptor-mediated pathway, co-transporting the ON. The AMs treated with the MPL:ON complex exhibited a significant increase in ON uptake over free ON-treated controls. The ON uptake was shown to require the recognition of the mannose moiety since unmodified polylysine was much less effective in promoting ON uptake. Following cellular internalization, the ON largely accumulated in endocytic vesicles (Liang et al. 1996).

46.2.3 Mannosylated Liposomes in Gene Delivery

Mannosylated Liposomes: Introduction of ligands for cell-surface receptors into liposomes has been continuing to improve transfection efficiency: as far as macrophages are concerned (Simões et al. 1999). In earlier studies, liposomes were coated with macromolecular ligands such as transferrin, immunoglobulins and asialoglycoproteins. Direct respiratory delivery via inhalation of mannose modified liposomal carriers to alveolar macrophages is of great interest. The success of targeting systems to alveolar macrophages depends on internalization into these cells for pharmacological intervention. Mannose grafted liposome intercalated Benzyl derivative of an antibiotic MT81 (Bz2MT81) eliminated intracellular amastigotes of *Leishmania donovani* within splenic macrophages more

efficiently than the liposome intercalated Bz2MT81 or free Bz2MT81. Both Man-liposomes and Bz2MT81 appeared to be non-toxic to the host peritoneal macrophages. Liver and kidney function tests (SGPT, alkaline phosphatase, creatinine and urea in blood plasma) showed that the toxicity of Bz2MT81 was reduced up to normal level when mannose grafted liposomal Bz2MT81 were administered (Mitra et al. 2005).

Using cell receptors on the surface of mononuclear phagocyte cells, which are important hosts for HIV, stavudine-loaded Man-liposomal formulations have been developed for targeting HIV-infected cells. Using Con A as a model system for in-vitro ligand-binding capacity, Man-liposomes showed potential applications for the site-specific and ligand-directed delivery systems with better pharmacological activity (Garg et al. 2006). Perhaps, clustering of mannose residues on liposomal surfaces is important in determining the binding affinity of Man-liposomes with MBP and related to the mannose density of Man-liposomes (Terada et al. 2006; Wijagkanalan et al. 2008).

Mannosylated Cationic Liposomes: Several strategies have been developed to transfer genes directly into macrophages. In recent years, complexes of polylysine linked to ligands such as mannose (Ferkol et al. 1996; Erbacher et al. 1996) with DNA have been reported to enhance gene expression in macrophages. But, the transfection efficiency of many of these vectors is handicapped due to endosomal or lysosomal degradation. A promising nonviral gene delivery system developed involves cationic liposomes. Various kinds of cationic lipids have been synthesized and shown to be able to deliver genes into cells both in vitro and in vivo. DC-Chol liposomes have been used in gene therapy applications in clinical settings (Gao and Huang 1991; Nabel et al. 1993). A galactosylated cholesterol derivative in combination with dioleoylphosphatidylethanolamine (DOPE) efficiently transferred a plasmid DNA into human hepatoma cells (HepG2) via an asialoglycoprotein receptor-mediated mechanism (Kawakami et al. 1998). However, cationic liposomes do not exhibit any cell specificity in vivo. Kawakami et al. (2004) developed a low-molecular weight lipidic ligand, a mannosylated cholesterol derivative, cholesten-5-yloxy-N-(4-(1-imino-2-β-D-thiomannosylethyl)amino)butyl)-formamide (Man-C4-Chol), for gene delivery to hepatocytes and compared with other types of liposomes prepared with various molar ratios of Man-C4-Chol and particle size of about 200 nm for transfection assays in hepatocytes and mouse peritoneal macrophages (Kawakami et al. 2000).

The gene expression with Man-C4-Chol/DOPE (6:4) liposome/DNA complexes in the liver was observed preferentially in the non-parenchymal cells and was significantly reduced by predosing with Man-BSA. The gene expression in the liver was greater following intraportal injection. These results suggested that plasmid DNA complexed with Man-liposomes exhibits high transfection activity due to recognition by mannose receptors both in vitro and in vivo. Intravenous injection of DNAcationic liposome complexes resulted in gene expression in many tissues including the heart, lung, liver, kidney and spleen (Mahato et al. 1995) through participation of mannose receptors in liver Kuppfer and or endothelial cells. The splenic macrophages may also be targeted by this approach.

