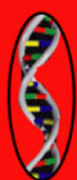




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HANDBOOK OF MOLECULAR CHAPERONES

Roles, Structures and Mechanisms

Piero Durante
Leandro Colucci
Editors

NOVA

CELL BIOLOGY RESEARCH PROGRESS SERIES

HANDBOOK OF MOLECULAR CHAPERONES: ROLES, STRUCTURES AND MECHANISMS

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CELL BIOLOGY RESEARCH PROGRESS SERIES

**HANDBOOK OF MOLECULAR
CHAPERONES: ROLES, STRUCTURES
AND MECHANISMS**

**PIERO DURANTE
AND
LEANDRO COLUCCI
EDITORS**

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Preface

Molecular chaperones are a ubiquitous class of proteins that play important roles in protein folding and in the protection of cells from several stresses associated with the disruption of three native dimensional structures of proteins. The most important of these proteins are the so-called heat shock proteins (HSPs), also known as stress proteins. This book examines some of the biological aspects of this intriguing family of proteins that are important for consideration of the "proteomics of HSPs." This book also reviews current research on protein folding in the endoplasmic reticulum (ER) and the functions of ER-resident molecular chaperones in protein folding in the ER. The biochemical, structural and functional information on Redox Enzyme Maturation Proteins (REMPs) are also reviewed in detail. Furthermore, recent progress in molecular biology has provided new insights into the molecular basis of diseases and molecular targets for diagnosis and therapy of human diseases. The role of molecular biology research in molecular imaging is examined, as well as the applications of molecular imaging in diagnostics, gene therapy and drug development. Other chapters in this book explore the role of protists as promising objects for the study of adaptive mechanisms at the biochemical and the molecular level, the different trends in the evolution of molecular adaptations to adverse environmental conditions, and a review of the molecular mechanisms of bicyclol in the protection against liver damage.

Chapter I - An important function of the endoplasmic reticulum (ER) is the folding of newly synthesized polypeptide chains. Several general and specialized chaperones operate in the ER to assist protein folding. The HSP70 class chaperones BiP and GRP170, their DnaJ and GrpE co-chaperones, the HSP90 class chaperone GRP94, and the lectin chaperones calnexin, calmeglin, and calreticulin are the major general chaperones of the ER. HSP47 is an example of a specialized chaperone required for collagen folding. Protein foldases such as protein disulfide isomerases and *cis-trans* peptidyl prolyl isomerase catalyze two otherwise rate-limiting steps in protein folding, formation and isomerization of disulfide bonds and the *cis-trans* isomerization of peptidyl-prolyl bonds. Chaperone-assisted folding of the polypeptide chain, disulfide bond formation and posttranslational modification of the polypeptide chain, most notably by addition of large oligosaccharides to exposed asparagine residues by oligosaccharyl transferase interact in the ER in a complex manner to produce properly folded proteins. In addition to functioning in protein folding chaperones participate in other vital functions of the ER, such as import of polypeptide chains by translocation,

quality control of protein folding status and targeting of permanently unfolded proteins for retrograde translocation and proteasomal degradation, and regulation of the activity of ER Ca^{2+} channels. In this chapter I review our current knowledge of protein folding in the ER and of the functions of ER-resident molecular chaperones in protein folding in the ER.

Chapter II - Common to many bacteria is the ability to establish a symbiotic relationship or to evade innate immune responses of an animal, plant, fish or insect host. Most often this capacity is mediated by a type III secretion system (T3SS). The function of these complex molecular machines is likened to a syringe-needle injection device that is dedicated to the translocation of effector proteins directly into target eukaryotic cells. Each translocated effector tends to possess a distinct enzymatic activity that aids in subverting host cell signaling for the benefit of the bacterium. Their translocation requires another class of secreted protein – the translocator – which form pores in the target eukaryotic cell plasma membrane through which the effectors may transit to gain entry into the cell interior. Most often, each secreted substrate requires a dedicated small, non-secreted cytoplasmic chaperone for their efficient secretion. Unlike traditional molecular chaperones, these specialized type III chaperones do not assist in protein folding and are not energized by ATP. Controversy still surrounds their primary role; as bodyguards to prevent premature aggregation or as pilots to direct substrate secretion through the correct T3SS. The later is supported by recent evidence that these chaperones can dock directly to the cytoplasmic face of the T3S machinery, possibly serving as a recognition motif for substrate secretion. Added to this functional complexity is their important contribution to system regulation, which can ultimately confer temporal order to substrate secretion. Moreover, some chaperones display a bewildering propensity to interact with several additional T3S-associated proteins – the relevance of which remains uncertain. Structural data has now appeared for several important type III chaperones, either alone or in complex with their cognate substrate. This is proving a fillip in our attempts to understand the mercurial ways in which these versatile proteins operate in nature. It is hoped that this article will provide information on type III chaperone function, as well as highlighting key recent advances in the field. May it also be a testament to the value of continued intense effort in unravelling the mysteries of type III chaperone biology.

Chapter III - Chaperones are a large group of unrelated protein families that stabilize unfolded proteins, unfold them for translocation across membranes or degradation, and assist in their correct folding and assembly. They represent one of the most ancient and evolutionarily conserved protective protein families found in nature. A fundamental group of molecular chaperones is the so-called heat shock proteins (HSPs), also known as stress proteins. Originally discovered as inducible molecules capable of maintaining cellular homeostasis against abrupt temperature changes, HSPs were later considered an adaptive physiological response that protects against a variety of different cellular proteotoxic stresses. Early in the study of these proteins, it was evident that these molecules also have physiological roles that facilitate the synthesis, folding, assembly, trafficking, and secretion of specific proteins in various cellular compartments in the absence of significant pathological processes. In summary, these proteins guard the cellular proteome against misfolding and inappropriate aggregation.

From a clinical point of view, modification of the chaperone proteome, mainly the induction of HSPs, has been observed in a wide spectrum of inflammatory and degenerative diseases, including cancer, infectious disease, autoimmune processes, neurodegenerative conditions, and prion disease. The involvement of HSPs in these diverse diseases highlights the importance of the chaperone machinery not only in cell biology, but also in pathophysiology. At the same time, the induction of HSPs in diseases suggests potential clinical applications for molecular chaperones, particularly HSPs, in the diagnosis, prognosis and, above all, therapy of different degenerative and inflammatory human diseases. On this basis, proteomic approaches represent a valuable method to study the roles, structural interrelationships, and intimate molecular mechanisms of the major chaperone families that have been insufficiently characterized, limiting their diagnostic and therapeutic potential.

Chapter IV - A group of bacterial system specific chaperones are involved with the maturation pathway of redox enzymes that utilize the twin-arginine protein translocation (Tat) system. These chaperones are referred collectively as REMPs (Redox Enzyme Maturation Protein). They are proteins involved in the assembly of a complex redox enzyme which itself does not constitute part of the final holoenzyme. These proteins have been implicated in coordinating the folding, cofactor insertion, subunit assembly, protease protection and targeting of these complex enzymes to their sites of physiological function. The substrates of REMPs include respiratory enzymes such as N- and S-oxide oxidoreductases, nitrate reductases, and formate dehydrogenases, which contain at least one of a range of redox-active cofactors including molybdopterin (MoPt), iron sulfur [Fe-S] clusters, and b- and c-type haems. REMPS from *Escherichia coli* include TorD, DmsD, NarJ/W, NapD, FdhD/E, HyaE, HybE and the homologue YcdY. The biochemical, structural and functional information on these REMPs are reviewed in detail here.

Chapter V - Recent progress in molecular biology has provided new insights into the molecular basis of diseases and molecular targets for diagnosis and therapy of human diseases. Molecular imaging is a research discipline aimed at development and testing of novel tools, reagents and methods to image specific molecular pathways *in vivo* that are key targets in disease process and appear much earlier than anatomical and physiological changes. The advancement in the field of imaging and therapy of diseases is mainly due to the vast information available from molecular biology research on new targets with specific ligands and methods to evaluate their application in *in vitro* and *in vivo* systems. Improvement in imaging modalities like single photon emission computed tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging (MRI), computed tomography (CT) and optical imaging (OI) has also contributed in the progress of molecular imaging. First section of this chapter is focused on molecular imaging, different approaches adopted for development of molecular imaging agents and recent imaging modalities and their applications.

Molecular biological techniques used in *in-vitro* diagnostics are being adapted to the special requirements of imaging diagnostics and high affinity imaging is achieved based upon receptor-ligand, antigen-antibody, transporter-substrate and enzyme-substrate interactions. Development of newer approaches based on reporter gene concept are solely dependent on molecular biology research tools. Small animal models of human diseases have become available after completion of human genome project. Noninvasive imaging of molecular,

genetic and cellular processes in animal models complements established *ex vivo* molecular biological assays and imaging provides a new dimension to understanding of various diseases. Role of molecular biology research in molecular imaging is discussed in second section of this chapter. Development of imaging agents based on peptides and role of molecular biology methods in identification of their targets to development of labelled ligands and their evaluation in *in-vitro* and *in-vivo* systems is also discussed.

Chapter concludes with the applications of molecular imaging in diagnostics, gene therapy and drug development. Advancements in biology and medicine is possible due to synergism between various new disciplines, especially molecular biology research has contributed significantly towards progress of molecular imaging.

Chapter VI - Three groups of proteins associated with misfolded protein depending aggregates were identified in *Saccharomyces cerevisiae* cells by using a new approach: comparative analysis of crude lysate pellets of isogenic yeast strains differing by their prion composition or adenine biosynthesis pathway characteristics. 2D electrophoresis followed by MALDI analysis of a recipient [*psi*⁻] strain and of [*PSI*⁺] cytoductant permitted identification of 53 proteins whose aggregation state depended on prion content or red pigment accumulation in yeast cells. Further studies allowed identifying an overlapping group of 38 proteins whose aggregation state responded to a shift of prion(s) content and also a rather similar group of more than 40 proteins whose aggregation state depended on accumulation of red pigment. In all these cases nearly one half of the identified proteins belonged to a functional group of chaperones and enzymes involved in glucose metabolism. Notable were proteins involved in oxidative stress response and in translation. The prion dependent group also contained a proteinase. These results are comparable with recent literature data on various misfolded proteins containing aggregates in yeast cells. Being not dependent on cloned heterologous genes, our approach permits a conclusion about universal presence of glucose metabolism enzymes in such aggregates. Most of the identified proteins, although behaving like prions in some experiments (for example, being “transmittable” by cytoduction), seem to be just amyloid-associated and mobilized to pellets in response to presence of prion fibrils. Our model experiments demonstrate that red pigment binds insulin fibrils and blocks their interaction with Thioflavine T. This allows concluding that red pigment impedes mobilization of some prion-associated proteins to prion-containing aggregates and so makes them to appear as pigment depending ones. Also there are some proteins (e.g. Sod1p and Cus1p) that themselves can be “clients” of a hypothetical prion generation pathway dealing with not NQ-rich proteins in yeast.

Chapter VII - The divergent loop cyclophilin, cyclophilin 40 (CyP40), initially discovered in association with the estrogen receptor, is now recognized as an immunophilin cochaperone common to all steroid receptors. This unique peptidylprolyl isomerase, the first tetratricopeptide repeat (TPR) cyclophilin to be identified, contains a C-terminal TPR domain through which it shares structural identity with FKBP52 and other partner cochaperones in steroid receptor-Hsp90 complexes. By dynamically competing for Hsp90 interaction, the cochaperones allow the receptors to establish distinct Hsp90-chaperone complexes, with the potential to exert tissue-specific control over receptor activity. CyP40 regulates Hsp90 ATPase activity during receptor-Hsp90 assembly. Functional deletion of the yeast CyP40 homologue, Cpr7, adversely affected glucocorticoid receptor and estrogen receptor α activity

that could be fully restored, either with wild type Cpr7 or Cpr7 with a cyclophilin domain lacking isomerase activity. We draw parallels with the mechanism already established for FKBP52 and propose that the CyP40 divergent loop, within the N-terminal cyclophilin domain, interfaces with a contact surface on the steroid receptor ligand-binding domain to achieve an optimal orientation for receptor activity. The cyclophilin domain also mediates association with dynein, suggesting a role for CyP40 in nuclear translocation of steroid receptor-Hsp90 complexes from the cytoplasm, as proposed for other immunophilin cochaperones. CyP40 chaperone function has been mapped to a central linker region separating the cyclophilin and TPR domains. Although not essential for viability, CyP40 homologues are important for normal cell growth and mitosis in yeast and in progression of vegetative growth in plants. These may be linked to CyP40 client proteins other than steroid receptors, including diverse kinases and transcription factors. The recent development of CyP40 knockout mouse models provides an attractive opportunity to address fundamental questions regarding the physiological role of CyP40 in mammals.

Chapter VIII - Heat shock proteins (HSP) are closely involved in response of organisms to adverse natural and anthropogenic factors. Within one of the recent directions of HSP studies, attempts are made to uncover mechanisms underlying the organisms' adaptation to various stresses. Promising model objects for this research are protists – lower eukaryotes that are at the same time a cell and a fully fledged organism.

In this paper, we present some results of our studies on HSP70 level in intact cells of several free-living protists and on the characteristics of its dynamics in the cells in response to the changes in natural environmental factors, salinity and temperature. The protists chosen for the study, the amoebae and the ciliates, possess an essentially different organization of the cell and belong to the most phylogenetically distant groups.

In many cases, a high constitutive level of HSP70 was recorded in intact cells under normal (non-stressful) conditions. It may be considered as a universal pre-adaptation of these protists to possible drastic environmental changes.

The strains of *Amoeba proteus* and several related species were very similar as to the level of HSP70 and the position of the stained zone on the blots, despite differences in geographic provenance, temperature conditions in natural habitats the strain was isolated from, and the strain age. Species of other genera of freshwater lobose amoebae studied, though close to *Amoeba*, differed considerably in the HSP70 level.

Out of the seven strains of the facultative parasites *Acanthamoeba*, only two had a noticeable constitutive level of HSP70. Differences in the constitutive level of HSP70 in the cells of different *acanthamoebae* strains may reflect their potential pathogenicity.

The ciliates used in the study represented various ecological groups different as to their attitude to environmental salinity. They were shown to employ various strategies of the chaperone system response to increasing and decreasing salinity of the medium. Constitutive level of HSP70 in their cells correlated with the degree of the salinity tolerance.

Chapter IX - The endoplasmic reticulum is an essential cellular compartment with many diverse functions. It is the main Ca^{2+} storage site and is involved in maintaining Ca^{2+} homeostasis. The endoplasmic reticulum is the first compartment of the secretory pathways, making it the entry compartment for approximately one-third of all the proteins synthesized by the eukaryotic cell. The endoplasmic reticulum contains unique enzymes, maintaining an

oxidative environment that allows co- and post-translational modifications such as glycosylation and disulfide bond formation, as well as molecular chaperones that assist in protein folding and quality control of newly synthesized membrane and secretory proteins. Protein folding in endoplasmic reticulum is controlled by endoplasmic reticulum quality control mechanisms. The primary players of endoplasmic reticulum quality control are the molecular chaperones like calnexin and calreticulin that reside in the endoplasmic reticulum. The mechanism of endoplasmic reticulum quality control assures that only correctly folded, functional proteins will exit the endoplasmic reticulum whereas non-native, misfolded proteins will be degraded via endoplasmic reticulum-associated degradation. ERAD pathway is closely connected to unfolded protein response pathway that is involved in its induction and regulation.

Chapter X - HspB8 is one of the recently described members of a large family of human small heat shock proteins. This family is presented by ten members that are characterized by a rather small molecular mass (16–28 kDa) and by the presence of a short (80-100 residues) α -crystallin domain. HspB8 is widely expressed in different human tissues and its content is especially high in muscles and nerves. Expression of HspB8 is dependent on many factors and is induced under certain unfavorable conditions. HspB8 tends to form small homooligomers and seems to interact with other members of the family of small heat shock proteins forming different heterooligomers. HspB8 is phosphorylated by a number of different protein kinases and phosphorylation might affect its structure and chaperone-like activity. HspB8 prevents aggregation of partially denatured proteins both *in vitro* and *in vivo*. The detailed mechanism of chaperone-like activity of HspB8 in the cell remains unclear, however the data of literature indicate that HspB8 is able to directly interact with denatured proteins, activates elimination of denatured proteins by proteasomes, activates autophagy interacting with Bag3 or regulates phosphorylation of α -subunit of the translation initiation factor eIF2. HspB8 interacts with glycolytic enzymes, amyloid proteins, different heat shock proteins, protein kinases, RNA-binding protein SAM68 and biomembranes. The molecular basis underlying interaction of HspB8 with so many diverse substrates remains unknown; however it is supposed that having an intrinsically disordered structure HspB8 can adopt different conformations suitable for interaction with different ligands. Interacting with so many substrates HspB8 seems to be involved in regulation of apoptosis, cell differentiation and proliferation and protects the cell from accumulation of denatured and aggregated proteins. Therefore point mutations of HspB8 correlate with development of certain neurodegenerative diseases and the level of HspB8 expression might affect cardiac hypertrophy and carcinogenesis.

Chapter XI - Small stress or heat shock proteins (sHSP) are involved in protective activity against some of the most important pathologies affecting human health including cancer and neurodegeneration. The fundamental molecular mechanism for the function(s) of small stress proteins (sHSPs or small heat shock proteins) are just beginning to be understood. The archetype for small stress proteins is human alphaB crystallin where multiple interactive sequences were identified using protein pin arrays and confirmed using site directed mutagenesis. A dynamic equilibrium between subunits of alphaB crystallin and with large polydisperse complexes has been described. The interactive domains were mapped to a homology model of the surface of the small stress proteins. The surface exposure of the

interactive domains varies with unfolding and self assembling proteins. Peptides were synthesized on the basis of the interactive sequences and were found to be active in assays for protection against proteins associated with protein unfolding diseases. The equilibrium between sHSP subunits and assembled complexes may be a dynamic mechanism for regulation of stress protein function. Characterization of the functional mechanism of small stress protein action is expected to lead to novel therapies for diseases of aging.

Chapter XII - The chaperonin containing TCP-1 (CCT) is found in the cytosol of all eukaryotic cells. It is an oligomer formed from two back to back rings, each containing eight subunits that surround a central cavity. Each CCT subunit contains three domains: an equatorial domain containing an ATP binding site, a substrate binding domain which displays the least sequence similarity between other CCT subunits and a linker domain. The folding cycle of CCT is ATP driven and the subunits of each chaperonin ring hydrolyse ATP in a sequential manner. For actin and tubulin it has been demonstrated that these proteins initially bind to CCT in an open conformation. Following the nucleotide cycle of CCT they become compact whilst remaining bound to one or more chaperonin subunits. This is in contrast to the mechanism of the prokaryotic chaperonins for which it is thought that folding substrates are encapsulated within the chaperonin cavity without direct interactions with chaperonin subunits.

The sequence diversity of the CCT substrate binding domains provides a complex binding interface for potential substrates. The major folding substrates of CCT are the abundant cytoskeletal proteins actin and tubulin, whilst other less abundant proteins such as the cell cycle regulating proteins Cdc20 and Cdh1 are known to require interactions with CCT to reach their native state. The numbers of CCT substrate proteins and the way in which CCT binds to its substrates is a matter for debate. At present it is estimated that in yeast up to 300 proteins may bind to CCT but it is not yet known how many proteins are obligate substrates. There are two major theories regarding how CCT recognises its folding substrates. The first proposes that CCT is a relatively general chaperone, recognising hydrophobic binding determinants, whilst the second, at least in the case of actin, proposes that binding to CCT is sequence specific.

The mechanisms of CCT action and the diversity of potential CCT substrates will be discussed in relation to the activity of CCT having far reaching implications on the many cellular functions that depend on the activity of its folding substrates.

Chapter XIII - Plant growth is greatly affected by abiotic stresses, such as drought, high salinity and cold. Therefore, plants need to have defending systems against many stresses for survival. Molecular chaperone interacts with unfolded or partially folded protein subunits, e.g. nascent chains emerging from the ribosome, leading to stabilizing native proteins, preventing aggregation of denatured proteins and degradation of defective or improperly folded proteins. Many molecular chaperones are stress proteins and most of them, but not all, have been identified as heat shock proteins (HSPs). HSPs/molecular chaperones and the heat stress elements in their promoters are conserved in all the eukaryotes, suggesting that they essentially have same function in response to high temperature stress. Participation of HSPs/molecular chaperones in other stresses has been also reported in plants. Furthermore, other types of proteins with chaperone functions, such as protein disulfide isomerase (PDI) and calnexin/calreticulin are up-

regulated by the stress. They play a role for preventing aggregation by assisting refolding of nonnative proteins. It has been shown that expression of HSPs/molecular chaperones increases for assisting the deposition/assembly of high abundant secretory storage proteins in endoplasmic reticulum (ER) lumen during seed development. HSPs/molecular chaperones in plants are not well understood, however, they are elucidated little by little.

In this review, we first describe the characteristics of HSPs/molecular chaperones in plants. Then we describe the roles of HSPs/molecular chaperones in abiotic stress (heat and cold etc.) and ER stress during seed development, including enhanced chaperones accompanying by deposition of recombinant products in transgenic plants.

Chapter XIV - Intrinsically disordered proteins (IDP) are unfolded/unstructured under native conditions. They play important roles in living organisms, mostly in signal transduction and regulation of biochemical pathways. Biochemical studies showed that some IDPs also have protective effect on partner molecules, or enhance folding of a specific protein, i.e. they have chaperone activity. In our studies we have analyzed the chaperone activity of six IDPs and found that all of them are active in a variety of assays employed. The studies were carried out with two plant stress proteins from the dehydrin family (ERD10 and ERD14, *A. thaliana*), and four *E. coli* ribosomal proteins (L15, L16, L18, L19). We have tested these proteins in both the prevention of substrate aggregation and deactivation, and in the refolding/reactivation of a denatured partner molecule. We found that ERD10 and ERD14 hinder aggregation and also deactivation, however they do not assist refolding. The ribosomal proteins significantly enhance the refolding and reactivation of a denatured protein and also prevent the deactivation of substrate molecules under denaturing conditions. A combination of their chaperone activities and molecular properties (tolerance against mutation, resistance against aggregation, etc.) elevate these IDPs to a unique level of functionality and stability, due to which they play an essential role in the survival of strong environmental stress conditions and probably also in sudden changes in protein stability caused by a mutation for example.