As far as the design of carriers for active targeting using receptor-mediated endocytosis was concerned, the density and stereochemistry of the ligand seemed to be important. It was demonstrated that galactosylated protein is recognized by liver cells in a manner directly related to the estimated surface density of the galactose residues. Similar strategy applies to Man-liposomes. The chemical structure and physicochemical characteristics of Man-C4-Chol seemed to satisfy the conditions for transfection in macrophages by offering a cationic charge and being recognized by the mannose structure on the liposomal surface.

Mannosylated-Emulsions: Carbohydrate grafted emulsions are one of the most promising cell-specific targeting systems for lipophilic drugs. Man-emulsions composed of soybean oil, EggPC and Man-C4-Chol with a ratio of 70:25:5 were significantly delivered to liver non-parenchymal cells (NPC) via mannose receptor-mediated mechanism after intravenous administration in mice and supported the design of pDNA/ligands-grafted cationic liposome complexes for cell-specific gene delivery (Kawakami et al. 2004). The in vitro study showed increased internalization of Man-5.0- and Man-7.5-emulsions and significant inhibition of uptake in the presence of mannan. The enhanced uptake of Man-emulsions was related to the increasing of Man-C4-Chol content. This suggested that the mannose density of Man-emulsions plays an important role in both cellular recognition and internalization via a mannose receptor-mediated mechanism (Yeeprae et al. 2006).

Wijagkanalan et al. (2008) demonstrated the efficient targeting to alveolar macrophages by the intratracheally administered Man-liposomes via ManR-mediated endocytosis in rats. The study involved Man-liposomes, with various ratio of mannosylated cholesterol derivatives, cholesten-5-yloxy-N-(4-((1-imino-2-D-thiomannosylethyl)-amino)alkyl)-formamide (Man-C4-Chol) and suggested that in vitro uptake of Man-liposomes occurs in a concentration-dependent manner. Through intratracheal route of administration of Man-7.5 and Man-5.0-liposomes, internalization was enhanced and selective to alveolar macrophages.

46.2.4 DC-Targeted Vaccines

A workshop on "Dendritic Cells: Biology and Therapeutic Applications," brought together basic and clinical research scientists to discuss the mechanisms underlying the control of immune responses and tolerance by DCs, as well as research in cancer immunotherapy based on DC vaccination (Ardavin et al. 2004).

Mannose Receptor Targeting Vaccine

DCs have a number of receptors for adsorptive uptake of antigens. Some are shared with other cells, such as Fcγ receptors, DEC-205, a type I membrane-integrated glycoprotein and the macrophage mannose receptor (MMR) (Chap. 35). Other receptors are more DC restricted, e.g., Langerin/CD207 (Chap. 35), DC-SIGN/CD209 (Chap. 36), asialoglycoprotein receptor or hepatic lectins (HL) (Chap. 33), and dendritic cell lectin (DLEC; also referred to as BDCA-2). Targeting antigens to endocytic receptors on professional APCs represents an attractive strategy to enhance the efficacy of vaccines. Such APC-targeted vaccines have the ability to guide exogenous protein antigens into vesicles that efficiently process the antigen for MHC class I and class II presentation. Efficient targeting not only requires high specificity for the receptor that is abundantly expressed on the surface of APCs, but also the ability to be rapidly internalised and loaded into compartments that contain elements of the antigen-processing machinery. The ManR and related C-type lectin receptors are especially designed to sample antigens, much like pattern recognition receptors, to integrate the innate with adaptive immune responses. A variety of approaches involving delivery of antigens to the ManR have demonstrated effective induction of potent cellular and humoral immune responses. ManR-targeted vaccines are likely to be most efficacious in vivo when combined with agents that elicit complementary activation signals. A better understanding of the mechanism associated with the induction of immune responses as a result of targeting antigens to the ManR, will be important in exploiting ManR-targeted vaccines not only for mounting immune defenses against cancer and infectious disease, but also for specific induction of tolerance in the treatment of autoimmune disease (Keler et al. 2004).

DEC-205 Mediated Cancer Immunotherapy

Anti α-DEC-205 antibodies target to the DEC-205 receptor that mediates antigen presentation to T cells by DC. DEC-205 is a mannose specific receptor, present on DC. One of the major functions of DEC-205 is to internalize the antigens and present to naïve T lymphocytes for development of T cell dependent immunity. To assess the potential of antigen targeting to DC, Bonifaz et al. (2004) incorporated ovalbumin into a mAb to the DEC-205 receptor, which is expressed on these cells in lymphoid tissues. A single low dose of antibody-conjugated ovalbumin initiated immunity from the naive $CD4^+$ and $CD8^+$ T cell repertoire. Unexpectedly, the αDEC-205 antigen conjugates targeted to DCs for long periods and ovalbumin peptide was presented on MHC class I. This was associated with stronger $CD8^+$ T cell-mediated immunity relative to other forms of antigen delivery, even when the latter was given at a 1,000 times higher doses. In parallel, the mice showed enhanced resistance to an established rapidly growing tumor and to viral infection at a mucosal site. By antibody-mediated antigen targeting via the DEC-205 receptor increased the efficiency of vaccination for T cell immunity, including systemic and mucosal resistance in disease models (Bonifaz et al. 2004; Mahnke et al. 2005). In preclinical studies, Badiee et al. (2007) prepared anti-human DEC-205 immunoliposomes (anti-hDEC-205 iLPSM) and compared their uptake by monocyte-derived DC and blood DC (BDC) with conventional liposomes (cLPSM). Confocal microscopy confirmed that the anti-hDEC-205 iLPSM were phagocytosed by DC and available for antigen processing. Thus, DEC-205 is one of effective targets for delivering liposomes to human DCs (Badiee et al. 2007).

Since, anti α-DEC-205 antibodies target to the DEC-205 receptor that mediates antigen presentation to T cells by DCs, these properties were exploited for immunization strategies by conjugating the melanoma antigen tyrosinase-related protein (TRP)-2 to α-DEC-205 antibodies and immunization of mice with these conjugates together with DC-activating oligonucleotides (CpG). Upon grafting of the melanoma cell line B16, α-DEC-TRP immunized mice were protected against tumor growth or showed substantially slow growth of implanted B16 cells into tumor bearing hosts. Approximately 70% of the animals were cured from existing tumors by treatment with α-DEC conjugates carrying two different melanoma antigens (TRP-2 and gp100). Thus, targeting of DCs in situ by antibody-antigen conjugates may be a novel approach to induce long-lasting antitumor immunity.

Mannan-Coated Nanoparticles in Vaccination: Genetic immunization using "naked" plasmid DNA (pDNA) has been used to elicit humoral and cellular immune responses. In search of a cell-targeted delivery system, cationic nanoparticles coated with pDNA for genetic immunization have been explored. Plasmid DNA-coated nanoparticles, especially with both an endosomolytic lipid and DC-targeting ligand, resulted in 16-fold increase in IgG titer and threefold release in Th1-type cytokine over "naked"

pDNA, indicating that the engineered pDNA-coated nanoparticles could enhance in vitro cell transfection and enhanced in vivo immune responses (Cui and Mumper 2002a). Furthermore, pDNA-coated nanoparticles, especially the mannan-coated pDNA-nanoparticles with DOPE, resulted in significant enhancement in both antigen-specific IgG titers and splenocyte proliferation over 'naked' pDNA alone (Cui and Mumper 2002b). The freeze-dried nanoparticles (prior to pDNA coating) showed potential application for cell-specific targeting of macrophages. Moreover, incubation of nanoparticles with ManR positive mouse macrophage cell line (J774E) showed that the uptake of mannan-coated nanoparticles by the cells was 50% higher than that of uncoated nanoparticles (Cui et al. 2003).

DC-SIGN-Mediated Targeting

Targeting DC surface proteins to deliver liposomes carrying antigens has demonstrated potential for eliciting antigen-specific immune responses. Myeloid dendritic cells (MyDCs) can serve as one of the major reservoirs for HIV-1. Using monocyte-derived MyDCs, Gieseler et al. (2004) presented the evidence for liposomal compound delivery to these cells by specifically addressing. DC-SIGN (CD209), a MyDC-associated C-type lectin implicated in the transmission of HIV-1 to T helper cells. The DC-SIGN was demonstrated as a superior target as compared with other MyDC markers (CD1a, CD4, CD45R0, and CD83). This study implied that liposomal targeting to DC-SIGN (CD209) and related C-type lectins may afford therapeutic intracellular drug delivery to MyDCs and other reservoir and nonreservoir cells susceptible to infection with HIV-1.