Chapter XV - Fluorescence spectroscopy of tryptophan residues and the 8-anilino-1-naphthalenesulfonate probe and light scattering were used to study some properties of bovine α -crystallin, in particular, its thermo- and photo-induced aggregation. The effective diameter of the native α -crystallin globule, calculated from the polarization and life-time of 8-anilino-1-naphthalenesulfonate (ANS) using the Levshin-Perrin equation, amounts to 90 Å and increases during aggregation to at least 140 Å. The decrease in the tryptophan fluorescence intensity in the course of α -crystallin thermo- and photo-denaturation and aggregation is caused by local conformational alterations in the environment of the tryptophan residues and by light scattering. Tryptophan residues in the aggregates are hidden in the interior. Thermal aggregation of the protein takes place not only at high temperatures. Extrapolation of the experimental time dependence of slow spontaneous aggregation to the long time range allows one to find the “denaturation time” t_e . The t_e value for α -crystallin (at a concentration of 0.8 mg/ml in phosphate buffer at pH 8.4) is about 100 h. Using steady-state, polarized, and phase-modulation fluorometry, the DTT-induced denaturation of insulin and formation of its complex with α -crystallin in solution were studied. Prevention of the aggregation of insulin by α -crystallin is due to formation of a chaperone complex, i.e. tight interaction of chains of the denatured insulin with α -crystallin. The conformational changes in α -crystallin that occur

during complex formation are rather small. It is unlikely that N-termini are directly involved in the complex formation. It has been shown that ANS is not sensitive to the complex formation. ANS emits mainly from α -crystallin monomers, dimers, and tetramers, but not from oligomers or aggregates. The possibility of highly sensitive detection of aggregates by light scattering using a spectrofluorometer with crossed monochromators is demonstrated.

Chapter XVI - Bicyclol is a new potent anti-hepatitis drug and has been approved to treat viral hepatitis in China since 2004. Pharmacologically, bicyclol protects against drug and chemical-induced liver injury, and inhibits hepatitis virus replication in duck viral hepatitis and in a HepG2.2.15 cell line. Bicyclol exerts most of its effects by eliminating free radicals, maintenance of mitochondrial glutathione redox status, anti-inflammation and anti-apoptosis. However, further elucidation of the molecular mechanism of bicyclol in protection against liver damage is still needed.

Heat shock proteins (HSPs) are a family of constitutive and inducible expressed gene products that collectively function to maintain cellular protein conformation during stressful conditions. HSPs can be induced during acute or chronic stress as a result of protein misfolding, aggregation or disruption of regulatory complexes. Prior induction of HSPs protects cells from subsequent lethal insults. The up-regulation of HSPs expression constitutes an important cellular defense mechanism. Therefore, it would be of great therapeutic benefit to discover compounds that are clinically safe and able to enhance the expression of HSPs.

Since bicyclol has cytoprotective action against liver injury, a question arises whether the effect of bicyclol on liver injury is mediated through induction of hepatic HSPs. Our study demonstrated that bicyclol significantly induces the hepatic HSP27 and HSP70, which play a key role in protection against liver injury.

Oral administration of bicyclol alone significantly induced hepatic HSP27 and HSP70 expression both in protein and mRNA levels in a time- and dose-dependent manner through activation of heat shock factor-1 (HSF1) in mice, but no inducing effect on HSP27 and HSP70 in mouse spleen and kidney was observed. In *in vitro* study of HepG2 cells, bicyclol can enhance HSP27 and HSP70 promoter activities, indicating that bicyclol induces transcription of the HSPs genes.

The hepatoprotective effect of HSP27 and HSP70 induced by bicyclol was confirmed in acetaminophen and concanavalin A (ConA) induced liver injury in mice. HSP27 and HSP70 suppressed the elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), liver tissue necrosis, the release of cytochrome c and apoptosis inducing factor (AIF) from mitochondria as well as hepatic DNA fragmentation caused by acetaminophen and ConA. Moreover, overexpression of HSP27 and HSP70 by bicyclol attenuated NF- κ B activation induced by ConA either by inhibiting I κ B degradation or by directly suppressing the NF- κ B transcriptional activity, and also inhibited D-galactosamine-induced activation of JNK signal transduction pathway. These results suggest that the protective action of bicyclol against liver injury is mediated by its induction of HSP27 and HSP70, which provide new evidence for elucidating the mechanism of cytoprotective effect of bicyclol against liver injury both in animals and patients.

Chapter XVII - Similarly to all other organisms plants are constantly exposed to different environmental abiotic and biotic factors. Some of these agents can exert strongly negative

influence upon crop growth. Heat shock protein synthesis appears to be one of the major anti-stress responses conserved among all known organisms on our planet -from bacteria, plants and animals to human, which has unique nuance in plants. Studying and understanding of this defensive mechanism, despite the difficulties in finding appropriate model systems in plants, have great importance for the detection of markers against different kind of stresses and their practical application in genetically transferred crops.

This review summarizes and comments the main part of the researches for different heat shock protein families that can act as molecular chaperones in maintaining the cellular homeostasis. This paper demonstrates the significant role of these proteins in normal and stress conditions in plants, which are discussed in parallel. Classification of heat shock protein families and subfamilies and regulation of heat shock protein expression are presented, and there is information about different kinds of stress inducers in plants. This review explains the understanding for the interaction between different heat shock proteins in one general molecular chaperone network. Additionally, the article discusses thermotolerance and the main participants in this acclimation process.

Chapter XVIII - Heat shock proteins (Hsps) provide cellular and whole body adaptation of animals to various adverse environmental conditions due to their diverse chaperone properties. Hsp70 is apparently the major player underlying biological adaptation in all organisms studied so far.

Therefore, in our experiments to analyze the patterns of heat shock system firing in different Diptera families we predominantly concentrated on the *hsp70* gene family expression at the transcription and translation levels. We have investigated several species in three families: Drosophilidae, Stratiomyidae and Chironomidae belonging to three suborders (Cyclorrhapha, Orthorrhapha and Nematocera).

All *Drosophila* species studied so far did not reveal any level of Hsps under normal physiological conditions but responded to heat shock (HS) by rapid synthesis of all Hsps. On the contrary, four Stratiomyidae species studied are characterized by extraordinary high concentration of Hsp70 in cells (phenomenon especially pronounced at the larval stage) under normal physiological conditions and hardly detectable in control but inducible synthesis of correspondent RNAs. Such a pattern, which implies high stability of Hsps in species belonging to this family, is probably responsible for wide range of Stratiomyidae species in various habitats, including highly aggressive ones. Preliminary studies of Chironomidae inhabiting cold running waters (larvae of two species in the subfamily Diamesinae) indicate that representatives of this subfamily exhibit high constitutive level of *hsp70* RNA in cells under normal conditions and seem to lose the ability to respond to heat shock treatment.

The data accumulated suggest different trends in the evolution of molecular adaptations to adverse environmental conditions occurring in the same insect order.

Chapter XIX - Heat shock proteins (Hsp) are considered cellular protective agents against a wide range of stressors; inducible expression of Hsp prevents misfolded proteins from aggregation, interfere with the cell death program and help cells recover and survive.

In normal resting cells Hsp are involved in various “house keeping” missions in the cell, form complexes with client proteins and act as molecular chaperones. This includes protein folding, assembly and translocation between compartments, degradation of misfolded or

aggregated proteins, maintenance of the cell cycle, interaction with steroid hormone receptors and signal transduction pathway for steroid hormone).

Over the last few years we have been focusing on various stress stimuli in homeothermic livestock, namely, environmental, nutritional and psychological. Our findings led us to hypothesize that upon stress, the Hsp response may be bi-directional regarding the type of stimulus and the tissue tested. While in some cases Hsp levels increase, in other instances it goes the other way round. In those situations (nutritional stress) where Hsp levels decrease, a concomitant induction of other genes, related to the function of the specific tissue has occurred. This is in marked difference from the classical Hsp response, in which, in parallel with the induction of Hsp the expression of other proteins is silenced.

Chapter XX - The chaperonin containing T-complex polypeptide (CCT) is the major cytosolic chaperonin in eukaryotes and has been estimated to interact with up to 15% of all cellular proteins. The CCT holoenzyme is a hexadecameric molecule comprised of two copies each of eight discrete subunits, each of which shares partial homology to the others, as they are thought to have evolved from a single common ancestor gene. CCT has principally been implicated in the folding of cytoskeletal proteins such as tubulin and actin, and has been noted as an important factor in a variety of cellular processes, including cell proliferation, embryogenesis, ciliary biogenesis, etc.

The majority of studies on CCT function have thus far focused on the 16-mer holoenzyme; however, an increasing body of evidence suggests that the individual subunits of CCT may have independent function and significance. This evidence derives from several different types of observations: the effects of naturally occurring (or experimentally-induced) mutations in individual CCT subunits, the differential patterns of expression and localization displayed by various CCT subunits, and direct experimental demonstration of individual CCT subunit physiology in a capacity without the CCT aggregate enzyme.

Multiple spontaneous and induced mutations in CCT subunits have been identified that lead to discrete phenotypes: in CCT-epsilon (causing mutilating sensory neuropathy with spastic paraplegia in humans), in CCT-delta (causing the mutilated foot phenotype in rats), and in CCT-gamma (leading to the *no tectal neuron* phenotype in zebrafish, interfering with eye development). Investigations on expression of CCT subunits have demonstrated that not all subunits are coordinately expressed, and that some subunits localize within cells as monomers or as microcomplexes much smaller than the 16-mer CCT enzyme. In some instances a specific biological activity has been imputed to a single CCT subunit. The CCT-eta has been found to be a biological partner for the soluble guanylyl cyclase, affording it a role within nitric oxide signaling, and CCT-alpha has been shown to preferentially inhibit the polyglutamine-mediated toxicity of the huntingtin protein. This seemingly disparate collection of observations together suggest that individual CCT subunits may have particular physiological roles apart from the CCT holoenzyme.

Chapter XXI - Hsp26 from *Saccharomyces cerevisiae* is represented by a 24-mer oligomer with the overall organization resembling that of a hollow globular sphere, which possesses co-chaperone activity. It functions in the recovery of misfolded proteins and prevent aggregation, but its *in vivo* role in protein homeostasis remains unclear. sHsps from some organisms are known to be posttranslational regulated by phosphorylation, where those covalent modifications regulate function and quaternary structure. Global analysis studies of

yeast phosphoproteome identified phosphorylated peptide sequences in Hsp26. To study the *in vivo* phosphorylation of yeast Hsp26, the gene encoding Hsp26 was overexpressed from a multicopy plasmid using its own promoter. Hsp26 was purified from stationary phase cells to homogeneity by a procedure already described in our lab. The purification method used consisting of three steps: ethanol precipitation, sucrose gradient ultracentrifugation, and heat inactivation of residual contaminants produced native Hsp26 protein. The purified Hsp26 was shown to be phosphorylated in its serine-peptides. Hsp26 was resolved in four isoforms, displaying the same molecular masses but different isoelectric points. A MALDI/MS analysis of the isoforms led to the identification of a phosphopeptide 37-QLANT(**p**)PAK-44 at the N-terminus of Hsp26. If the isoforms represent multiple phosphorylated forms of Hsp26 is an open question.

Chapter I

Molecular Chaperones of the Endoplasmic Reticulum

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Abstract

An important function of the endoplasmic reticulum (ER) is the folding of newly synthesized polypeptide chains. Several general and specialized chaperones operate in the ER to assist protein folding. The HSP70 class chaperones BiP and GRP170, their DnaJ and GrpE co-chaperones, the HSP90 class chaperone GRP94, and the lectin chaperones calnexin, calmeglin, and calreticulin are the major general chaperones of the ER. HSP47 is an example of a specialized chaperone required for collagen folding. Protein foldases such as protein disulfide isomerases and *cis-trans* peptidyl prolyl isomerase catalyze two otherwise rate-limiting steps in protein folding, formation and isomerization of disulfide bonds and the *cis-trans* isomerization of peptidyl-prolyl bonds. Chaperone-assisted folding of the polypeptide chain, disulfide bond formation and posttranslational modification of the polypeptide chain, most notably by addition of large oligosaccharides to exposed asparagine residues by oligosaccharyl transferase interact in the ER in a complex manner to produce properly folded proteins. In addition to functioning in protein folding chaperones participate in other vital functions of the ER, such as import of polypeptide chains by translocation, quality control of protein folding status and targeting of permanently unfolded proteins for retrograde translocation and proteasomal degradation, and regulation of the activity of ER Ca^{2+} channels. In this chapter I review our current knowledge of protein folding in the ER and of the functions of ER-resident molecular chaperones in protein folding in the ER.

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Introduction

Molecular chaperones are a ubiquitous class of proteins that play important roles in protein folding and protection of cells from several stresses associated with disruption of the native three dimensional structures of proteins [1-3]. Protein folding is governed by the hydrophobic effect resulting in burial of hydrophobic amino acid side chains in the center of the protein [4-6]. Exposure of these hydrophobic stretches at the surface of a protein is highly toxic to cells as it leads to aggregation of proteins within a cell, aberrant protein-protein interactions that inactivate essential components of the cellular machinery, and disruption of phospholipid bilayers [7]. Molecular chaperones detect these hydrophobic surface areas and shield them from interaction with other proteins and biological membranes [8-10]. Constitutive expression of molecular chaperones in the two major protein folding compartments of an eukaryotic cell, the cytosol and the endoplasmic reticulum (ER), reflects their function in folding of newly synthesized polypeptide chains and translocation of polypeptide chains across lipid bilayers. Many molecular chaperones have been identified as proteins induced upon heat stress, a condition leading to protein unfolding, and have originally been classified as heat shock proteins (HSPs) [11]. Several classes of molecular chaperones or HSPs have been distinguished based on the molecular masses of their founding members, for example small HSPs (sHSPs, IbpA/IbpB in *Escherichia coli*), HSP60/HSP10 (GroEL/GroES in *E. coli*), HSP70/HSP40 (DnaK/DnaJ in *E. coli*), HSP90 (HtpG in *E. coli*), and HSP100/CLP (ClpB in *E. coli*). Heat shock also induces expression of several proteases which by degrading unfolded proteins protect cells from accumulation of unfolded proteins. Induction of chaperones and proteases by heat shock has been termed the heat shock response. Other stresses, such as oxidative stress or viral infections associated with immense production of viral proteins, also cause a heat shock response [12, 13]. The heat shock response is ubiquitous and affects all organelles of an eukaryotic cell. However, organelle-specific heat shock responses, such as a mitochondrial heat shock or unfolded protein response (UPR), or the UPR of the ER, selectively induce chaperones and proteases of these organelles.

The ER is a major protein folding compartment in eukaryotic cells second only to the cytosol. Its folding clients are secretory proteins, transmembrane proteins of the plasma membrane, and proteins residing in the secretory pathway. Not surprisingly, the ER harbors many chaperone classes that are also found in the cytosol, for example the HSP70 chaperones heavy chain binding protein (BiP) [14]/glucose-regulated protein of 78 kDa (GRP78) [15-17]/karyogamy 2 protein (Kar2p) [18], luminal HSP seventy 1 protein (Lhs1p) [19]/chaperone in the ER 1 protein (Cer1p) [20]/Ssi1p [21]/oxygen-regulated protein of 150 kDa (ORP150) [22-24]/GRP170 [25-28], the HSP90 chaperone GRP94 [15, 16, 29]/adenotin [30, 31]/endoplasmic reticulum protein of 96 kDa (gp96) [33, 34]/ER protein of 99 kDa (ERp99) [35]/HSP108 [36]/Ca²⁺-binding protein 4 [37]/protein kinase of 80 kDa [38], and the HSP40 chaperones ER DnaJ 1 (ERdj1)/murine DnaJ-like protein 1 (MTJ1) [39, 40], ERdj2/secretory 63 protein (Sec63p) [41-44], ERdj3 [45, 46]/human ER associated DnaJ (HEDJ) [47]/*Saccharomyces cerevisiae* DnaJ 1 protein (Scj1p) [48, 49], ERdj4 [50]/microvascular

endothelial differentiation gene 1 (MDG1) [51-53], ERdj5/Erj5p/JPDI [54-57], DnaJ-like protein of the ER membrane (Jem1p) [58], and p58^{IPK}/DnaJc3 [59, 60]. sHSPs or HSP60s of the ER are currently unknown. Two major differences between protein folding in the ER and the cytosol exist. First, many proteins targeted to the ER undergo several post-translational modifications, of which *N*-linked glycosylation plays important roles in protein folding in the ER. Therefore, the ER contains specialized lectin chaperones – calnexin [61]/p88 [62, 63], calreticulin [64, 65]/Ca²⁺-binding protein of 63 kDa (CAB-63) [66]/calregulin [67]/Ca²⁺-regulated protein of 55 kDa (CRP55) [65, 68]/high affinity Ca²⁺-binding protein (HACBP) [69]/Ca²⁺-binding protein 3 (CaBP3) [70], and the testis-specific lectin chaperone calnexin [71-73]/calnexin-t [74, 75] – that recognize the *N*-linked glycosylation status of proteins folding in the ER. Second, disulfide formation between two cysteine thiol groups occurs in the ER, a protein folding reaction catalyzed by a large family of protein disulfide isomerases (PDIs, Figure 1) [76, 77]. PDIs and *cis-trans* peptidyl prolyl isomerases (PPIs), a ubiquitous family of proteins whose members catalyze the *cis-trans* isomerization of peptidyl-prolyl bonds (Figure 2), are often called protein foldases. Conformational, chaperone-assisted, and oxidative folding intersect [78], which is reflected by several PDIs possessing chaperone activity. These ER luminal chaperones and foldases also play important roles in cellular processes closely associated with protein folding: Translocation of newly synthesized polypeptide chains into the ER [79, 80], recognition of unfolded polypeptide chains for retrograde translocation out of the ER and degradation by the proteasome in a process called ER associated protein degradation (ERAD) [81-83], and activation of signaling pathways termed the UPR that coordinate protein folding demand and capacity of the ER (Figure 3) [84, 85]. Here I will review how molecular chaperones of the ER assist protein folding and detect unfolded proteins in the ER.

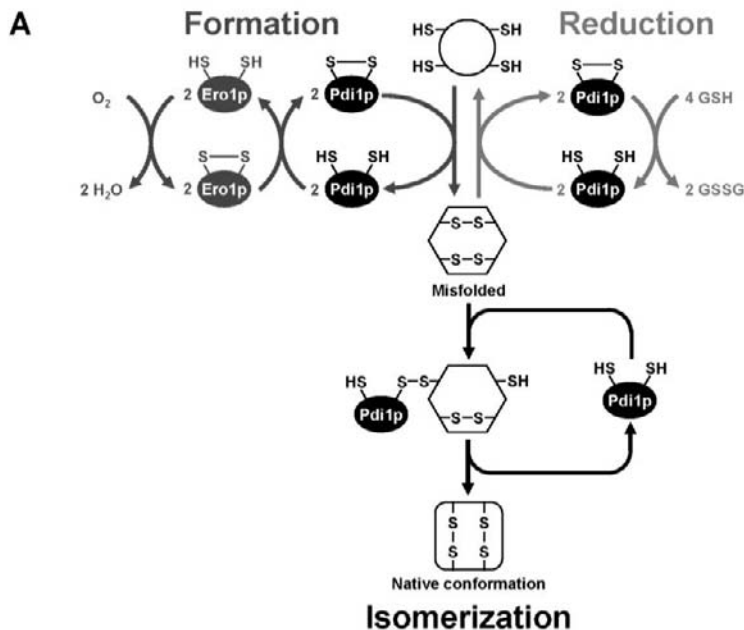


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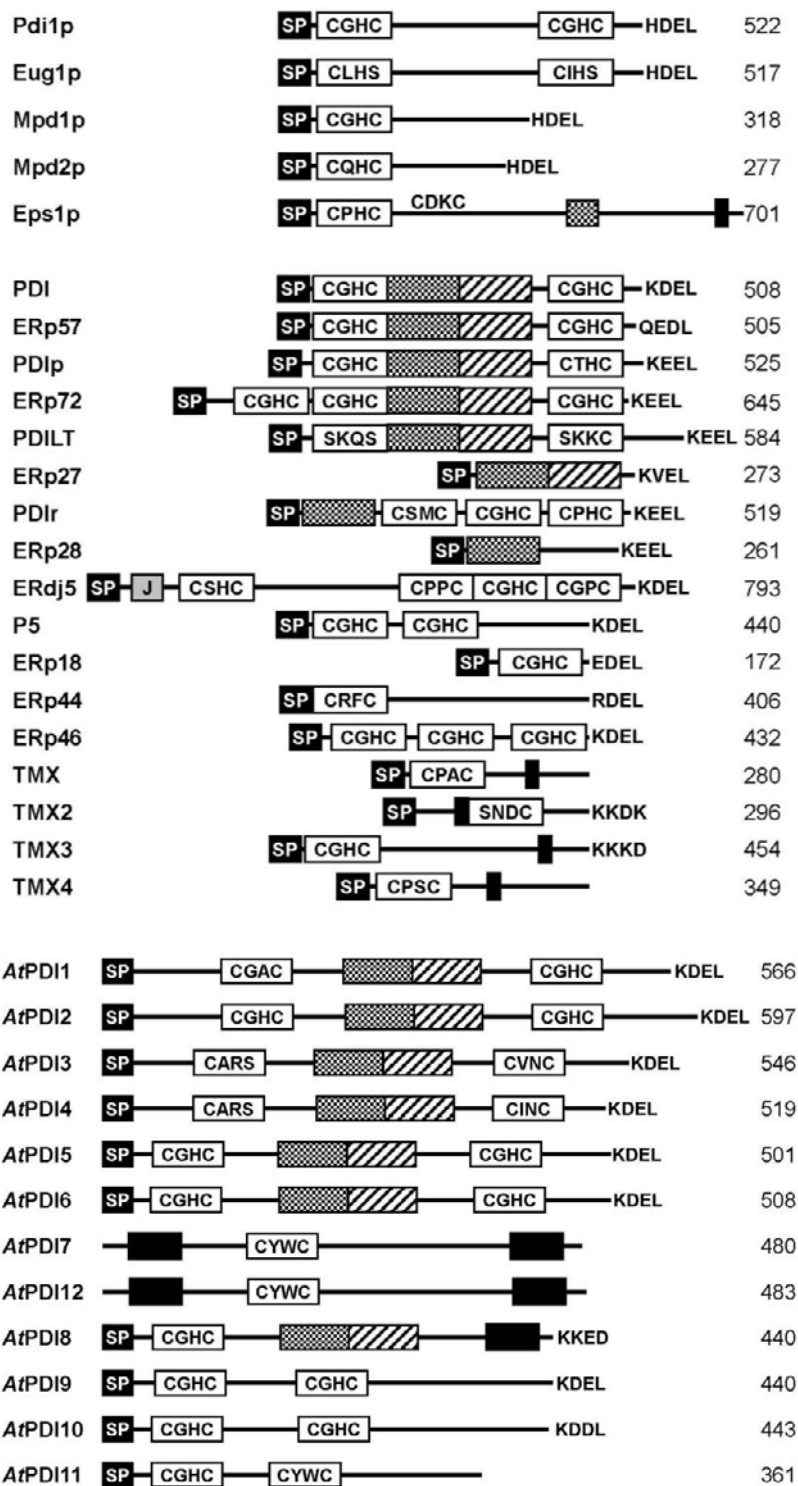
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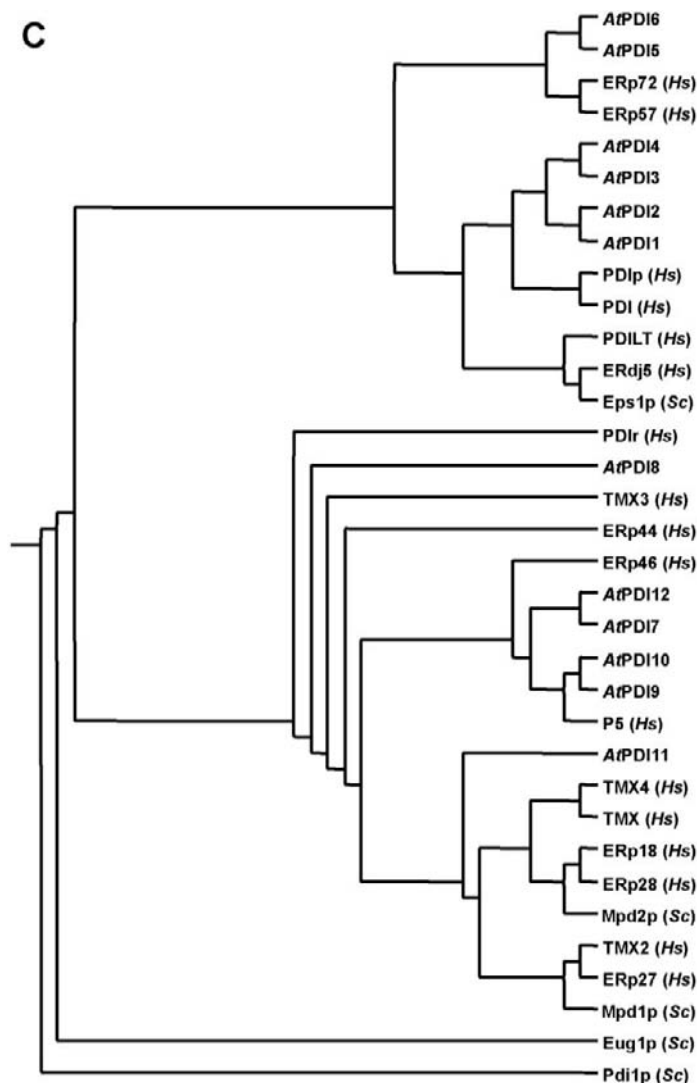


Figure 1. Oxidative protein folding in the ER. (A) Disulfide bond formation and isomerization reactions catalyzed by prototypic PDI. (B) PDI families in *Saccharomyces cerevisiae* (top), *Homo sapiens* (middle), and *Arabidopsis thaliana* (bottom). Thioredoxin-like domains are indicated by white or hatched rectangles. Catalytic centers are indicated in single letter amino acid code in the a and a' domains. The b and b' domains are indicated by hatched rectangles. Black rectangles represent transmembrane domains, and black rectangles with the lettering 'SP' signal peptides. ER retrieval sequences are indicated at the C-terminus in single letter amino acid code. Numbers represent the lengths of the proproteins. (C) Rooted, phylogenetic tree of *A. thaliana* (At), *H. sapiens* (Hs), and *S. cerevisiae* (Sc) PDIs generated with PHYLIP 3.68. The GenBank accession numbers are: AtPDI1 (CAB41088), AtPDI2 (BAB09837), AtPDI3 (AAG51554), AtPDI4 (BAB02677), AtPDI5 (AAD41430), AtPDI6 (NM_106400), AtPDI7 (Q9T042), AtPDI8 (AAK62431), AtPDI9 (AAB91984), AtPDI10 (AAF40463), AtPDI11 (NP_001078074), AtPDI12 (NP_566664), Eps1p (P40557), ERdj5 (Q8IXB1), ERp18 (O95881), ERp27 (Q96DN0), ERp28 (P30040), ERp44 (Q9BS26), ERp46 (Q8NBS9), ERp57 (P30101), ERp72 (P13667), Eug1p (P32474), Mpd1p (Q12404), Mpd2p (Q99316), P5 (Q15084), PDI (P07237), Pdi1p (P17967), PDILT (Q8N807), PDIp (Q13087), PDIr (Q14554), TMX (Q9H3N1), TMX2 (Q9Y320), and TMX3 (Q96JJ7). *A. thaliana* PDIs are labeled according to [86].

Physicochemical Principles Governing Protein Folding

As for any other chemical reaction protein folding reactions have to be energetically favorable under biochemical conditions, i.e. characterized by a negative free Gibbs enthalpy, and have to be reasonably fast to be productive in the life time of a cell. Thermodynamic and kinetic implications on protein folding in a cellular environment are summarized in the following paragraphs.

Thermodynamics of Protein Folding

Each conformation of a given polypeptide chain is characterized by a certain free Gibbs enthalpy. In this way the primary structure of a protein determines the protein's folding landscape in which the free enthalpy is plotted against each conformation, characterized by the fractions of native and non-native interactions of its amino acid side chains [5]. In an aqueous environment the Gibbs free energy of the water-protein system is largely determined by the number of hydrophobic amino acid residues exposed at the surface of the protein [87, 88]. The more hydrophobic residues are exposed at the surface, the higher the Gibbs free energy of the water-protein system. This effect – also known as the hydrophobic effect - is largely an entropic effect, because minimization of the surface between hydrophobic molecules and the bulk water phase liberates water molecules from structured 'cages' formed at this surface to maximize hydrogen bonding between water molecules close to the surface. With increasing temperature the entropic consequences of the hydrophobic effect become less pronounced, because water molecules become more mobile as hydrogen bonds between water molecules are broken. The cellular consequence of elevated temperature is the heat shock response caused by entropic destabilization of protein structure. Exposure of polar groups at the surface, on the other hand, results in a much less pronounced decrease in the Gibbs free energy of the system, because the energetic and entropic differences between interactions of a water molecule with other water molecules or hydrophilic amino acid side chains are small. To be energetically favorable, protein folding hides as many hydrophobic amino acids in the center of the protein as possible, thereby decreasing the surface between hydrophobic amino acid side chains and the surrounding water and, in turn, the Gibbs free energy of the water-protein system by increasing its entropy. On an energy landscape a polypeptide chain folds along pathways leading to ever decreasing free energies until the conformation with the lowest free energy is reached. Theoretical calculations support the idea that this conformation is the native conformation for most proteins [89, 90]. These biophysical considerations on how the thermodynamics of the water-protein system energetically govern protein folding are summarized by Anfinsen's dogma, stating that the information for the native conformation of a protein is encoded by its primary structure [91].

Kinetics of Protein Folding

Thermodynamic considerations do not inform on the kinetics of protein folding. Levinthal's paradox states that even for a protein consisting of only 100 amino acids too many conformations are possible if a random sampling mechanism probing each conformation for only 10^{-11} s is considered to allow the protein to fold in the life time of the universe [92-94]. A solution to Levinthal's paradox is constriction of the folding choice of the unfolded protein by assuming that proteins fold along discrete pathways very much as small molecule reactions proceed along a few pathways. However, the complex nature of proteins and the similar, but not identical, physiochemical properties of their constituents, make such an easy picture unlikely. On the energy landscape discussed above a hydrophobic collapse initiates protein folding, especially in larger proteins. By progressing toward conformations with decreasing free energies sufficient conformational constraints are retained to solve Levinthal's paradox. In the hydrophobic collapse hydrophobic side chains are buried in the center of the protein shielding them from surrounding water [6]. An important feature of the hydrophobic collapse is burial of charged and hydrophilic amino acids in the hydrophobic core of the initial structure formed by the hydrophobic collapse. These charged and hydrophilic residues provide energy signatures to individual conformations and influence the directionality of further folding of this intermediate. Without formation of salt bridges, disulfide bonds, or hydrogen bonds in the hydrophobic core energetic differences between individual conformations would be small and a thermodynamic driving force and directionality of further folding would not exist [6].

Secondary structure elements in proteins fold very fast. For example, β -turns or α -helices are formed within 0.1-10 μ s [95]. Structurally simple and small proteins fold in less than 50 μ s [96-98], whereas more complex structures, e.g. β -sheets, fold more slowly [99]. Modules or domains of larger proteins fold independently of each other into near native structures [100]. In a final cooperative folding event water is excluded from the protein core and the native structure formed [101]. Translation of mRNAs and proceeds at $\sim$ 2-8 amino acid residues per second [102-107] which is slow compared to formation of secondary structure elements. To form secondary and tertiary structural elements in which residues far apart in the amino acid sequence interact, e.g. β -sheets or disulfide bonds, the preceding residues must be maintained in a folding competent state until the interacting partners are added to the polypeptide chain. The high protein concentration *in vivo* exacerbates this problem. The protein concentration in the ER reaches $\sim$ 100 g/l ($\sim$ 2 mM). Assembly of IgG heavy and light chains present in the ER of a plasma cell at concentrations of $\sim$ 4-6 μ M can, in principle, be a diffusion-controlled reaction [6]. The hydrophobic effect is not affected by structural differences in different polypeptide chains and does not distinguish between interactions of hydrophobic amino acid side chains with other hydrophobic amino acid side chains or lipids. As a consequence, aggregation triggered by aberrant hydrophobic interactions between two polypeptide chains and disruption of phospholipid bilayers by unfolded proteins is a significant problem for all cells. To solve these problems cells invented molecular chaperones, classes of proteins that interact with unfolded proteins and shield unfolded proteins from interaction with hydrophobic interaction partners, i.e. other proteins and phospholipid bilayers (Figure 4).

Protein aggregates or partially folded folding intermediates are extremely toxic to cells. Several mechanisms of protein aggregate toxicity are recognized. Protein aggregates may interact with other proteins whose biological function is to interact with other proteins. For example, huntingtin protein interacts with the transcriptional co-activator CBP through glutamine-rich stretches present in both proteins, leading to downregulation of transcription of genes controlled by cAMP response elements (CREs) [7, 108-113]. Sequestration and inactivation of other transcription factors by polyglutamine stretches has also been reported for other polyglutamine diseases, such as spinocerebellar ataxias 1 and 3, dentatorubral pallidoluysian atrophy, and Kennedy disease [7, 114-116]. A second class of proteins affected in this way are molecular chaperones and proteins of the protein folding quality control machinery, for example the proteasome. HSP70 chaperones are associated with huntingtin [117-119], Lewy bodies formed by aggregated α -synuclein in Parkinson's disease [7, 120], and intracellular amyloid β (A β) aggregates [121]. Protein aggregate toxicity may be mediated by sequestration and inactivation of molecular chaperones. Depletion of molecular chaperones in turn impairs folding of essential, metastable proteins [122]. Consistent with this view is that overexpression of chaperones mitigates protein aggregate toxicity [7, 123]. In addition, one would expect that anterograde and retrograde protein translocation mechanisms at the ER and the mitochondria are affected, because of the involvement of cytosolic chaperones in these processes. Second, proteasomal function is impaired by Lewy bodies and α -synuclein protofibrils [124, 125], in Alzheimer's disease by A β and phosphorylated tau [126-129], Parkinson's disease [130], and by polyglutamine proteins [131-134], which can be at least in part attributed to direct inhibition of proteasome activity by aggregated proteins. Inhibition of the proteasome abolishes ERAD. Likewise, depletion of cytosolic chaperones may interfere with retrotranslocation of unfolded proteins out of the ER and extraction of stalled, translocating polypeptide chains out of the translocation channel in a process called pre-emptive quality control [135], again inhibiting ERAD and causing ER stress.

The ER

Protein folding in the ER is more complex than in the cytosol mainly because the ER is a separate metabolic compartment surrounded by a phospholipid bilayer [136]. Newly synthesized proteins are transported into the ER by translocation across a phospholipid bilayer and co- and posttranslationally modified by addition of oligosaccharides to asparagine residues in consensus Asn-X-Ser/Thr (X = any amino acid except proline) motifs and by oxidation of cysteine thiol groups to form disulfide bonds. Disulfide bond formation increases the structural rigidity of extracellular proteins and increases their half-life, whereas glycosylation significantly increases the hydrophilicity of proteins. These modifications affect the conformational folding pathway and folding choices of the polypeptide backbone of the protein.

Topology

The luminal space of the ER is topologically equivalent to the extracellular space. A hydrophobic signal sequence targets proteins to the ER membrane for co-translational crossing of the ER membrane through the Sec61p complex [137-140]. Posttranslational entry into the ER, once the complete polypeptide chain has been synthesized, is an alternative mechanism to enter the ER. Signal peptidase co-translationally cleaves off the signal peptide. Bacterial signal peptidases process their substrates after translation of ~80% of the polypeptide chain [141, 142], suggesting that the signal peptide is present during initial folding steps of the protein. Indeed, the signal sequence influences the timing of *N*-linked glycosylation and signal sequence cleavage [143]. Inefficient cleavage can result in prolonged interaction of the protein with ER chaperones [144].

Chemical Composition

The pH in the ER is near neutral [145] and comparable to the cytosol. In mammalian cells the ER or its specialized counterpart, the sarcoplasmic reticulum (SR) in muscle cells, is the major site for Ca^{2+} storage. ER luminal free Ca^{2+} concentrations reach 1-5 mM, compared to 0.1 μM in the cytosol [146, 147]. ER luminal Ca^{2+} concentrations rapidly and frequently fluctuate as the ER Ca^{2+} pool is mobilized during intracellular signaling (Figure 5) [148]. Ca^{2+} release and entry channels in the ER membrane control the ER luminal Ca^{2+} concentration. Ca^{2+} release channels are the ryanodine receptors (RyR) [149-152] and the inositol 1,4,5-trisphosphate receptors (IP₃R) [153-155]. IP₃R and RyRs can coexist in the same cell [156]. Three isoforms (RyR1, RyR2, and RyR3) [157-161] and several splice variants of the RyR are found in many tissues. RyRs function as homotetramers [162], and show highest expression in striated muscle [150, 158, 163-171]. In skeletal muscle RyR1 is the predominant isoform in mammals [167, 172-174], in cardiac muscle RyR2 is predominant. Excitation-contraction coupling through interaction with voltage-dependent Ca^{2+} channels in the t-tubule membrane, i.e. the dihydropyridine receptors (DHPRs) [175-178], activate RyR in the SR. Depolarization of the t-tubule membrane induces a conformational change in the DHPR that activates RyRs stimulating RyR-mediated Ca^{2+} release from the SR [150]. IP₃R also exist in three isoforms with one to two alternative splice variants per isoform and also form heterotetramers [179]. Binding of the second messenger inositol 1,4,5-trisphosphate, which is generated at the plasma membrane, opens the IP₃R to release Ca^{2+} from the ER. The action of the Ca^{2+} release channels is countered by ATP-dependent Ca^{2+} pumps in the ER membrane, the sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA) 1-3 [180-182]. SERCA pumps mediate most of Ca^{2+} influx into the ER. ATP hydrolysis is coupled to Ca^{2+} transport by these pumps because SERCA pumps transport Ca^{2+} against a Ca^{2+} gradient from the cytosol into the ER [180-182]. SERCAs can operate in reverse mode in which they synthesize ATP and contribute to Ca^{2+} leakage from the ER [183]. Three *SERCA* genes are found in humans, *ATP2A1* encoding SERCA1, *ATP2A2* encoding SERCA2, and *ATP2A3* encoding SERCA3 [181]. All genes possess several splice variants with characteristic tissue-specific expression patterns [181]. SERCA2b and SERCA3

are nearly ubiquitously expressed [181]. Binding of calreticulin [184] or calnexin [185] to a 12 amino acid luminal extension tail specific to SERCA2b inhibits SERCA2b when the ER luminal Ca^{2+} store is full [186]. In addition to Ca^{2+} channels and pumps in the ER membrane Ca^{2+} -binding proteins of the ER increase the capacity of the ER luminal Ca^{2+} store allowing to store up to 20 mM Ca^{2+} [187, 188] while maintaining a free Ca^{2+} concentration of 1-5 mM [189]. The most abundant Ca^{2+} -binding protein in the SR is calsequestrin [187], followed by the 165 kDa histidine-rich Ca^{2+} -binding protein (HRC) [187, 190, 191] and sarcalumenin [187, 191, 192]. Calsequestrin has ~50 Ca^{2+} -binding sites and the K_d for Ca^{2+} is ~1 mM [193-195]. Calsequestrin is anchored via the transmembrane proteins triadin [196-199] and junctin [200, 201] to the RyR. At low (< 10 mM) Ca^{2+} concentrations calsequestrin inhibits the RyR [187, 202].

Ca^{2+} homeostasis of the ER is important for protein folding in the ER because many chaperones of the ER are Ca^{2+} binding proteins. In fact, many ER luminal chaperones have first been identified as Ca^{2+} -binding proteins [37, 68, 70, 203, 204]. Further, the activity of several chaperones is regulated by alterations in Ca^{2+} concentration. Depletion of Ca^{2+} dissociated BiP from T-cell antigen receptor α -chain [205]. Lectin chaperones bind to oligosaccharides at high Ca^{2+} concentrations, but not at Ca^{2+} concentrations representing a depleted ER Ca^{2+} store (~100 μM) [206]. These examples suggest that depletion of ER luminal Ca^{2+} inactivates ER chaperones and causes accumulation of unfolded proteins in the ER. This conclusion is supported by strong induction of the UPR by the irreversible SERCA inhibitor thapsigargin [207-210]. Ca^{2+} dissociates oligomeric calnexin [204], whereas low Ca^{2+} concentrations promote interaction of calreticulin with PDI [211, 212]. Likewise, interaction of calreticulin with ERp57 is affected by changes in the Ca^{2+} concentration [211, 213]. It is likely that fluctuations in ER luminal Ca^{2+} concentration also affect protein-protein interactions in other chaperone complexes [214-218] and regulate interaction of chaperones with SERCA pumps and Ca^{2+} release channels. The physiological consequences of regulation of chaperone interactions by Ca^{2+} remain to be elucidated. Ca^{2+} also participates in protein folding reactions. Ca^{2+} can engage in formation of salt bridges in proteins affecting protein folding in a protein specific manner [215, 219]. The folding of several proteins, for example apo- α -lactalbumin, requires Ca^{2+} [220].

Redox Buffering of the ER

Two redox buffer systems exist in the ER, the reduced/oxidized glutathione redox pair and the redox pairs of reduced and oxidized nicotinamide dinucleotide (NADH/NAD^+) and $\text{NADPH}/\text{NADP}^+$. These redox pairs are separate from their counterparts in the cytosol, and can exist in a different redox status. For example, the ratio of reduced (GSH) to oxidized (GSSG) glutathione is 30:1 to 100:1 in the cytosol, whereas in the ER this ratio is 1:1 to 3:1 [221] making this redox pair more oxidized in the ER. Generation of one molecule H_2O_2 per formed disulfide bond [222] may contribute to the oxidizing character of the ER [223]. Fenton decay of H_2O_2 at cytochromes and other metal ion-containing proteins of the ER membrane generates the highly oxidizing and toxic hydroxyl radical [224, 225]. Thioredoxin reductase [226] and glutathione reductase [227] appear to be absent from the ER, indicating

that the glutathione and nicotinamide dinucleotide redox pairs are uncoupled in the ER. Net reduction of cortisone to cortisol by 11 β -hydroxysteroid dehydrogenase 1 (11 β HSD1), which catalyzes a reversible reaction, indicates that the redox potential of the NAD(P)H/NAD(P)⁺ redox pairs in the ER is reducing. Only the GSH/GSSG redox pair is linked to oxidative protein folding. The ER membrane is virtually impermeable for GSSG, but possesses saturable transport systems for GSH [228], indicating that maintaining the luminal GSH/GSSG ratio requires additional redox systems. The Foyer-Halliwell-Asada cycle (Figure 6) connects the GSH/GSSG redox pair to the ascorbate/dehydroascorbate redox pair. Ascorbate is synthesized in the ER lumen by a membrane-bound flavoprotein, gulonolactone oxidase, in most animals, but not humans [229, 230]. A byproduct of this reaction is H₂O₂ [231]. The ER membrane is permeable for dehydroascorbate, but not ascorbate [232]. Ingested ascorbate is oxidized by ascorbate oxidases allowing uptake of dehydroascorbate by the ER. Dehydroascorbate is an electron acceptor in oxidative protein folding and accepts electrons from PDI [233] and may also function in maintaining the GSH/GSSG redox buffer of the ER. Ascorbate is also a cofactor for luminal enzymes such as prolyl- and lysyl hydroxylases [234, 235], which play important roles in collagen synthesis. Lipid-soluble electron carriers, especially the tocopherol/tocopheryl radical redox system may relay dehydroascorbate into the ER lumen [236, 237]. The vitamin K redox cycle (Figure 7) participates in γ -carboxylation of glutamate side chains in secretory proteins [238]. Vitamin K epoxide reductase (VKORC1) regenerates vitamin K from vitamin K 2,3 epoxide. VKORC1 contains a thioredoxin-like CXXC center and forms a stable complex with PDI [239], suggesting that VKORC1 accepts electrons from cysteinyl thiols of folding cargo proteins of the ER.