Since DCs are a central element in the development of antigen-specific immune responses, current DC-based vaccines are based on ex vivo-generated autologous DCs loaded with antigen prior to re-administration into patients. A more direct and less laborious strategy is to target antigens to DCs in vivo via specific surface receptors. Therefore, a humanized antibody, hD1V1G2/G4 (hD1), directed against the DC-SIGN was explored for its capacity to serve as a target receptor for vaccination purpose. hD1 was cross-linked to a model antigen, keyhole limpet hemocyanin (KLH). The chimeric antibody-protein complex (hD1-KLH) bound specifically to DC-SIGN and was rapidly internalized and translocated to the lysosomal compartment. Antibody-mediated targeting of antigen to DCs via DC-SIGN effectively induces antigen-specific naive as well as recall T-cell responses. This identifies DC-SIGN as a promising target molecule for DC-based vaccination strategies (Gupta et al. 2009; Tacken et al. 2005). Optimal HIV vaccines should elicit $CD8^+$ T cells specific for HIV proteins presented on MHC class I products, because these T cells contribute to host resistance to viruses. Based on humans, studies with highly polymorphic MHC products reveal that DCs and DEC-205 can cross-present several different peptides from a single protein. Because of the consistency in eliciting $CD8^+$ T cell responses these data support the testing of αDEC-205 fusion mAb as a protein-based vaccine (Bozzacco et al. 2007).

The efficacy of adenoviral (Ad) vectors can be enhanced through alterations in vector tropism such that DC-targeted transduction is achieved. The efficiency of DC transduction by Ad vectors retargeted to DC-specific DC-SIGN was studied and compared to that of Ad vectors retargeted through CD40 (Korokhov et al. 2005). A comparable and significant enhancement of gene transfer to monocyte derived DCs (MDDCs) was accomplished by means of an Ad vector harboring the Fc-binding domain of *S. aureus* protein A in combination with antibodies to DC-SIGN or to CD40 or with fused complexes of human Ig-Fc with their natural ligands, i.e., ICAM-3 or CD40L, respectively. Results demonstrated the usefulness of DC-SIGN as a DC-restricted targeting motif for Ad-mediated vaccination strategies.

Targeted Oral Vaccine Delivery to M Cells: A strategy for mucosal vaccination and drug delivery: In the intestine, the delivery of antigens across the epithelial barrier to the underlying lymphoid tissue is accomplished by M cells, a specialized epithelial cell type that occurs only in the lymphoid follicle-associated epithelium. Selective and efficient transport of antigen by M cells is considered an essential requirement for effective mucosal vaccines. Therefore, particulate antigen formulations are currently being developed to take advantage of the capacity of M cells to endocytose particles. Delivery may be achieved using synthetic particulate delivery vehicles including poly(DL-lactide-co-glycolide) microparticles and liposomes. M cell interaction of these delivery vehicles is highly variable, and is determined by the physical properties of both particles and M cells. Delivery may be enhanced by coating with reagents including appropriate lectins, microbial adhesins and immunoglobulins which selectively bind to M cell surfaces (Brayden 2001; Clark et al. 2001). In an alternative approach, antigens are coupled to or encapsulated in particulate synthetic carriers. To enhance binding and uptake of such nonviable vectors, ligands are being attached which direct the vaccine particle to receptors on the M cell surface. While binding and uptake of M cell-targeted latex particles and stable liposomes by mouse M cells has been shown using the mouse M cell-specific lectin, Ulex europaeus 1 (UEA-1), a direct relationship between M cell particle uptake and immune outcome was reported by Brayden (2001).

Since various lectins and lectin containing pathogens bind specifically to oligosaccharides including mannose on intestinal cells, exploiting this specificity, lectins have been

used as a ligand for targeted oral vaccine delivery to M cells (antigen-presenting cells) in follicle-associated epithelium. The antigen-sampling M cells offer a portal for absorption of colloidal and particulate delivery vehicles, including bacteria, viruses and inert microparticles. Consideration is also given to lectin-mediated targeting in non-intestinal sites and to the potential application of other bioadhesins to enhance M cell transport (Jepson et al. 2004). While ManR is found on lymphatic endothelial cells of small intestine, the intestinal serosa revealed a regular, dense, planar network of cells with prominent dendritic morphology within the external muscular layer and with increasing frequency along the length of the intestine (Flores-Langarica et al. 2005). The serosal-disposed layers show a significant fraction of DCs that express DEC-205, Langerin, CD14 and various other molecules. In vivo, these DCs responded to two microbial stimuli, systemic LPS and oral live bacteria, by up-regulating DEC-205, and Langerin within 12 h. This network of DCs, representing a unrecognized APC system in the intestine, needs to be explored for drug targeting and mucosal vaccination through ManR, DEC-205, and langerin present on intestinal cells (Flores-Langarica et al. 2005).