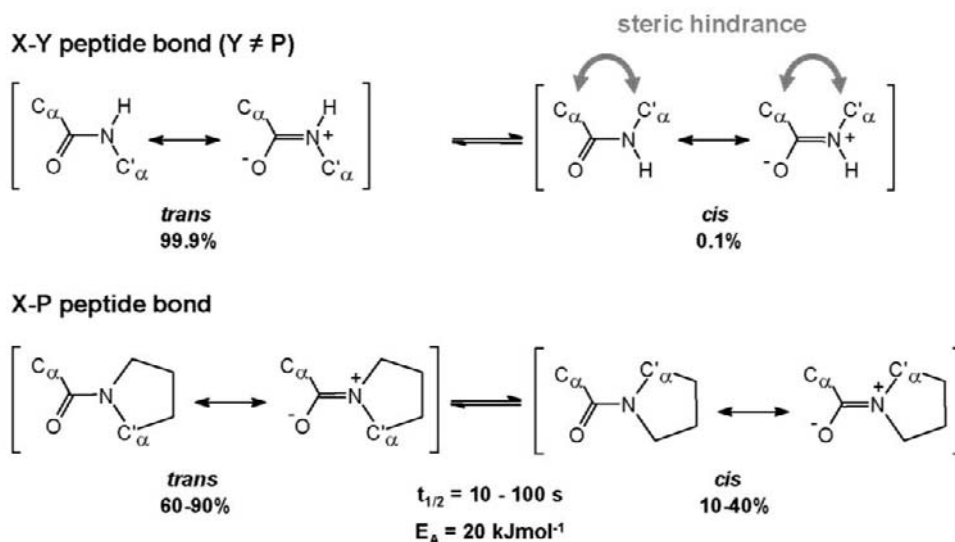


Figure 2. *Cis-trans* peptidyl prolyl bond isomerization. For most peptide bonds the *cis* configuration is disfavored because of steric hindrance between the two C α atoms. If the C-terminal amino acid is proline, this energetic difference between the *cis* and *trans* configurations is largely eliminated. Therefore, peptidyl-prolyl bonds exist both in the *trans* and *cis* configuration. The partially double bonded character of the peptide bond hinders free rotation around the carbonyl C – N bond (E_A – activation energy, $t_{1/2}$ – half life). Catalysis by *cis-trans* peptidyl prolyl isomerases decreases E_A and increases the rate of conversion of the *trans* into the *cis* configuration and *vice versa*.

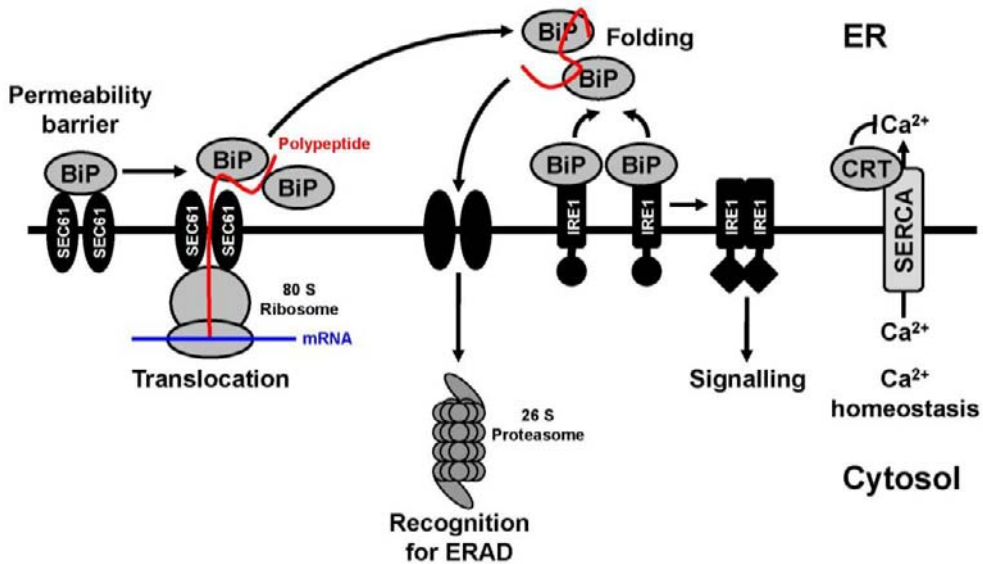


Figure 3. Biological functions of molecular chaperones in the ER.

N-linked glycosylation is initiated by transfer of a core oligosaccharide from a membrane-bound dolichol phosphate anchor to consensus Asn-X-Ser/Thr residues in the polypeptide chain (Figure 8) [240, 241]. Glycosylation serves several purposes in protein folding. First, due to the hydrophilic nature of carbohydrates, glycosylation increases the solubility of glycoproteins and defines the attachment area for the surface of the protein. Second, due to their large hydrated volume oligosaccharides shield the attachment area from surrounding proteins and thus act as a chaperone. Third, oligosaccharides interact with the peptide backbone and stabilize its conformation [242]. Fourth, sequential trimming of sugar residues is monitored by the lectin chaperone machinery to report on the folding status of the protein (Figure 3; [243, 244]) and will be discussed in detail below.

ER Luminal Molecular Chaperones

Two classes of proteins assist protein folding in the ER: molecular chaperones and protein foldases. Foldases catalyze protein folding reactions that otherwise would be too slow to allow for productive protein folding in a cellular environment. Prominent examples for foldases are peptidyl-prolyl *cis-trans* isomerases (Figure 2) and PDIs (Figure 1). Molecular chaperones facilitate protein folding by shielding unfolded hydrophobic regions from surrounding proteins and are further divided in chaperone foldases and chaperone holdases. Chaperone foldases consist of at least two domains, a substrate binding domain (SBD) and a nucleotide binding domain (NBD). They employ ATP hydrolysis cycles to cycle through ATP- and ADP-bound states that in- or decrease their affinities for unfolded substrates by altering the conformation of their SBD [245, 246]. In the high affinity state the unfolded

substrate is bound to the chaperone and conformationally locked because of the interaction of the unfolded domain with the chaperone. The low affinity state terminates the substrate-chaperone interaction (Figure 9). Released substrates kinetically partition between folding to the native state, aggregation, and rebinding to the chaperone (Figure 4). Preferential interaction of the chaperone with the unfolded protein provides directionality to the folding of the substrate. ATP hydrolysis and ADP/ATP exchange provide the energy to drive conformational changes in the SBD. The only clear example for a chaperone foldase with an ATP hydrolysis cycle in the ER is the HSP70 class chaperone BiP. The HSP90 class chaperone GRP94 has been suggested to be a chaperone holdase due to its barely detectable ATPase activity, but elucidation of its three dimensional structure together with clear demonstration of ATPase activity for GRP94 have now shifted the discussion toward GRP94 being a chaperone foldase [247-249]. The lectin chaperones calnexin, calreticulin, and calmeglin may also be considered to be chaperone foldases in which substrate recognition and ATP consumption cycles have been uncoupled (Figure 10). The function of chaperone foldases is tightly regulated by co-chaperones [250-252]. In the case of HSP70 class chaperones HSP40 or DnaJ co-chaperones stimulate the ATPase activity of HSP70 class chaperones and load substrates onto their HSP70 partners [252], whereas GrpE co-chaperones are nucleotide exchange factors for HSP70 class chaperones [251]. For cytosolic HSP70s several DnaJ co-chaperones and nucleotide exchange factors have been well characterized. For their ER luminal counterparts at least six DnaJ co-chaperones (ERdj1/MTJ1 [39, 40], ERdj2/Sec63p [41-44], ERdj3/HEDJ/Scj1p [45-49], ERdj4 [50]/MDG1 [50-53], ERdj5/Erj5p [54, 55], Jem1p [58], and p58^{IPK}/DnaJc3 [59, 60]) and one nucleotide exchange factor for BiP (suppressor of Ire1/Lhs1 synthetic lethality 1 protein (Sil1p)/synthetic lethal with the 7S RNA mutation 1 protein (Sls1p)/BiP-associated protein (BAP) [253-258]) have been identified. Further, the HSP70 chaperone Lhs1p has been shown to function as a nucleotide exchange factor for BiP [259]. In addition, several auxiliary co-chaperones for cytosolic HSP70s have been described for which ER luminal counterparts have not been identified [260-265]. For HSP90 chaperones the functional diversity of co-chaperones is even more complex. HSP90 co-chaperones that stimulate the ATPase activity of HSP90 chaperones, co-chaperones that are PPIs, or that may be involved in targeting proteins for degradation, such as the ubiquitin ligase carboxyl terminus of heat shock-cognate (Hsc) protein of 70 kDa-interacting protein (CHIP), or in bridging the HSP70 and HSP90 chaperone systems have been described [266]. GRP94 co-chaperones remain unknown.

Chaperone holdases do not employ ATP hydrolysis cycles. Examples for chaperone holdases are sHSPs, HSP33, DnaJ proteins, and the chaperone activity of PDI. Chaperone holdases interact with substrate proteins through hydrophobic interactions [267-269] but do not support folding of the substrate to its native state [268, 270-273]. Structural maturation of the substrate may terminate the substrate-holdase interaction [274]. Some holdases provide a chaperone buffer to chaperone foldases and closely cooperate with certain chaperone foldases [271, 275-277]. Under conditions unfavorable for protein folding the substrate is transferred from the foldase to the holdase to which they remain bound until conditions become favorable for folding again. Transfer of the substrate back to the foldase reinitiates folding of the substrate thus preventing futile, ATP-consuming folding attempts under stress conditions. Nevertheless, chaperone holdases may also cycle between a high and low affinity

conformation, in which case the transition is triggered by changes in the redox status of the cell or other environmental factors such as temperature [274]. In the following chapters the molecular function of the major ER luminal chaperones will be discussed in the context of the biochemical features of the ER. Because of the more advanced state of research into cytosolic chaperones information on the function and regulation of these chaperones will be incorporated into these discussions to highlight potential gaps in knowledge and mechanistic understanding of chaperone-mediated protein folding in the ER.

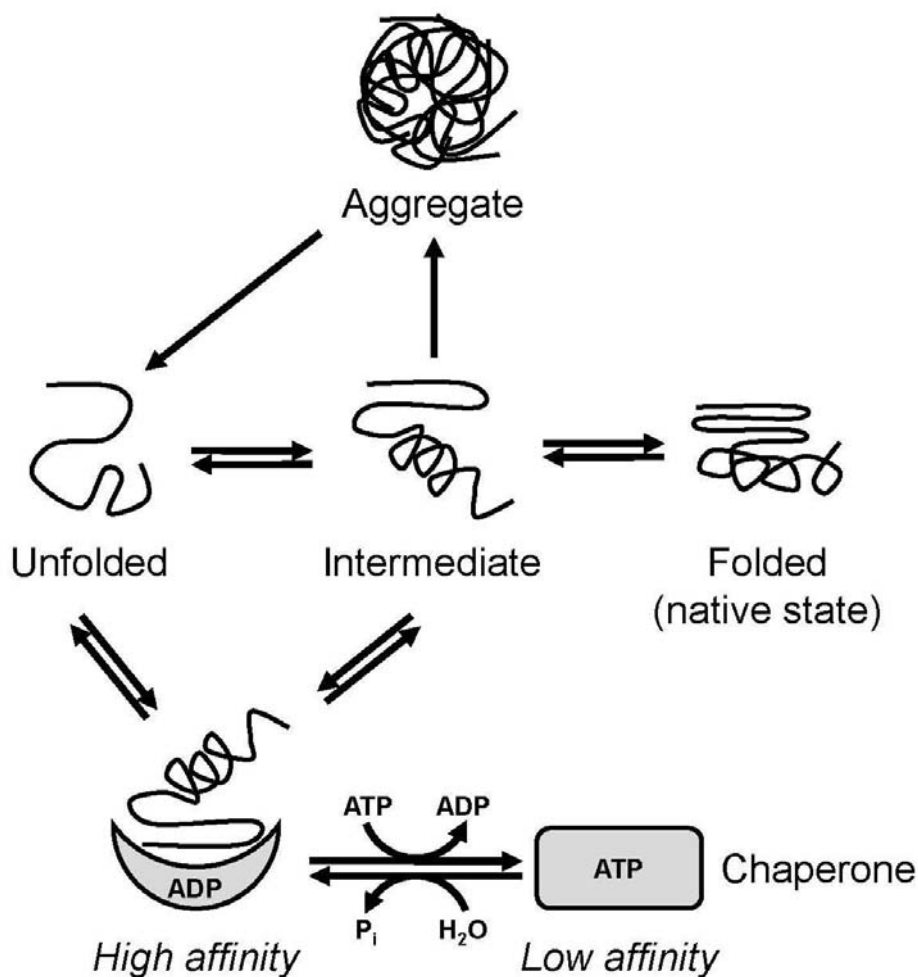


Figure 4. Competition between folding, chaperone interaction and aggregation of an unfolded protein. An unfolded protein folds through several intermediates into its native state. Aggregation kinetically competes with productive folding, because protein folding is governed by the hydrophobic effect. Interaction with chaperones stabilizes the unfolded conformation and kinetically partitions the unfolded protein toward productive folding. Chaperones recognize hydrophobic surface patches on unfolded proteins and, therefore, efficiently compete with aggregation. Chaperones exist in a high and low affinity state for the substrate. In HSP70 chaperone foldases the high affinity state is terminated by ATP for ADP exchange, resulting in release of the substrate from the chaperone. This allows the unfolded substrate, which is conformationally locked when bound to the chaperone, to continue on its folding pathway.

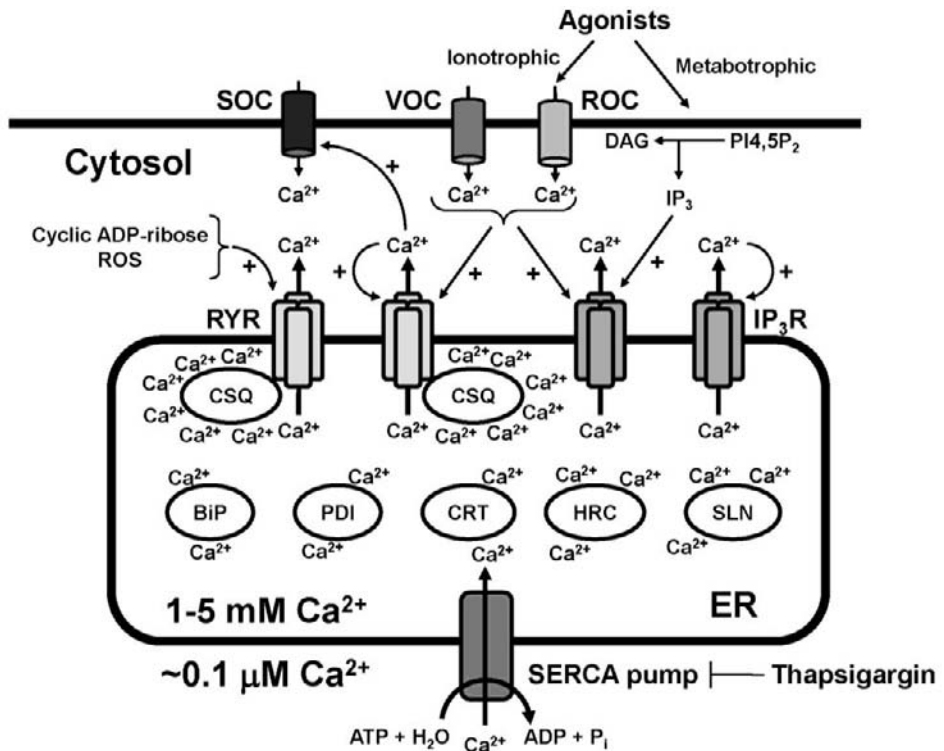


Figure 5. ER Ca^{2+} homeostasis. Abbreviations: CRT – calreticulin, CSQ – calsequestrin, DAG – diacylglycerol, HRC – 165 kDa histidine-rich Ca^{2+} -binding protein, IP_3 – inositol 1,4,5-trisphosphate, PI4,5P_2 – phosphatidylinositol 4,5-bisphosphate, ROC – receptor-operated channel, ROS – reactive oxygen species, SLN – sarcalumenin, SOC – store-operated channel, VOC – voltage-operated channel.

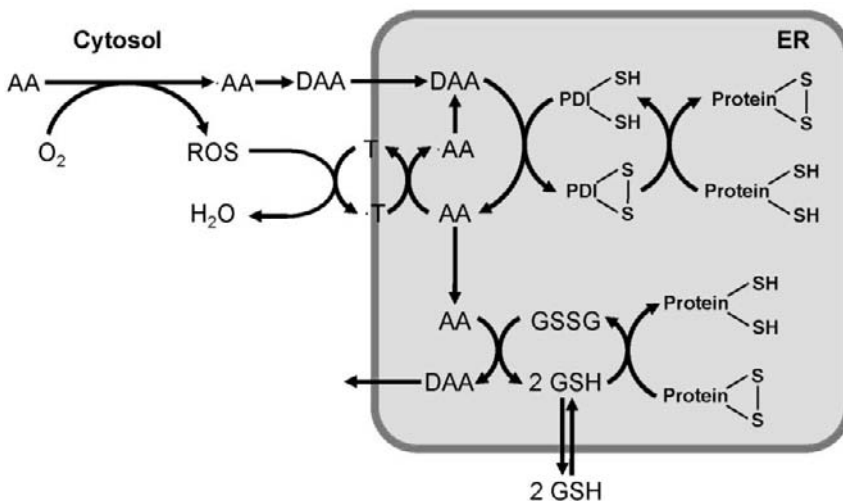


Figure 6. Foyer-Halliwell-Asada cycles in the ER contribute to disulfide bond formation and may contribute to maintaining the redox potential of the GSH/GSSG redox pair in the ER because the ER membrane is nearly impermeable for GSSG.

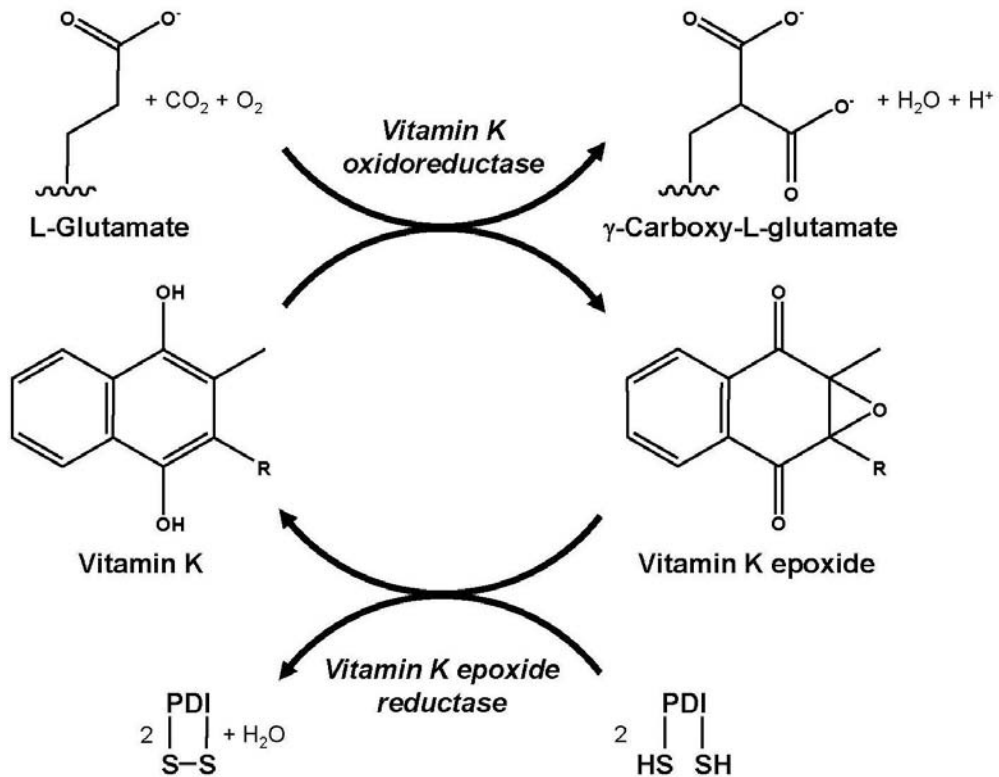


Figure 7. Coupling of L-glutamate γ -carboxylation to oxidative protein folding in the endoplasmic reticulum.