46.3 Asialoglycoprotein Receptor (ASGP-R) for Targeted Drug Delivery

46.3.1 Targeting Hepatocytes

Asialoglycoprotein receptor (ASGP-R) is predominantly expressed on the sinusoidal surface of mammalian hepatocytes and is responsible for the clearance of glycoproteins with desialylated galactose or acetylgalactosamine residues from the circulation by receptor-mediated endocytosis. The ASGP-R provides a unique means for the development of liver-specific carriers, such as liposomes, recombinant lipoproteins, and polymers for drug or gene delivery to the liver, especially to hepatocytes (Cawley et al. 1981; Wu et al. 1998) (Chap. 33). Another study used glycolipids containing a cluster galactoside moiety for targeting to ASGP-R. The liver uptake of the glycolipid-liposomes exceeds 80% compared to less than 10% for conventional liposomes after injection. The abundant receptors on the cells specifically recognize ligands with terminal galactose or N-acetylgalactosamine residues, and endocytose the ligands for an intracellular degradation process. The use of its natural ligand, i.e. asialofetuin, or synthetic ligands with galactosylated or lactosylated residues, such as galactosylated cholesterol, glycolipids, or galactosylated polymers has achieved significant targeting efficacy to the liver. There are several examples of successful targeted therapy for acute liver injury with asialofetuin-labeled and vitamin E-associated liposomes or with a caspase inhibitor loaded in sugar-carrying polymer particles, as well as for the delivery of an antiviral agent, 9-(2-phosphonylmethoxyethyl)adenine. Liposome-mediated gene delivery to the liver is still in its infancy due to difficulties in solving general issues, such as stability of liposome-DNA complexes in circulation, and lysosomal or endosomal degradation of plasmid DNA. Although, galactosylated polymers are promising for gene delivery, but require further studies to verify their potential applications (Wu et al. 2002).

Cationic liposomes and polymers have been accepted as effective non-viral vectors for gene delivery with low immunogenicity unlike viral vectors. Galactosylated liposomes and poly(amino acids) are selectively taken up by the ASGP-R-positive liver parenchymal cells in vitro and in vivo after intravenous injection. DNA-galactosylated cationic liposome complexes show higher DNA uptake and gene expression in the liver parenchymal cells in vitro than DNA complexes with bare cationic liposomes. In the in vitro gene transfer experiment, galactosylated liposome complexes are more efficient than DNA-galactosylated poly (amino acids) complexes but they have some difficulties in their biodistribution control. On the other hand, introduction of mannose residues to carriers resulted in specific delivery of genes to non-parenchymal liver cells (Hashida et al. 2001). Drugs conjugated with galactosyl-terminating macromolecules selectively enter hepatocytes after interaction of the carrier galactose residues with the ASGP-R present in large amounts and high affinity on hepatocytes. Within hepatocytes the conjugates are delivered to lysosomes where enzymes split the bond between the carrier and the drug, allowing the latter to become concentrated in the liver. The validity of this chemotherapeutic strategy has been endorsed by a clinical study (Fiume et al. 1997).

The results obtained reveal tremendous promise and offer enormous options to develop novel DNA based pharmaceuticals for liver disorders in near future. The ^{99m}Tc-labeled asialoglycoprotein analog, TcGSA (galactosyl-human serum albumin-diethylenetriamine - pentaacetic acid) has been applied to human hepatic receptor imaging. This method is unique and provides information that is totally independent of the ICG test or Child-Turcotte Score (Kokudo et al. 2003; Pathak et al. 2008).