The HSP70 Class Molecular Chaperones Bip and Lhs1p

BiP accounts for ~5% of the soluble, luminal protein of the ER. It belongs to the well-characterized class of HSP70 chaperones which are found in all eukaryotic organelles and the cytosol of bacteria (Figure 11) [278, 279]. Much of what we know about BiP has been learnt from work on cytosolic HSP70s and its bacterial ortholog, DnaK. BiP was originally identified as GRP78 [15-17], a protein induced by glucose starvation and, independently, as the immunoglobulin heavy chain-binding protein [14]. BiP is a chaperone foldase of broad substrate specificity and assists in the folding of newly synthesized protein in the ER [280, 281]. BiP also has important functions in co- and posttranslational translocation of nascent polypeptide chains across the ER membrane [79, 139], sealing the luminal end of the translocon [282], recognition of damaged or misfolded proteins for retrograde translocation and degradation [283, 284], and regulation of unfolded protein response signaling (Figure 3) [285-288]. A common denominator in most of its biological functions is preferential interaction with unfolded protein substrates and prevention of aggregation of these unfolded proteins. The budding yeast ortholog, Kar2p, is required for karyogamy, the fusion of the two nuclei of haploid **a** and **α** cells during mating of haploid yeast cells [18, 289, 290]. The

second ER luminal HSP70 protein, GRP170/Lhs1p [19-21], belongs to the HSP70 subfamily of HSP110/GRP170 chaperones (Figure 11). These chaperones stimulate the ATPase activity of other HSP70 chaperones [20, 256, 259, 291-301]. A mammalian ortholog of Lhs1p has been identified as a protein induced by glucose starvation (GRP170, [25-28]) and hypoxia (ORP150, [22-24]).

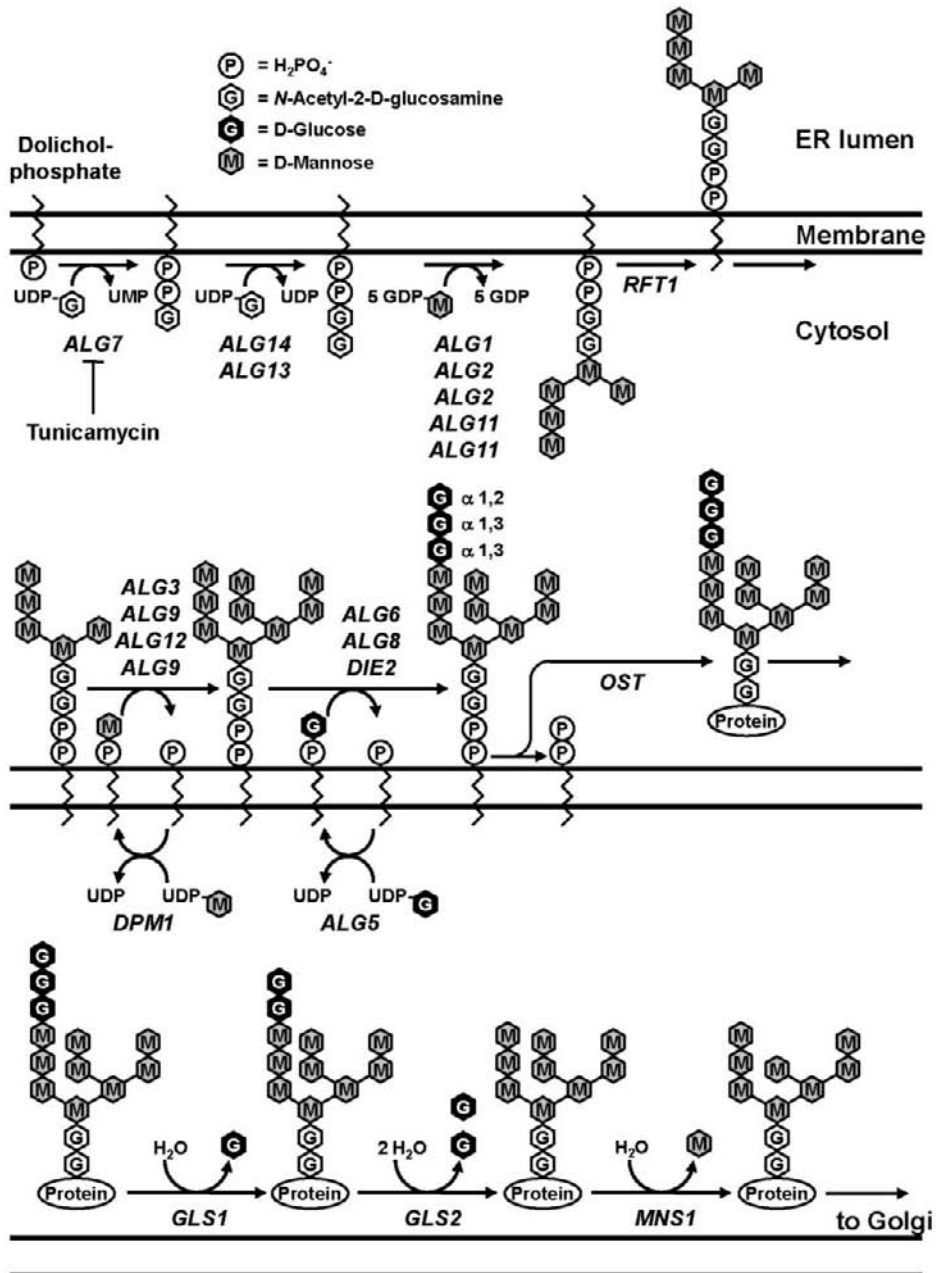


Figure 8. The pathway of N-linked glycosylation.

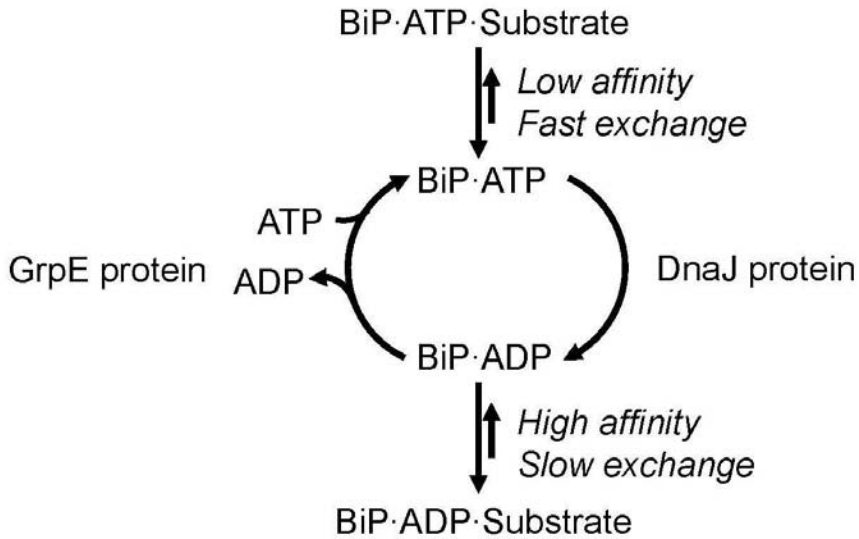


Figure 9. HSP70 chaperone ATPase cycle. HSP70 chaperones, such as BiP, cycle between an ADP- and ATP-bound state. Chaperone-bound ATP is hydrolyzed by the ATPase activity of the chaperone. Binding of DnaJ proteins to the HSP70 chaperone stimulates the ATPase activity of the HSP70 chaperone. GrpE co-chaperones stimulate the nucleotide exchange reaction. The ATP-bound state is characterized by low affinity for the substrate and fast exchange of substrates on the chaperone. The ADP-bound state is characterized by high substrate affinity and slow exchange of substrates.

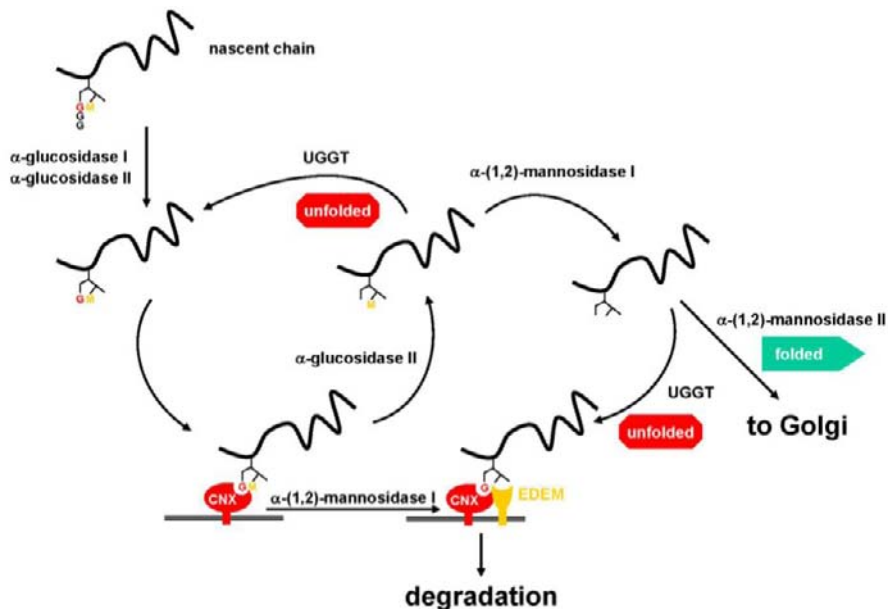


Figure 10. The calnexin cycle. Abbreviations: CNX – calnexin, EDEM – ER degradation enhancer, mannosidase α -like, G – D-glucose, M – D-mannose, and UGGT - uridine diphosphate (UDP)-glucose:glycoprotein glucosyl transferase. Reprinted from Mutation Research, volume 569, authors M. Schröder and R. J. Kaufman, title “ER stress and the unfolded protein response”, pages 29-63, Copyright (2004), with permission from Elsevier.

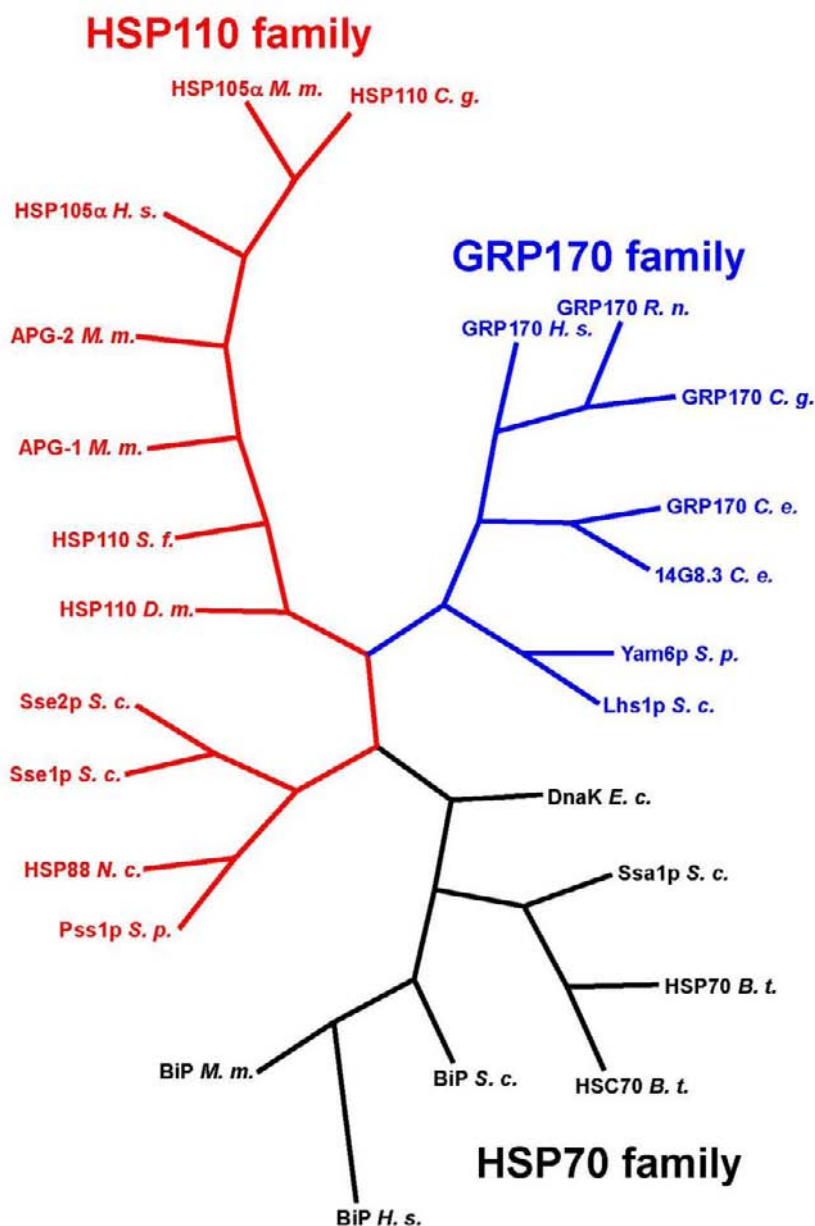


Figure 11. Phylogenetic tree of the HSP70 superfamily created with PHYLIP 3.68. Abbreviations: *B. t.* - *Bos taurus*, *C. e.* - *Caenorhabditis elegans*, *C. g.* - *Cricetulus griseus*, *D. m.* - *Drosophila melanogaster*, *E. c.* - *Escherichia coli*, *H. s.* - *Homo sapiens*, *M. m.* - *Mus musculus*, *N. c.* - *Neurospora crassa*, *R. n.* - *Rattus norvegicus*, *S. c.* - *Saccharomyces cerevisiae*, *S. f.* - *Stringylocentrotus franciscanus*, *S. p.* - *Schizosaccharomyces pombe*. Genbank accession numbers: 14G8.3. *C. e.* (CAA91809), APG-1 *M. m.* (NP_035150), APG-2 *M. m.* (Q61316), BiP *S. c.* (NP_012500), BiP *H. s.* (CAA61201), BiP *M. m.* (NP_071705), DnaK *E. c.* (NP_285706), GRP170 *C. e.* (CEESE89FB), GRP170 *C. g.* (Q60432), GRP170 *H. s.* (NP_006380), GRP170 *R. n.* (AAB05672), HSC70 *B. t.* (P19120), HSP70 *B. t.* (P34933), HSP88 *N. c.* (AAC23862), HSP105a *H. s.* (NP_006635), HSP105a *M. m.* (BAA11035), HSP110 *C. g.* (Q60446), HSP110 *D. m.* (CAB38172), HSP110 *S. f.* (AAB09038), Lhs1p *S. c.* (NP_012850), Pss1p *S. p.* (AAC18441), Ssa1p *S. c.* (NP_009396), Sse1p *S. c.* (P32589), Sse2p *S. c.* (P32590), and Yam6p *S. p.* (Q10061).

Structure and Function of the SBD and the NBD

BiP follows the structural blueprint of all HSP70 chaperones. They consist of a conserved 44 kDa N-terminal NBD and a 25 kDa C-terminal domain divided into a conserved SBD of 15 kDa followed by a 10 kDa immediate C-terminal domain (Figure 12) [8-10, 279, 302]. Conformational coupling of both domains by a flexible hinge region connecting both domains is the basis for ATP hydrolysis driven cycling of HSP70 chaperones through two alternating states of high (ADP-bound) and low (ATP-bound) affinity for unfolded substrates [303-308]. X-ray crystallography has provided insight into the three dimensional structures of the NBD of the 70 kDa bovine heat shock cognate protein (HSC70) [309-311] and the SBD of DnaK [312]. The structure of the NBD of DnaK closely resembles the structure of the NBD of HSC70 [313]. The N-terminal domain of BiP shares 66% sequence identity and 81% homology with HSC70 [279] and can easily be modeled onto the HSC70 structure [314], suggesting that structural lessons learnt from HSC70 and DnaK are valid to understand BiP. The K_M of HSP70 chaperones for ATP is $\sim 1\text{-}2\text{ }\mu\text{M}$ and is well below the cytosolic ATP concentration of $1\text{-}2\text{ }\mu\text{M}$ [250, 303, 306, 315-323]. For ER luminal HSP70s the ATP concentration in the ER lumen is of consequence, which remains unknown. ATP is transported into the ER by an ADP/AMP antiport mechanism [324]. Therefore, it is possible that the protein folding capacity of the ER is limited by ATP import, especially under conditions of high secretory activity or ER stress.

The NBD consists of two large, globular lobes (I and II) each containing two subdomains (A and B). The two lobes are connected by two crossed α -helices and are separated by a deep cleft to which ATP binds and in which ATP hydrolysis takes place (Figure 13) [309]. Both lobes and the connecting helices contribute to forming the binding pocket for ATP, one Mg^{2+} and two K^+ ions at the bottom of the cleft [309, 325]. ATP is positioned through a hydrophobic adenosine binding pocket [309] and interactions of one Mg^{2+} and two K^+ ions with the triphosphate group [325]. The carboxyl groups of three aspartates (D10, D199, and D206 in bovine HSC70) are involved in coordination of the metal ions [325, 326]. Mutagenesis of any of these aspartates to asparagine or serine decreases k_{cat} to 1-10% of the wild-type enzyme [323, 327]. A conserved lysine (K71) and a glutamic acid (D175) are positioned opposite to the β -phosphate group of ATP in the crystal structure of HSC70 [309]. Mutation of K71 to a neutral amino acid completely destroys ATPase activity [326], suggesting that this lysine positions a H_2O molecule or OH^- anion for in-line nucleophilic attack on the γ -phosphate group or acts as a base to increase the nucleophilicity of the nucleophile [326]. In contrast, the effect of mutation of E175, which may participate in correct positioning of the water molecule for nucleophilic attack on the γ -phosphate group, to glutamine or serine is not that dramatic. Taken together, these data suggest the following mechanism for ATP hydrolysis (Figure 14) [311, 325-327]: The MgATP complex binds initially through coordination of the Mg^{2+} ion with the carboxyl groups of D10 and D199 and a non-bonded oxygen atom of the β -phosphate group of ATP. Rearrangement leads to formation of a β , γ -bidentate complex in which the Mg^{2+} ion is coordinated by the carboxyl groups of D10 and D199 and one oxygen atom of the β - and γ -phosphate groups of ATP. The two K^+ ions stabilize the β , γ -bidentated complex, explaining the absolute dependence of ATP hydrolysis on the presence of K^+ ions [328]. The β , γ -bidentated complex aligns the

terminal phosphate group for in-line nucleophilic attack by a water molecule or OH⁻ anion. Lysine 71 participates in the nucleophilic attack by stabilizing or accepting a proton from the OH⁻ anion or water molecule (Figure 14). The rate-limiting step of ATP hydrolysis is the chemical, hydrolytic step [318].

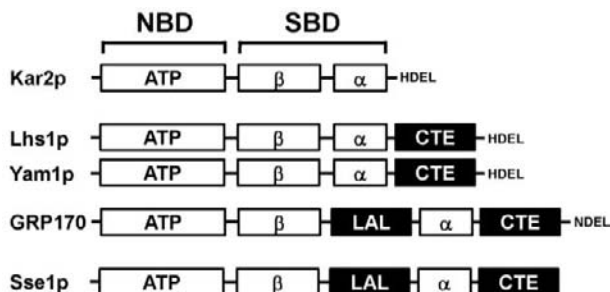


Figure 12. Domain architecture of the HSP70 family showing the relative arrangement of the nucleotide-binding domain (NBD), the β -sandwich and α -helical lid of the substrate binding domain (SBD), the C-terminal extension characteristic to HSP110 and GRP170/Lhs1p family members, and a long acidic loop (LAL) inserted between the β -sandwich and α -helical lid of the substrate binding domain present in some HSP110 and GRP170 chaperones. For ER luminal HSP70s the C-terminal ER retrieval sequence is shown. Kar2p is shown as an example for an archetypal HSP70 and Sse1p as an example for a HSP110 family member. The drawing is not to scale.

The SBD is formed by two structural units, a β -sandwich consisting of two sheets of four antiparallel β -strands with a hydrophobic groove on its upper side and a lid formed by four α -helices located above the hydrophobic groove. The two units are connected via an additional α -helix (Figure 15). The α -helices are making direct contacts to the substrate. The hinged lid has been proposed to gate access to the hydrophobic groove. ATP binding to the NBD opens the peptide binding pocket, whereas ATP hydrolysis closes the pocket to increase the affinity of the chaperone for its substrates. Two loops emanating from the top sheet of the β -sandwich form the hydrophobic pocket in DnaK. The hydrophobic pocket consists of a floor of hydrophobic amino acid residues surmounted by an hydrophobic arch. Both, the hydrophobic floor and arch engage in hydrophobic interactions with the substrate and determine substrate specificity. The hydrophobic pocket forms a channel with a cross section of $\sim 5 \times 7$ Å. In DnaK negatively charged residues flank the ends of the hydrophobic channel thus favoring the presence of basic amino acids at the end positions of interacting peptides. The main chain of the bound peptide also forms several hydrogen bonds with the main chain of the chaperone. This geometry and enclosure of the bound substrate by lid closure requires that the remainder of the substrate protein is separated by ~ 10 Å from its region interacting with the chaperone, suggesting that DnaK substrates are considerably unfolded. Analysis of peptide libraries has identified consensus motifs for interaction with DnaK [329-331] and BiP [304, 305]. The consensus peptide interacting with DnaK consists of 4-5 hydrophobic residues, preferably leucines, flanked by basic residues. Acidic residues are not present in the peptide core, and disfavored in the flanking regions, which is in good agreement with the structure of the DnaK peptide binding pocket. These sequences are often buried in the hydrophobic core of a protein and found in β -sheets. Similar approaches with BiP have established that BiP displays selectivity for hydrophobic peptides with the consensus

sequence HyXH₂YXH₂YXH₂ (Hy = large hydrophobic amino acid, X = any amino acid). High K_M values (1-100 mM) [304, 305] of HSP70 chaperones suggest a wide substrate spectrum.

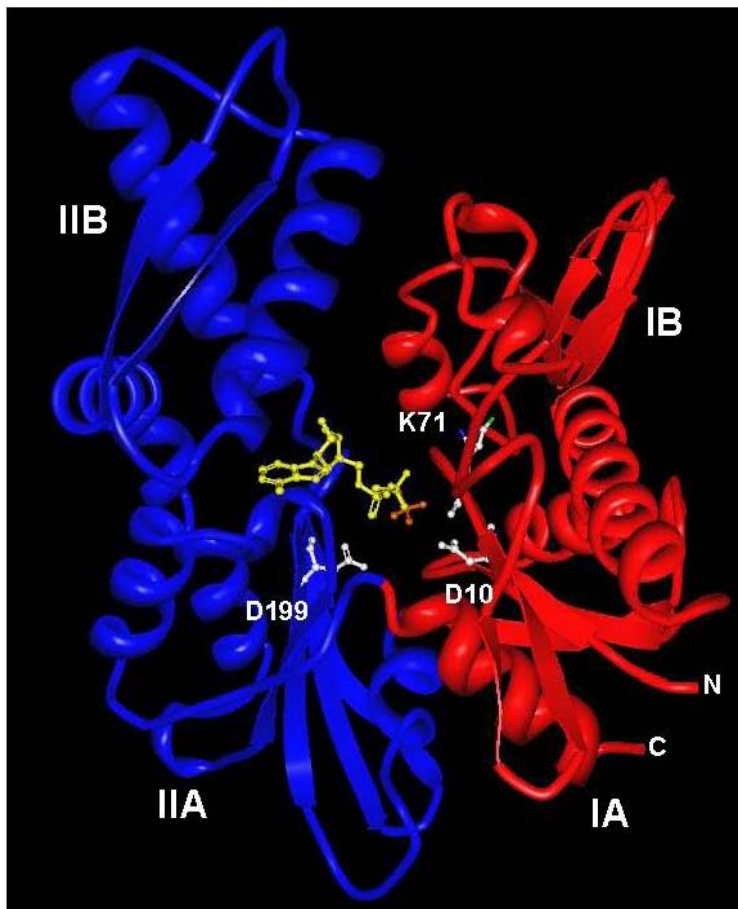


Figure 13. Crystal structure of the ATPase domain of bovine HSC70 (PDB entry no 1ba0). The two lobes are colored red and blue, ADP yellow, P_i orange, and several residues involved in MgADP coordination (D10 and D199) and catalysis (K71) are colored white. The N- and C-termini are labelled 'N' and 'C', respectively. The numbering of the two lobes of the ATPase domain is indicated [334].

Protein aggregation occurs on a time scale of seconds. If binding of unfolded substrates to HSP70 chaperones is to compete successfully with their aggregation, unfolded substrates must interact with HSP70 chaperones on a similar, if not faster, timescale as protein aggregation. The rate constant for association of nucleotide-free HSP70 chaperones with peptide substrates are $\sim 10\text{-}100\text{ M}^{-1}\text{s}^{-1}$ [332]. At this rate, complex formation would take at least several minutes. ATP binding accelerates this rate constant at least ~ 10 fold, but substrate binding requires conversion of the ATP to the ADP-bound state. The turnover number for unstimulated ATP hydrolysis by HSP70 chaperones is $\sim 0.1\text{ min}^{-1}$ [250, 303, 306, 318-323, 333], making ATP hydrolysis the rate-limiting step for the HSP70 chaperone substrate interaction. Substrate binding to HSP70 chaperones stimulates the ATPase activity of HSP70 chaperones [303, 304], but is not considered to sufficiently elevate the rate of ATP

hydrolysis to allow HSP70 chaperones to kinetically compete with aggregation of the substrate. To make HSP70 chaperones productive chaperones *in vivo*, ATP hydrolysis is stimulated by substrate binding [303, 304] and specialized co-chaperones of the HSP40 family.

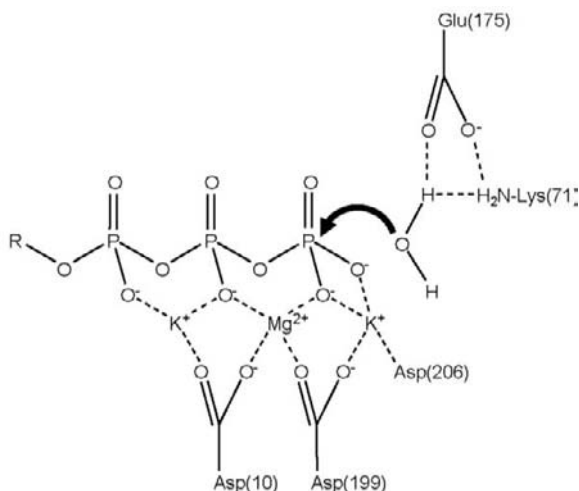


Figure 14. Simplified model of the β , γ -bidentated complex in the catalytic center of the NBD of bovine HSC70.

Communication between the SBD and NBD in BiP

The crystal structure of the SBD suggests that lid closure induced by ATP hydrolysis in the NBD locks the substrate in the substrate binding pocket and increases the affinity of HSP70 chaperones for their substrates. Insight into interdomain communication between the NBD and SBD has been provided by the 2.6 Å structure of bovine HSC70 in the nucleotide-free state lacking ~10 kDa at the C-terminus (Figure 16) [335]. This structure encompasses the β -sandwich forming the SBD and the α -helix connecting the hinged lid to the bottom of the substrate binding pocket. The two domains are connected by a flexible linker of 10 aa. The hinge helix of the SBD contacts a β -strand and a loop in the groove opposite to the ATP binding cleft of the NBD. Closing of the ATP binding cleft upon nucleotide binding/release or ATP hydrolysis impinges on the groove interacting with the hinge helix of the SBD, transducing ATP hydrolysis cycle induced movements in the ATP binding cleft to the lid of the SBD (Figure 16). Intrusion of the linker into the interdomain space may result in considerable conformational changes in this region, which may lead to differential movement of the helical lid and the β -sandwich of the SBD. An important role of linker movement in communication between both domains is supported by biochemical and crystallographic data [312, 335-340]. A conserved arginine exposed at the surface of this groove is involved in a hydrogen bond relay originating from the ATP binding cleft and may contribute to conformational responses of the groove to the nature of the bound nucleotide [341]. A smaller interaction surface formed by a loop of the SBD and a loop in the NBD is also important for conformational communication between both domains [342].

HSP40/DnaJ co-Chaperones for BiP

Without stimulation of ATP hydrolysis and ADP-ATP exchange HSP70 chaperones cannot kinetically compete with protein aggregation. HSP40 co-chaperones stimulate the ATPase activity of HSP70 chaperones, whereas GrpE co-chaperones are adenine nucleotide exchange factors for HSP70s (Figure 17). HSP40 co-chaperones are characterized by a conserved J domain of ~70 amino acids. The J domain is critical for stimulation of HSP70 ATPase activity. The J domain is formed by four α -helices connected via three loops (Figure 18). The J domain is stabilized by hydrophobic interactions between amino acid residues protruding from helices 2 and 3. The loop between helices 2 and 3 contains a conserved HPD sequence motif distinguishing J proteins from J-like proteins. The HPD motif is important for interaction of J proteins with HSP70 chaperones and stimulation of their ATPase activity. The J domain contacts the groove between lobes IA and IIA of the HSC70 NBD. Stimulation of ATPase activity is achieved by directing the linker toward a hydrophobic patch on the surface of the NBD. The SBD is displaced upon J protein binding from the NBD, suggesting that the SBD gains conformational freedom to capture substrates presented to the HSP70 chaperone by the J protein [343]. Interaction of DnaJ co-chaperones also increases the stability of the chaperone-substrate complex [344] and affects the substrate specificity of HSP70 class chaperones [344, 345].

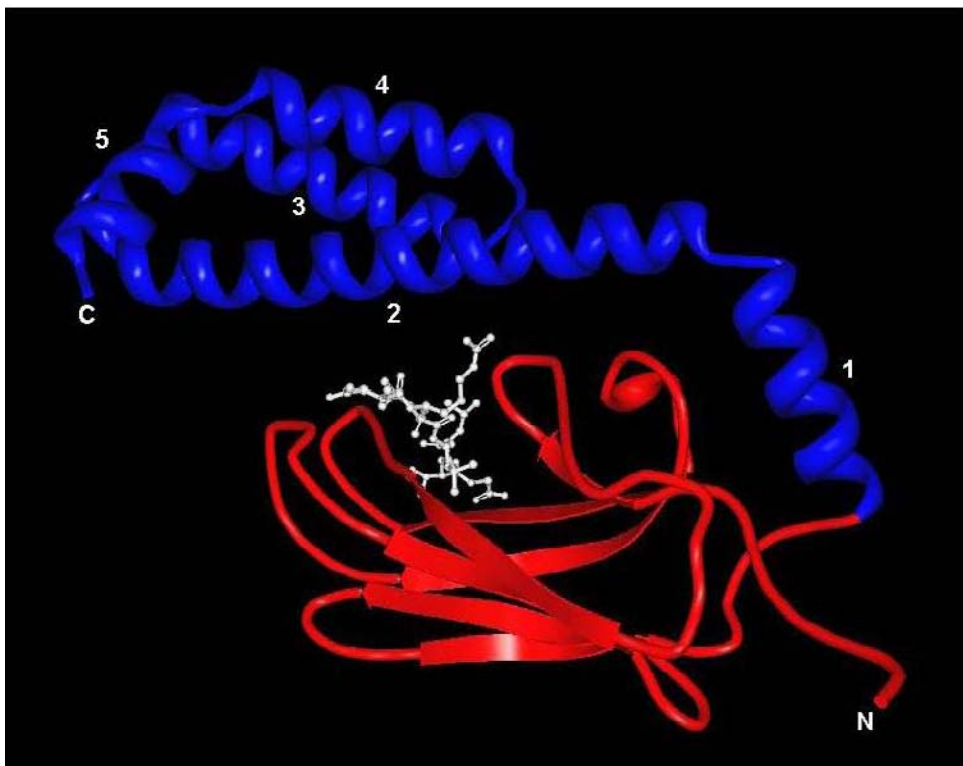


Figure 15. Crystal structure of the SBD of DnaK (PDB entry no. 1dkx). The β -sandwich and hydrophobic groove are colored red, the α -helical lid is shown in blue, and the peptide in white. The N- and C-termini are labelled 'N' and 'C', respectively, the α -helices 1 to 5 [312].

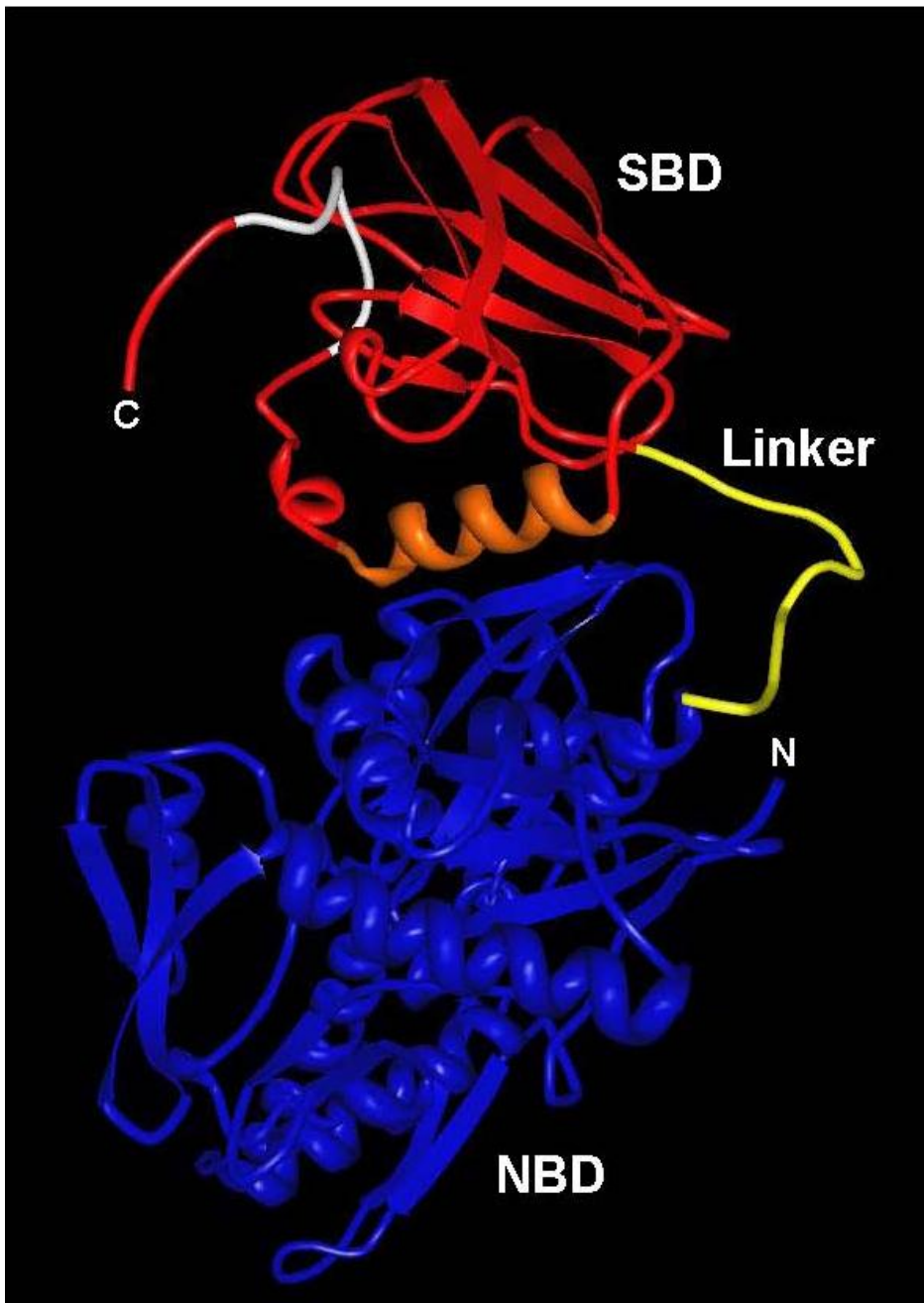


Figure 16. Crystal structure of amino acids 1-554 of bovine HSC70 (1yuw) [335]. Coloring: NBD – blue, SBD – red, linker – yellow, helix 1 of the SBD – orange, and portion of the SBD mimicking a substrate – white.

J proteins are classified into three categories based on the nature of domains they share with *E. coli* DnaJ, the founding member of this protein family (Figure 19) [346]. Class I J proteins contain a glycine and/or phenylalanine rich region and a Zn^{2+} -finger in addition to the J domain, class II J proteins only the glycine/phenylalanine rich region, and class III J proteins only a J

domain. Class I J proteins, such as ER luminal Scj1p [48] and HEDJ [347], interact with unfolded proteins through their Zn²⁺-fingers [348, 349]. This function may have been taken on by other domains such as glutamine-rich regions or tetratricopeptide binding motif repeats (TPR) in some class III J proteins, for example p58^{IPK} (Figure 19) [350, 351]. Class II J proteins also interact with unfolded proteins [56, 352]. As a consequence class I and II J proteins load HSP70 substrates onto HSP70 chaperone foldases (Figure 17). DnaJ proteins associate with these substrates fast enough to kinetically compete with aggregation successfully [332, 353, 354] and act catalytically in loading substrates onto HSP70 chaperones [355], suggesting that the majority of HSP70 substrate interactions are mediated by DnaJ proteins. The cytosolic J protein TPR2/TTC2/DJC7 can offload unfolded polypeptide chains from the HSP90 foldase machinery onto the HSP70 foldase machinery [356]. A J protein specific to the ER is Erj5p, which contains four thioredoxin-like domains and probably is involved in coordinating oxidative folding driven by disulfide bond formation and isomerization with conformational folding of the polypeptide backbone. A second function of J proteins is to target HSP70 chaperones to specific sites of action. For example, yeast Sec63p recruits BiP to the translocon to participate in translocation of nascent polypeptide chains into the ER [357]. Mammalian SEC63 and MTJ1 also function in translocation [358, 359]. Hlj1p, whose J domain is exposed to the cytosol, recruits the HSP70 chaperones Ssa1p and Ssa2p to the ER membrane to participate in ERAD. All mammalian ER luminal J proteins interact with BiP and stimulate its ATPase activity *in vitro* [40, 44, 46, 57, 347, 360, 361]. As J proteins are believed to form equimolar complexes with HSP70 chaperones the large structural diversity of J proteins suggests that these co-chaperones tether BiP to sub-ER localizations to participate in specific processes such as translocation, specify substrate selectivity of BiP, and the fate of the interacting substrate.

Nucleotide Exchange Factors (NEFs)

Nucleotide exchange factors are the second class of HSP70 co-chaperones. Several protein families provide NEF function to HSP70 chaperones. The archetypal NEF is *E. coli* GrpE. Two other dedicated NEFs that are structurally different from GrpE, are the BAG-1 family of NEFs [362], and NEFs characterized by several α -helical armadillo repeats. The latter class includes cytosolic HSP70 binding protein 1 (HspBP1) [363] and ER luminal BAP [253]/Sil1p [256, 364]/Slp1p [254, 255, 258]. Finally, the HSP70 subfamilies of HSP110 and GRP170/Lhs1p chaperones provide NEF services to HSP70 chaperones. GrpE is a 197 aa protein consisting of a central α -helical dimerization domain and a C-terminal β -sheet domain (Figure 20) [250, 365, 366]. GrpE accelerates ADP release from DnaK-ADP ~5,000 fold by decreasing the affinity of DnaK for ADP ~200-fold [367]. Together, GrpE and DnaJ increase ATP hydrolysis by DnaK several hundred-fold [252], which makes HSP70 proteins efficient chaperones despite their intrinsic low turnover number for ATP. GrpE-catalyzed nucleotide release may be a two step process, in which facilitated ADP release precedes release of GrpE from the chaperone, which is triggered by ATP binding to the chaperone (Figure 17) [368, 369]. Structural analysis of three HSP70-NEF complexes, GrpE-DnaK [313], Bag-1-HSC70 [362], and HspBP1-HSP70 [363], has revealed diversity in how different NEFs catalyze ADP release from HSP70 chaperones (Figure 20). The β -sheet

domain of one GrpE subunit contacts the nucleotide binding cleft of DnaK and induces a rotation of 14° of subdomain IIB of the DnaK NBD. BAG-1 also induces a 14° rotation of subdomain IIB, but by another mechanism. GrpE contacts the back of the NBD and reaches into the nucleotide binding cleft with its β -sheets to open subdomain IIB. In contrast, BAG-1 opens subdomain IIB by contacting both sides of the central cleft from above via α -helices. While different in structure and their mode of interaction with HSP70s, the mode of action of GrpE and BAG-1 is conserved. In contrast, HspBP1 uses a different mechanism. HspBP1 contacts subdomain IIB of HSP70 via its four α -helical armadillo-like domains. Sterical clashes with subdomain IB are resolved by movement of the two lobes of the NBD in relation to each other, leading to opening of the nucleotide binding cleft. The ER possesses one NEF, BAP [253]/Sil1p [256, 364]/Sls1p [254, 255, 258], which may adopt an HspBP1-like fold and, therefore, may open the nucleotide binding cleft of BiP in an analogous way to HspBP1.

Mutations in *SILI* cause Marinesco-Sjögren syndrome, a neurodegenerative disease, in humans [370, 371] and ataxia in the woozy mouse [257]. Further, deletion of *SILI* in yeast is not lethal [256]. These observations suggest that additional NEFs are present in the ER [293]. Synthetic lethality of deletion of the HSP70 Lhs1p with *SILI* suggests that Lhs1p and Sil1p share a common function [256]. Indeed, Lhs1p stimulates nucleotide exchange on BiP *in vitro* [259] and is present in the ER in complexes with BiP [214]. Recent work has shown that NEF activity for the HSP70 chaperone Lhs1p reflects on a common function of HSP70 chaperones belonging to the GRP170/Lhs1p and HSP110 subfamilies. The HSP70 protein family can be divided further into two subfamilies, archetypal and atypical HSP70s (Figures 11 and 12). Atypical HSP70s can be further divided into HSP110 and ER luminal GRP170/Lhs1p HSP70s [301, 372]. Atypical HSP70s are distinguished from archetypal HSP70s by the presence of a C-terminal extension and a long acidic loop insertion between the β -sandwich and α -helical lid of the SBD [301, 372]. The cytosolic HSP110 Sse1p and Lhs1p form 1:1 stoichiometric complexes with Ssa [294, 295, 373] and BiP [259], respectively. Complex formation with nanomolar affinity is independent of other co-chaperones and can be reconstituted *in vitro* [259, 292, 374]. This interaction is dependent on the ATP-bound state of the HSP110 chaperone [294, 295, 373]. The interaction is mediated by the NBDs of both chaperones [292, 373-376]. Like Lhs1p HSP110s are nucleotide exchange factors for their interacting HSP70 partners [259, 294, 295, 373]. The cytosolic HSP110 Sse1p is a NEF for the cytosolic HSP70s Ssa1-4p and ribosome-associated Ssb chaperones [292, 374]. A C-terminal truncation of Sse1p did not complement growth phenotypes of a *sse1 Δ* strain and was ineffective as a NEF *in vitro*, suggesting that the C-terminal extension mediates nucleotide exchange. Crystal structures for two HSP110·HSP70, a complex between yeast Sse1p and the NBD of human HSP70 [375] and between yeast Sse1p and the C-terminal ~10 kDa deletion of bovine HSC70 [376] have revealed extensive interactions between both NBDs (Figure 21). In these complexes the C-terminal 3 helix bundle domain (3HBD) of Sse1p contacts subdomain II of the HSP70 NBD and opens the nucleotide-binding cleft in the NBD. As a consequence ADP can no longer make contact to both binding pockets for the adenine moiety and the diphosphate group, resulting in a decrease in affinity of ADP and dissociation of ADP from HSP70.