The Specificity of ASGP-R for D-Galactose: The specificity of the receptor for D-galactose or D-mannose is accomplished by specific hydrogen bonding of the 3 and 4-hydroxyl groups with carboxylate and amide side-chains. Therefore, mutation of the amino acid sequence in the CRD results in a conversion of its specificity (Feinberg et al. 2000; Meier et al. 2000; Wu et al. 2002). The crystal structure provides a direct confirmation for the conversion of

the ligand-binding site of mannose-binding protein to an ASGP-R-like specificity (Meier et al. 2000). A number of functional mimics for the CRDs of these lectins have been developed by modification of the domain amino acid residues. The modified CRD displayed 40-fold preferential binding to N- acetylgalactosamine compared with galactose, making it a good functional mimic for ASGP-R (Feinberg et al. 2000). Mannose-labeling shifted the ratio to more non-parenchymal cell incorporation (the majority to Kupffer cells) (Feinberg et al. 2000). Therefore, alternative approaches are needed to target liposomes to hepatocytes via ASGP-R (Wu et al. 2002; Yamazaki et al. 2000).

46.4 Siglecs as Targets for Immunotherapy

46.4.1 Anti-CD33-Antibody-Based Therapy of Human Leukemia

Targeting CD33 (Siglec-3) or CD45 is currently exploited for immunotherapy of acute myeloid leukemia (AML). In normal myelopoiesis, expression of CD33 is restricted to advanced stages of differentiation, whereas primitive stem cells do not express CD33. Leukaemic stem cells in patients with $CD33^+$ AML express CD33 (Hauswirth et al. 2007). Antibody-targeted chemotherapy is a therapeutic strategy in cancer therapy that involves a monoclonal antibody specific for a tumor-associated antigen, covalently linked via a suitable linker to a potent cytotoxic agent. The restricted expression of several siglecs (Chaps. 16 and 17) to one or a few cell types makes them attractive targets for cell-directed therapies. The anti-CD33 (Siglec-3) antibody gemtuzumab (Mylotarg) is approved for treatment of acute myeloid leukemia, and antibodies targeting CD22 (Siglec-2) are currently in clinical trials for treatment of B cell non-Hodgkins lymphomas and autoimmune diseases. Since siglecs are endocytic receptors, they are very well suited for a 'Trojan horse' strategy, whereby therapeutic agents conjugated to an antibody, or multimeric glycan ligand, bind to the siglec and are efficiently carried into the cell. Although the rapid internalization of unmodified siglec antibodies reduces their utility for induction of antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity, antibody binding of Siglec-8, Siglec-9 and CD22 (Siglec-2) has been demonstrated to induce apoptosis of eosinophils, neutrophils and depletion of B cells, respectively. The properties of siglecs that make them attractive for cell-targeted therapies have been reviewed in Chaps. 16 and 17.

Gemtuzumab Ozogamicin (GO): Anti-CD33 antibodies have been used alone-and more effectively, attached to chemotherapy agents or radioisotopes-to treat those with AML (Nemecek and Matthews 2002). Antibody-targeted chemotherapy with gemtuzumab ozogamicin (GO) (CMA-676, a CD33-targeted immunoconjugate of N-acetyl-γ-calicheamicin dimethyl hydrazide [CalichDMH], a potent DNA-binding cytotoxic antitumor antibiotic) is a clinically validated therapeutic option for patients with AML. Calicheamicin is a cytotoxic natural product isolated from Micromonospora echinospora that is at least 1,000-fold more potent than conventional cytotoxic chemotherapeutics. Calicheamicin binds DNA and causes double-strand DNA breaks, leading to cell death. Gemtuzumab ozogamicin is the first clinically validated cytotoxic immunoconjugate in which a humanised anti-CD33 antibody is linked to a derivative of calicheamicin. A similar conjugate, inotuzumab ozogamicin, is being evaluated in Phase I clinical trials in patients with non-Hodgkin's lymphoma. GO is part of clinical practice for AML, but is frequently associated with severe side effects. A number of tumor-targeted immunoconjugates of calicheamicin are being explored preclinically at present for their therapeutic applications (Damle 2004).

Immunoconjugates of calicheamicin targeted against tumor-associated antigens exhibit tumor-specific cytotoxic effects and cause regression of established human tumor xenografts in nude mice. CD33-specific binding triggers internalization of GO and subsequent hydrolytic release of calicheamicin. The histone deacetylase inhibitor valproic acid potently augments gemtuzumab ozogamicin-induced apoptosis in AML cells. The synergistic proapoptotic activity of cotreatment of AML cells with VPA and GO indicates the potential value of this strategy for AML (Ten Cate et al. 2007). Simultaneous targeting of CD45 could improve GO cytotoxicity against AML cell lines and primary AML cells. Further study of this antibody combination for clinical use in AML is warranted (Walter et al. 2008).