In yeast, BiP/Kar2p is present in ~2500 fold excess over Lhs1p [377]. Further, the yeast ER possesses a ~20 fold excess of Sil1p over Lhs1p [377], suggesting that Lhs1p participates

only in a fraction of the chaperoning activities of BiP. Deletion of *LHS1* and *SIL1* is synthetic lethal, showing that these two NEFs are the only NEFs in a yet to be identified specific chaperoning activity of BiP and can substitute for each other in this chaperoning activity. In translocation of nascent polypeptide chains across the ER the activities of Sil1p and Lhs1p can be separated. Sil1p functions in co-translational translocation [255, 256], whereas Lhs1p appears to function only in posttranslational translocation of polypeptide chains into the ER [19, 21, 291, 378]. In this regard viability of *sil1*Δ and *lhs1*Δ strains suggests the existence of additional NEFs in the yeast ER that substitute for Lhs1p and/or Sil1p in translocation. Nucleotide exchange releases substrates from HSP70 chaperones. Therefore, stimulation of nucleotide exchange on BiP by Lhs1p may simply facilitate substrate release from BiP (Figure 22). Alternatively, but non-mutually exclusively, Lhs1p may take over substrates from BiP, or both chaperones may co-ordinate their chaperoning activities on a particular substrate molecule (Figure 22). Intriguingly, the relationship between BiP and Lhs1p is reciprocal in that BiP stimulates the ATPase activity of Lhs1p [259]. Action of BiP on Lhs1p in a J protein-like fashion suggests that BiP may by transferring substrates onto Lhs1p. Lhs1p, therefore, may be involved in folding of partially folded substrates, a subset of substrates, or in targeting of terminally misfolded substrates toward degradation.

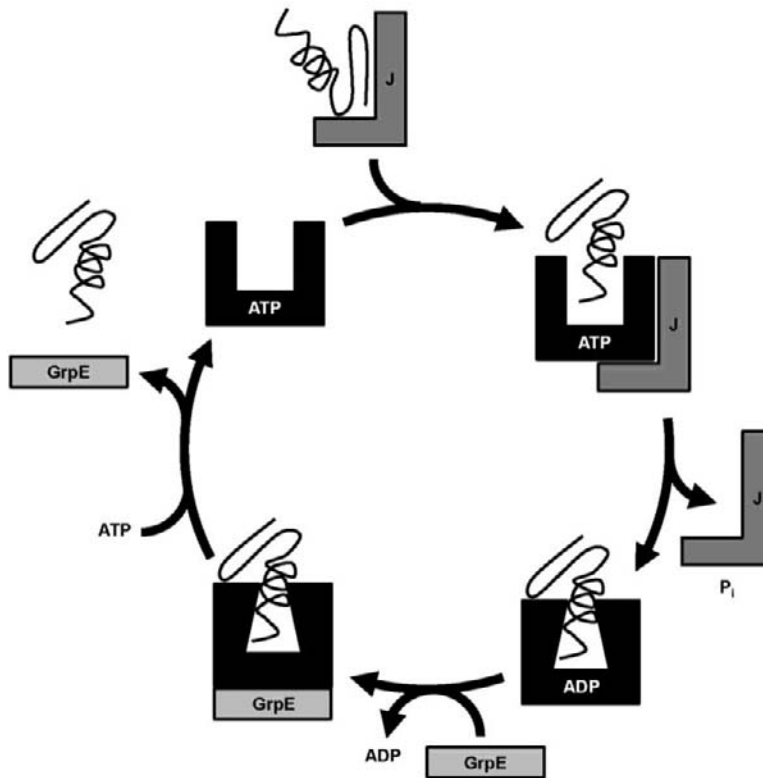


Figure 17. Revised HSP70 ATPase cycle incorporating loading of substrates onto HSP70 chaperones by DnaJ proteins. The HSP70 chaperone is shown in black. J protein-mediated substrate loading overcomes the slow association kinetics of HSP70 chaperones with their substrates *in vitro* and enables HSP70 chaperones to kinetically compete with protein aggregation.

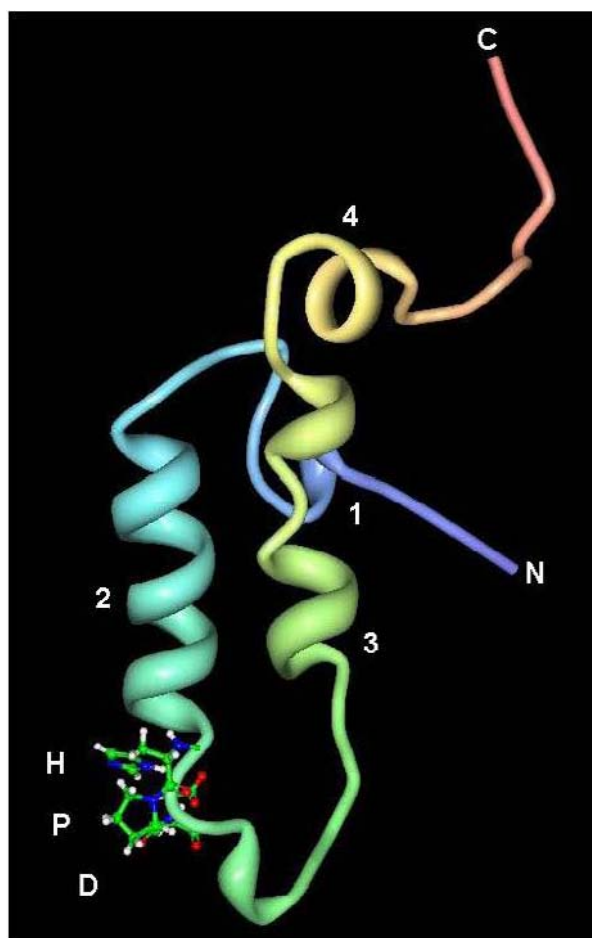
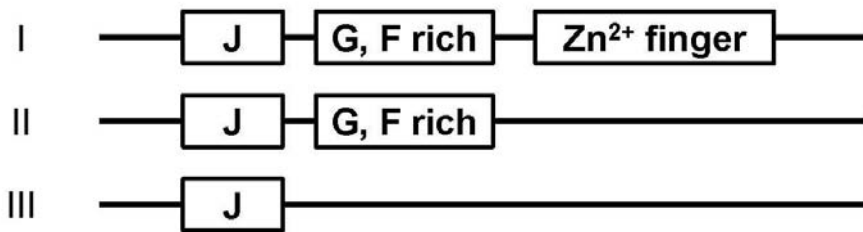


Figure 18. NMR solution structure of the J domain of *E. coli* DnaJ (residues 1-78) (PDB entry no. 1bqz) [379]. The *N*- and *C*-termini are labeled 'N' and 'C', respectively. The four α -helices are labeled 1 to 4. The HPD motif is indicated as a ball and stick model.

Regulation of BiP by Posttranslational Modification

In an inactive state, HSP70 chaperones, including BiP, form stable dimeric and oligomeric complexes [306, 315, 380, 381]. In the oligomeric state, HSP70 chaperones are bound to ADP, phosphorylated in their SBD [315, 380-386], and ADP-ribosylated [381, 382, 387-389]. These oligomeric forms do not interact with unfolded substrates and have been proposed to function as a reserve pool. In *in vitro* experiments both peptides [306] and ATP [380] dissociate the oligomeric form of BiP. The monomeric and oligomeric BiP pools are readily interconvertible. *In vivo*, conversion of BiP to the monomeric, unmodified form precedes induction of expression of its gene [381, 390]. However, it is currently unknown if these posttranslational modifications are required for BiP function, or are solely coinciding. Autophosphorylation of purified BiP preparations was suggested [384, 385], but phosphorylation due to a contaminating kinase was not convincingly ruled out as a source for phosphate incorporation into BiP.

A Class



B

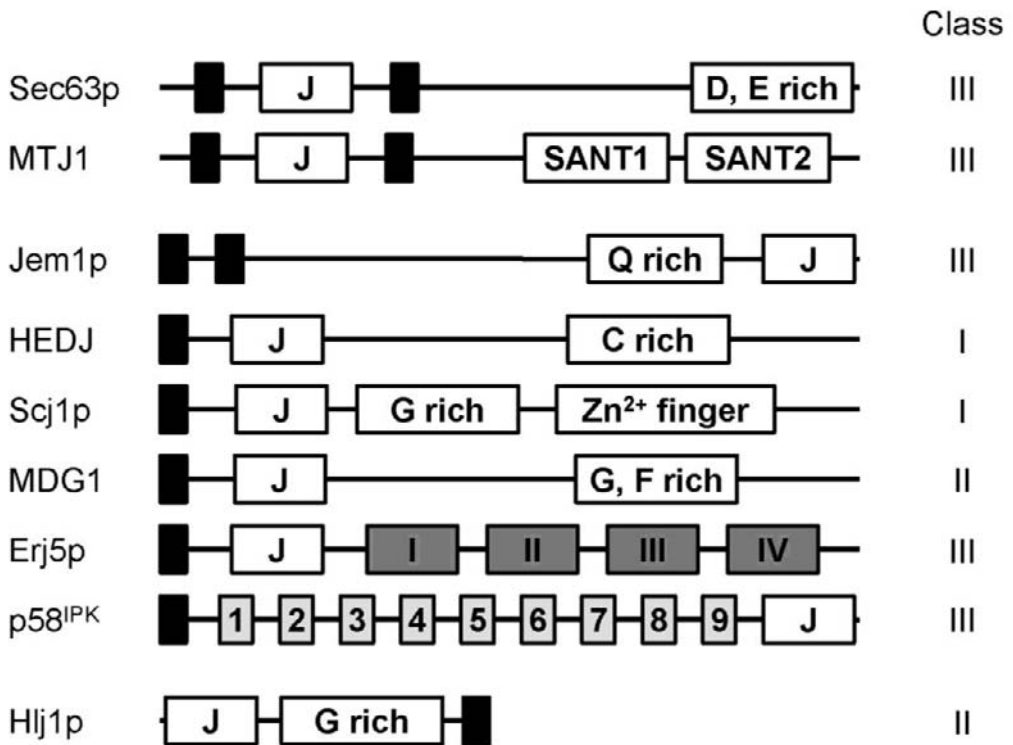


Figure 19. DnaJ proteins. (A) Domain structure of class I, II, and III J proteins. (B) J proteins of the ER. The J domain of Hlj1p is localized in the cytosol. Abbreviations and symbols: F – L-phenylalanine, G – glycine, J – J domain, SANT – SANT domain, I to IV – thioredoxin-like domains, 1 to 9 – TPR repeats. Black boxes in the middle of the protein represent transmembrane segments, directly at the *N*-terminus signal peptides. The drawings are not to scale.

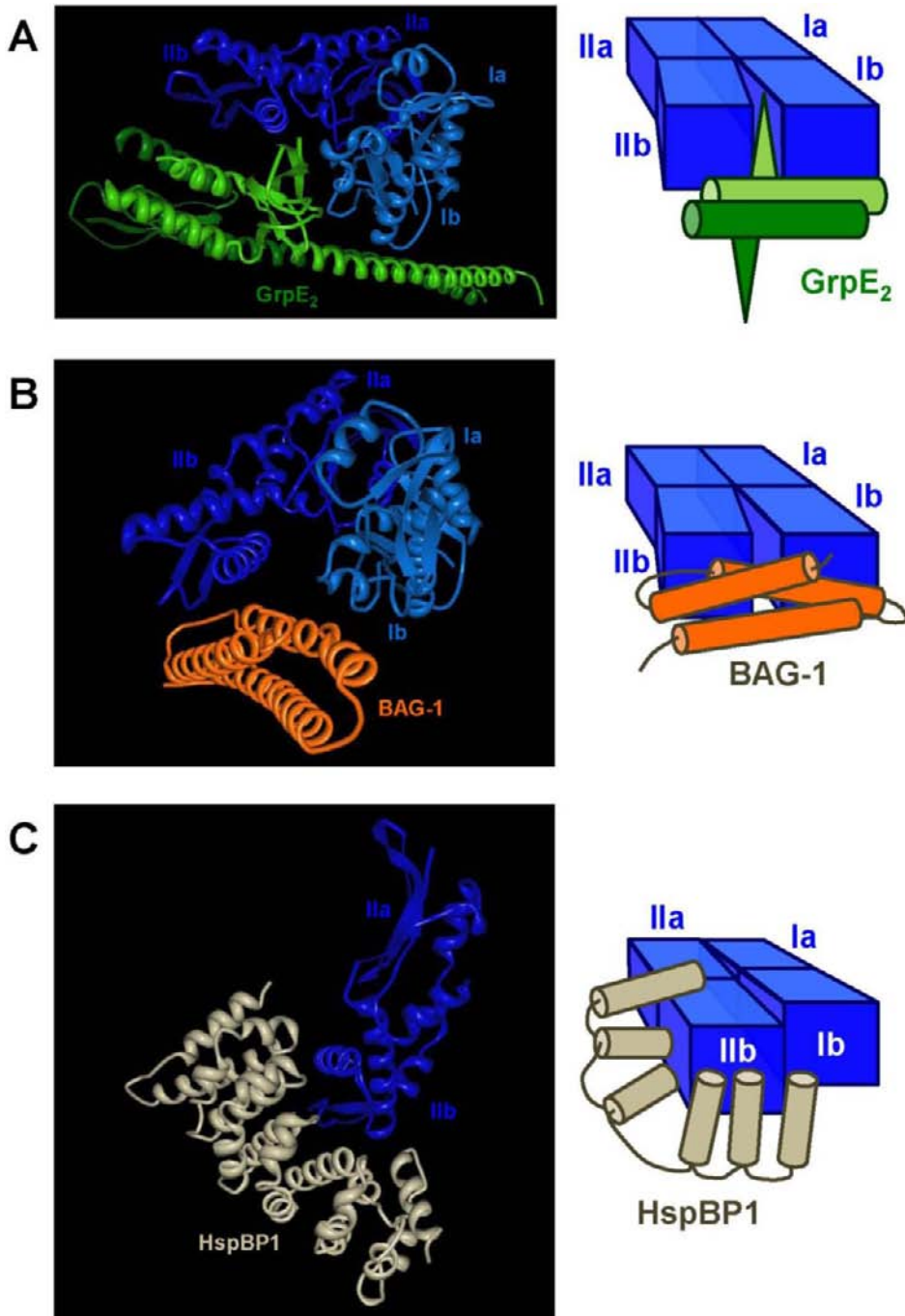


Figure 20. Molecular action of NEFs on the NBD of HSP70 chaperones. (A) The β -sheet of a GrpE dimer reaches deep into the nucleotide binding cleft between the two lobes of the NBD. A 14° rotation of subdomain IIb opens the cleft and facilitates nucleotide release (PDB entry 1dkg, [313]). (B) The three helix bundle of BAG-1 contacts subdomains Ib and IIb. In analogy to GrpE a 14° rotation of subdomain IIb opens the cleft and facilitates nucleotide release (PDB entry 1hx1, [362]). (C) The four armadillo repeats of HspBP1 contact subdomain IIb inducing vertical movement of subdomain IIb in relation to subdomain Ib to open the nucleotide binding cleft (PDB entry 1xqs, [363]).

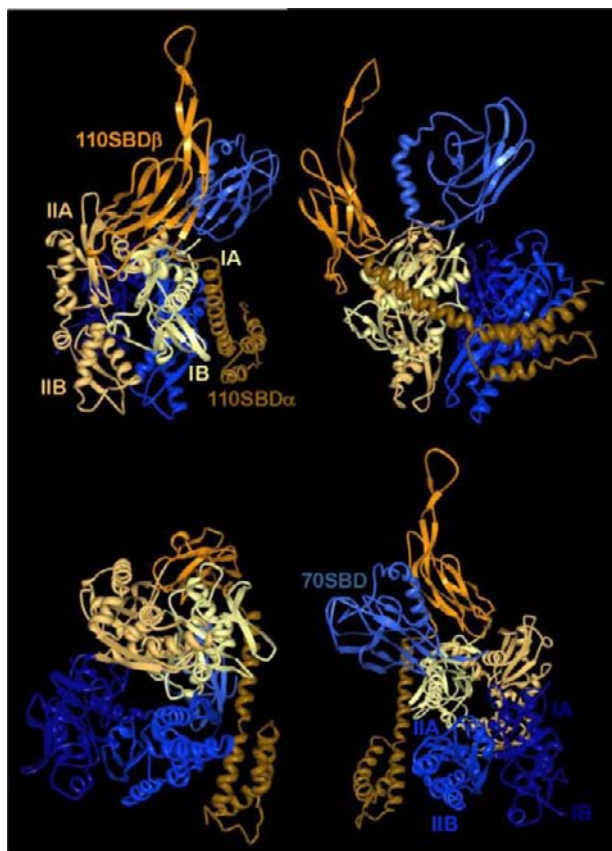


Figure 21. Crystal structure of the HSP110-HSP70 complex (PDB entry 3c7w, [376]). Sse1p is shown in yellow to brown tones and HSC70 in blue tones. The SBDs and the two lobes of the NBDs of Sse1p and HSC70 are labeled.

The HSP90 Class ER-Resident Molecular Chaperone GRP94

GRP94 is a member of the HSP90 class of chaperones and consists of an *N*-terminal NBD (N domain), followed by a charged region (M domain), a dimerization domain (C domain) and C-terminal ER retention motif (Figure 23) [391]. HSP90 chaperones are obligate dimeric chaperones and belong to the GHKL superfamily of ATPases and histidine kinases [392]. GHKL protein family members are DNA topoisomerases of the gyrase class, MutL-like DNA mismatch repair proteins, histidine kinases of the His-Asp signal transduction system, and HSP90 chaperones. These proteins share a unique nucleotide binding domain which is characterized by the so called Bergerat fold [393-399]. ATP is bound to the Bergerat fold in an unusual, kinked conformation. The Bergerat fold is characterized by four conserved motifs, motif I (also called ‘N box’, uubEuuaNouDA), II (G1 box, uxuxDNGxGuxbaauxxu), III (G2 box, uGxxGxouxSxxxuoxbuTuxT), and IV (G3 box, Tx_nGT, a – acidic residue, b – basic residue, o – small residue, u – bulky hydrophobic residue) [392]. The Bergerat fold consists of a sandwich formed by a bottom of four-stranded antiparallel β -sheets and a top formed by three

α -helices [392] (Figure 24A). Helices $\alpha 1$ and $\alpha 3$ are oriented parallel to the strands of the β -sheet, whereas helix $\alpha 2$ is perpendicular to the strands of the β -sheet. A loop (loop 4) between helices $\alpha 2$ and $\alpha 3$ functions as a flexible lid that can close over the ATP binding pocket. The N box lies at the C-terminus of helix $\alpha 1$. Its conserved asparagine (N107 in canine/human GRP94) co-ordinates an octahedrally co-ordinated Mg^{2+} ion that contacts all phosphate groups of the bound nucleotide through solvent-mediated hydrogen bonds. The G1 box lies in loop 3 connecting the $\beta 2$ sheet and helix $\alpha 2$. The conserved aspartate (D149) of the G1 box forms a hydrogen bond with the N6-amine of the adenine moiety. The conserved glycines of the G1 and G2 box form flexible hinges for the ATP lid. The ATP lid in HSP90 chaperones consists of two helices and one loop. The conserved glycine of the G1 box and the threonine/serine (S213) at the end of strand $\beta 3$ make solvent-mediated contacts with the N1 atom and the N6-amine of the adenine moiety (Figure 24B). A conserved lysine (K168) and asparagine (N162) in the ATP lid contact the β -phosphate of ATP and the adenine and ribose moieties, respectively. Glutamate 103 in the N box is involved in nucleophilic attack of a water molecule on the γ -phosphate group of ATP [393, 400–402]. It acts as a base that positions the water molecule in line for attack and participates in abstracting a proton from the water molecule. An important feature of all GHKL ATPases is that a second conserved lysine or arginine residue residing in a domain immediately adjacent to the NBD, the M domain in HSP90 proteins, also hydrogen bonds to this water molecule in the hydrolytically active state. In yeast Hsp82 this is R380 [403], in human HSP90 $\alpha 2$ R400, and in GRP94 R448 [247]. Mutation of this basic residue in *E. coli* GyrB [25], a *Drosophila* homolog of DNA gyrase [404], MutL [394], yeast Hsp82 [403], and GRP94 [247] decreases or completely destroys ATPase activity. A consequence of this ‘split ATPase’ arrangement is that substantial conformational rearrangements are necessary to bring this basic residue in line for formation of a hydrolysis-competent ATPase domain.

In cytosolic HSP90s and bacterial HtpG these domain movements are initiated upon ATP binding (Figure 25). In the nucleotide free, apo form HSP90 chaperones assume an open V-shaped conformation (Figure 25). ATP binding triggers closure of the ATP lid. Lid closure facilitates dimerization of the NBD domain by inducing conformational changes in a short N-terminal segment. These conformational changes in the N domain trigger further substantial changes in the rest of the HSP90 dimer including a $\sim 90^\circ$ rotation of the N and M domains. This brings the catalytic arginine in the M domain in proximity of the catalytic aspartate and activates the water molecule for in line attack on the γ -phosphate group of ATP. Overall, the ATPase competent chaperone assumes a closed, twisted V conformation (Figure 25). This conformation is metastable and relaxes upon ATP hydrolysis into the open, V-shaped conformation (Figure 25). A protein kinase client, CDK4, binds to the concave surface of the closed, twisted V conformation [405]. Several hydrophobic amino acid residues may be involved in substrate recognition. A methionine pair at the top of the C domain (M658 and M663 in human GRP94) [406], surface-exposed aromatic side chains of Y652, W654 and Y678 in GRP94, and W300 in yeast Hsp82 (W320 in human HSP90 $\alpha 2$) [407] may be involved in making hydrophobic contacts to unfolded substrates. W300 is replaced in GRP94 by a hydrophilic amino acid. A stretch of alternating basic and acidic residues, called a KEKE motif [408], may also be involved in protein-protein interactions. These first insights into the substrate-HSP90 chaperone interaction require extension to further substrates, but already indicate that HSP90 class chaperones use an extensive interaction surface to

recognize their substrates. As a consequence it becomes clear why HSP90 class chaperones prefer partially folded substrates over completely unfolded substrates and hierarchically act after HSP70 chaperones in protein folding.