Lintuzumab: Lintuzumab (HuM195) is an unconjugated humanized murine mAb directed against cell surface myelomonocytic CD33. The efficacy of lintuzumab in combination with induction chemotherapy has been compared with chemotherapy alone in adults with first relapsed or primary refractory AML. The percent CR plus CRp with MEC plus lintuzumab was 36% vs. 28% in patients treated with MEC alone. The overall median survival was 156 days and was not different in the two arms of the study. The addition of lintuzumab to salvage induction chemotherapy was safe, but did not result in significant improvement in

response rate or survival in patients with refractory/relapsed AML (Feldman et al. 2005).

46.4.2 CD22 Antibodies as Carrier of Drugs

The CD22 antigen is a viable target for therapeutic intervention for B-cell lymphomas. Several therapeutic anti-CD22 antibodies and an anti-CD22-based immunotoxin (HA22) are currently under investigation in clinical research. Coupling of anti-CD22 reagents with a nano-drug delivery vehicle is projected to significantly improve treatment efficacies. A mutant of the targeting segment of HA22 (a CD22 scFv), mut-HA22 conjugated to the surface of sonicated liposomes to generate immunoliposomes (mut-HA22-liposomes) may serve as promising carriers for targeted drug delivery to treat patients suffering from B-cell lymphoma (Loomis et al. 2010).

46.4.3 Immunogenic Peptides

Identification of immunogenic peptides for the generation of cytotoxic T lymphocytes (CTLs) may lead to the development of novel cellular therapies to treat disease relapse in AML patients. Bae et al. (2004) identified immunogeneic HLA-A2.1-specific CD33(65–73) peptide (AIISGDSPV) that was capable of inducing CTLs targeted to AML cells. The CD33-CTLs displayed HLA-A2.1-restricted cytotoxicity against both mononuclear cells from AML patients and the AML cell line. The peptide- was specific to CD33-CTLs that secreted IFN-γ in response to CD33(65–73) peptide stimulation. Alteration of native CD33(65–73) peptide at first amino acid residue from alanine (A) to tyrosine (Y) (YIISGDSPV) enhanced the HLA-A2.1 affinity/stability of the peptide and induced CTLs with increased cytotoxicity against AML cells. These results demonstrate the potential application of immunogenic HLA-A2.1-specific CD33 peptides in developing a cellular immunotherapy for treatment of AML patients (Bae et al. 2004). Bakker et al. (2004) selected a single chain Fv fragment that broadly reacted with AML samples and with myeloid cell lineages within peripheral blood. Expression cloning identified an antigen recognized as C-type lectin-like molecule-1 (CLL-1), and a transmembrane glycoprotein. CLL-1 showed variable expression in $CD34^+$ cells in chronic myelogenous leukemia and myelodysplastic syndrome but was absent in 12 of 13 cases of acute lymphoblastic leukemia. The AML reactivity combined with restricted expression on normal cells suggests CLL-1 as a novel potential target for AML treatment.

46.4.4 Blocking of CD33 Responses by SOCS3

CD33-related Siglecs 5–11 are inhibitory receptors that contain a membrane proximal ITIM, which can recruit SHP-1/2. The suppressor of cytokine signaling (SOCS) proteins, particularly SOCS1, are essential for regulating the inflammatory process. Gene-targeting studies revealed that SOCS play a nonredundant role in limiting the inflammatory response. SOCS expression is induced by cytokines, infective pathogen-associated molecular patterns, and other stimuli. They regulate cytokine signal transduction via a negative feedback loop. They are characterized by a phosphotyrosine binding SH2 domain and the SOCS box motif (Elliott and Johnston 2004; Krebs and Hilton 2001). SOCS3 binds phosphorylated ITIM of Siglec 7 and targets it for proteasomal-mediated degradation, suggesting that Siglec 7 is a SOCS target. SOCS3 can interact with a number of phosphorylated receptors and appears to potently inhibit JAKs in the presence of these receptors (Fujimoto and Naka 2003). Following ligation, the ECS E3 ligase is recruited by SOCS3 to target Siglec 7 for proteasomal degradation, and SOCS3 expression is decreased concomitantly. In addition, SOCS3 expression blocks Siglec 7-mediated inhibition of cytokine-induced proliferation. This may be a mechanism by which the inflammatory response is potentiated during infection (Orr et al. 2007a, b).

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