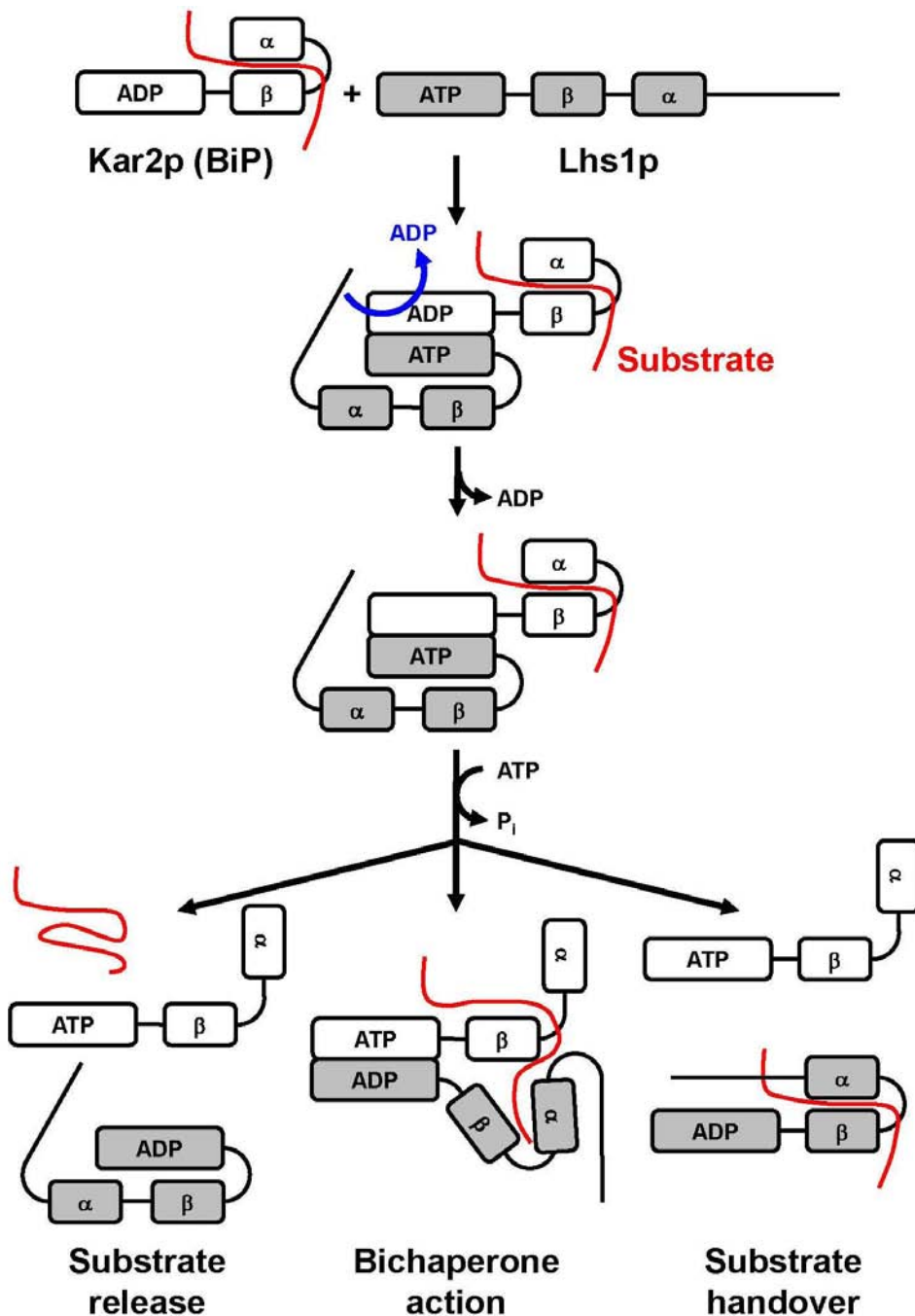


Figure 22. Model for function of the atypical HSP70 chaperones HSP110 and GRP170/Lhs1p as NEFs for archetypal HSP70 chaperones.

Identification of the CDK4-HSP90 interaction surface couples the ATP hydrolysis cycle of HSP90 chaperones to substrate recognition and interaction. HSP90 chaperones, including GRP94, have low ATP turnover numbers allowing them to hydrolyze one molecule of ATP every 1-2 min in case of yeast Hsp82 [401, 409] and GRP94 [247, 248] and every ~20 min in case of human HSP90 [410]. This low ATPase activity is essential for function of yeast HSP90 [400, 401]. The extensive conformational rearrangements triggered by ATP binding may be even slower than ATP hydrolysis. Along the same argumentation as for HSP70 chaperones, HSP90 chaperones by themselves should not be able to compete kinetically with substrate aggregation *in vivo*. Therefore, several co-chaperones for cytosolic HSP90s exist that regulate their ATPase activities, their conformational transitions, and load substrates onto cytosolic HSP90s, thus bridging the HSP70 and HSP90 machineries [411-413]. No co-chaperones for GRP94 have been identified. Sequences involved in co-chaperone interactions in cytosolic HSP90s are not conserved in GRP94, suggesting at the very least that if GRP94 co-chaperones exist that these will not simply be ER luminal paralogs of cytosolic HSP90 co-chaperones. GRP94 is insensitive to the nature of the bound adenine nucleotide and assumes an open, V-shaped conformation in the apo form, bound to ADP or non-hydrolyzable 5'-adenylyl- β,γ -imidodiphosphate. The crystal structure of full length GRP94 reveals that the γ -phosphate group of bound 5'-adenylyl- β,γ -imidodiphosphate makes no direct or water-mediated contacts with any parts of GRP94 [247]. Point mutations in the NBD inactivate GRP94 resulting in defects in galactosyl transferase activity in *Leishmania donovani* [414] and surface expression of toll-like receptors 1, 2, and 4 [415]. However, mutations that diminish nucleotide binding more severely compromise GRP94 function than point mutations predicted to render the NBD inactive [415]. Demonstration of ATPase activity for GRP94 *in vitro* shows that GRP94 can cycle between an open and closed conformation, but whether cycling is of importance *in vivo*, and how cycling is controlled, remains to be addressed. One possibility is that GRP94 substrates function as GRP94 co-chaperones that regulate its conformational transitions and its ATPase activity. In this regard it is worth noting that GRP94 appears to possess a limited substrate spectrum including immunoglobulin G molecules, some integrins, and all of the toll-like receptors [415-417]. This may explain why GRP94 is not found in all eukaryotes and why *grp94*^{-/-} mouse embryonic fibroblasts are viable [418].

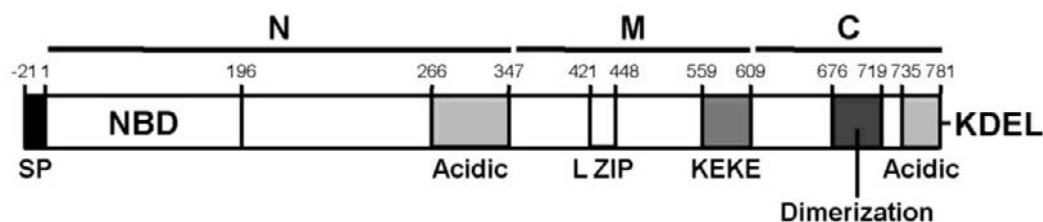


Figure 23. Domain structure of GRP94. The boundaries of the N, M, and C domains are indicated. KEKE – potential protein-protein interaction motif, L ZIP – leucine zipper, NBD – nucleotide binding domain, and SP – signal peptide.

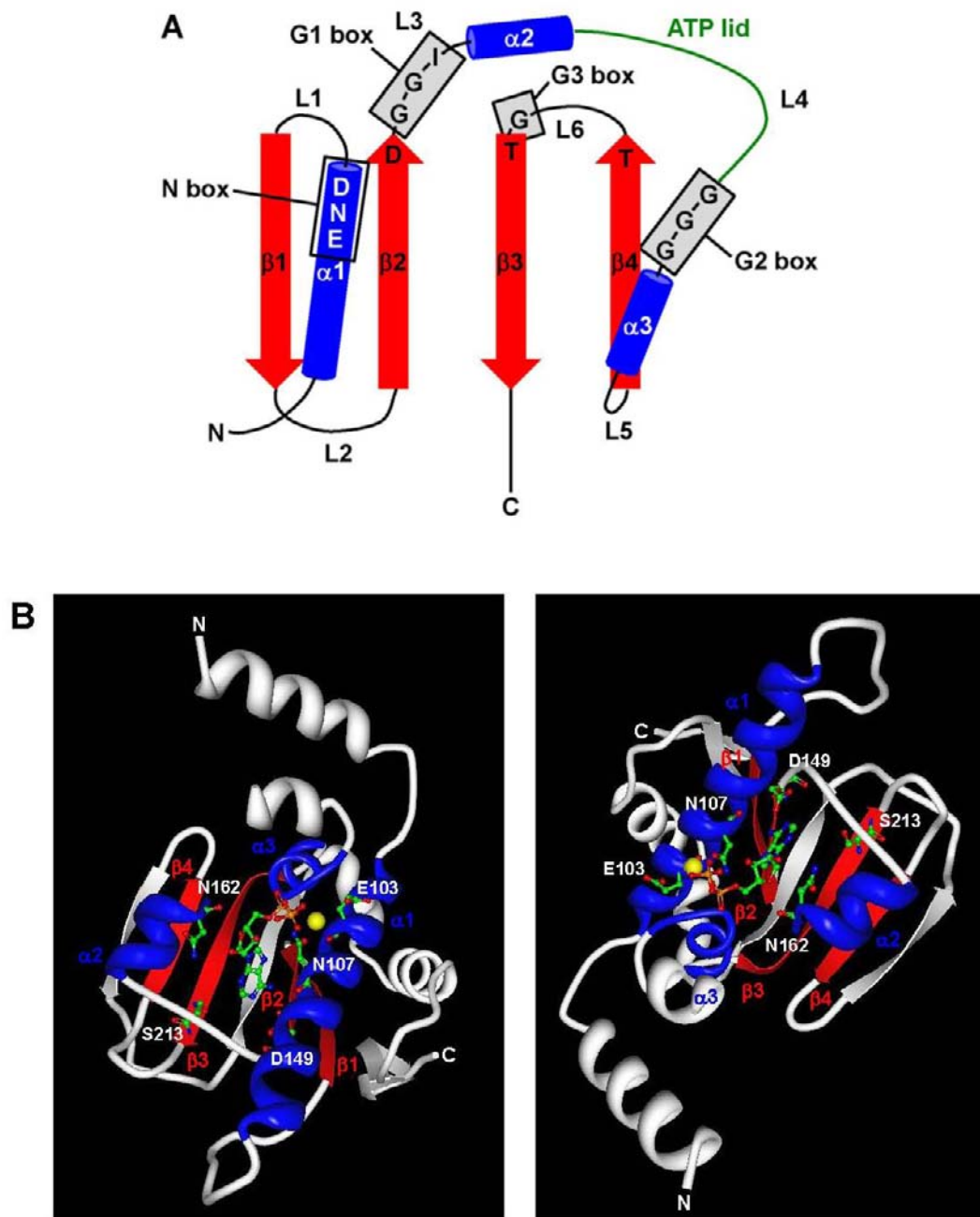


Figure 24. Structure of the NBD of GRP94. (A) Schematic of the Bergerat ATP-binding fold. (B) Two views of the crystal structure of canine GRP94 (PDB no. 1tc0 [419]). α -helices and β -strands of the Bergerat fold are shown in blue and red, respectively. Amino acids making contacts with bound nucleotide and bound ADP are shown in a ball and stick model and are labeled. The Mg^{2+} ion is shown in yellow.

The Lectin Chaperones Calnexin, Calreticulin and Calmegin

Proteins entering the ER are modified co-translationally by transfer of the oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (Glc - D-glucose, GlcNAc – 2-*N*-acetylamide-D-glucose, Man – D-mannose) from dolicholpyrophosphate- $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ located in the ER membrane to consensus Asn-X-Ser/Thr residues by oligosaccharyltransferase (OST, Figure 8) [240, 241]. The two terminal D-glucose moieties are rapidly ($t_{1/2} < 3$ min [420]) removed by sequential action of α -glucosidases I and II [421]. The monoglucosylated form interacts with the lectin chaperones calnexin, calreticulin, and the testis-specific calnexin homolog calmegin/calnexin-t (Figure 26) [422]. Removal of this glucose by α -glucosidase II releases the unfolded substrate from calnexin/calreticulin/calmegin (Figure 10) [423]. If still unfolded, the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT) reglucosylates the $\text{Man}_9\text{GlcNAc}_2$ oligosaccharide. This triggers a second round of interaction with the lectin chaperones [243, 244, 424]. This glycoprotein folding cycle is known as the calnexin/calreticulin cycle [421, 423-425]. The only component of this cycle for which evidence is available that it recognizes hydrophobic amino acid stretches is UGGT [426, 427]. Demannosylation events catalyzed by ER resident α -mannosidases and α -mannosidases residing in the ER Golgi intermediate compartment and the early cisternae of the Golgi complex are critical for extraction of permanently misfolded proteins from the calnexin cycle. In this way the glycosylation status of a newly synthesized protein functions as a molecular clock determining the fate of the polypeptide chain to which the oligosaccharide is attached.

Structure and Function of Calnexin and Calreticulin

Calreticulin, calnexin, and calmegin display a high degree of functional and structural similarity (Figure 26). Calreticulin and calnexin are chaperones for ~100 newly synthesized glycoproteins. Their substrate spectra are overlapping, but not identical [428]. Several viral glycoproteins that preferably interact with calnexin displayed increased interactions with calreticulin in calnexin knock-out cells [22], providing evidence for redundancy between calreticulin and calnexin. However, cellular glycoproteins did not switch over from calnexin to calreticulin and instead were found to be associated with BiP [429]. Calreticulin is a soluble, 46 kDa Ca^{2+} -binding protein of the ER, whereas calnexin and calmegin are 90 kDa and 70 kDa type I transmembrane proteins of the ER (Figure 26). Both proteins consist of a *N*-terminal N domain, a middle proline-rich P domain, and a *C*-terminal C domain. The NMR structures of the P domain of calreticulin and the crystal structure of the N and P domains of calnexin have been solved [430-433]. The N domain consists of a globular β -sandwich formed by eight antiparallel β -strands, has similarity to legume lectin, and functions as a lectin recognizing the monoglucosylated *N*-linked oligosaccharide $\text{GlcMan}_9\text{GlcNAc}_2$ [434, 435]. In both proteins several residues have been identified that participate in binding to the D-glucose moiety. In calreticulin these are Y109, D135 [435], and possibly K111, Y128, and D317 [436, 437]. In calnexin Y164, K166, Y185, M188, E216, and E425 are important for oligosaccharide binding [438]. Oligosaccharide binding to the N domain induces conformational changes in these

chaperones which are important for substrate binding [439]. Both the N and P domains contribute to the chaperone function [434, 435, 440, 441]. The P domain is characterized by two repeat sequences (repeat A, IXDPXA/DXKPEDWDX, and repeat B, GXWXPPIXNXPXYX, X = any amino acid). Calnexin harbors four [61] and calreticulin three repeats (Figure 26) [64, 65]. The P domain forms an extended structure stabilized by three antiparallel β -sheets [430–433]. Calnexin and calreticulin closely co-operate with the PDI ERp57 in protein folding [434, 442]. The P domain mediates the interaction with ERp57 [432, 440, 443, 444]. Mutagenesis experiments have confirmed that the extreme tip of the P domain is important for interaction of calreticulin with ERp57 [440]. Both the N and P domains bind Ca^{2+} , but the major Ca^{2+} binding domain in calreticulin is its C domain. The C domain of calreticulin binds $>50\%$ of ER luminal Ca^{2+} with low affinity ($K_d \sim 2 \text{ mM}$), but high capacity (25 mol Ca^{2+} /mol of protein) [441]. In contrast, the C domain of calnexin consists of a transmembrane domain, followed by stretches of negatively charged amino acids capable of binding Ca^{2+} and a C-terminal ER retention motif [61, 445]. Ca^{2+} binding to and phosphorylation of the C domain of calnexin may regulate interaction of calnexin with other proteins [446–448]. For example, interaction of calnexin with SERCA2b may be regulated by phosphorylation of S562 and contribute to coupling of ER luminal chaperone function to Ca^{2+} signaling. Phosphorylation of S554 and S564 regulates interaction with the cytosolic sorting protein phosphofurin acidic cluster sorting protein 2 (PACS2), which localizes to ER-mitochondrial junctions [449].

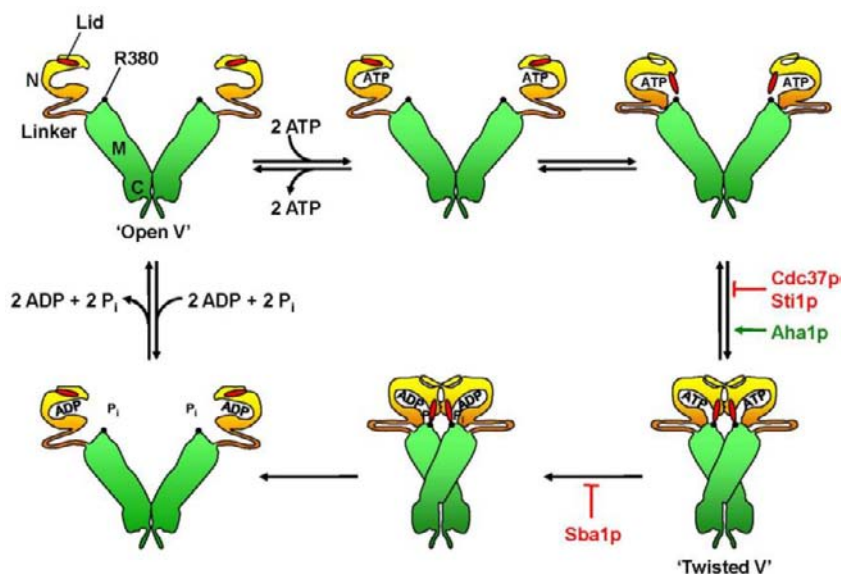


Figure 25. ATPase cycle of the cytosolic chaperone HSP90. ATP binding to the N domain (N) induces conformational changes in the N domain leading to lid closure and dimerization of the two N domains of both protomers in a HSP90 dimer. This brings R380 in the middle (M) domain in proximity of the γ -phosphate group of ATP generating active ATPase centers. Upon ATP hydrolysis the strained ‘twisted V’ conformation relaxes into the ‘open V’ conformation and releases P_i and ADP. The C domain (C) mediates dimer formation of HSP90. GRP94 undergoes a similar ATPase cycle as HSP90 *in vitro*, but the importance of this ATPase cycle for the *in vivo* function of GRP94 is not clear. Cytosolic HSP90 co-chaperones such as Aha1p, Cdc37p, Sba1p, and Sti1p regulate conformational changes in the HSP90 dimer. Similar GRP94 co-chaperones are currently unknown.

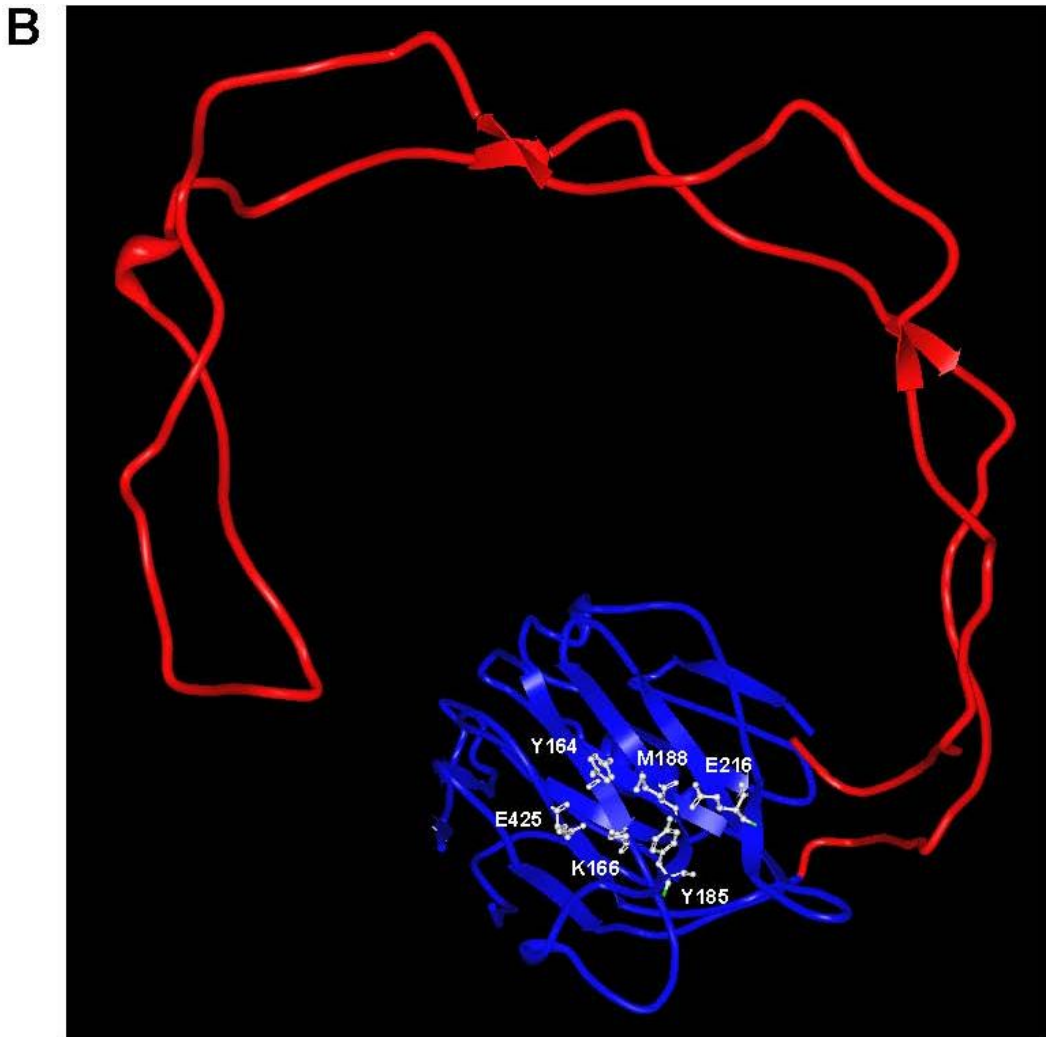
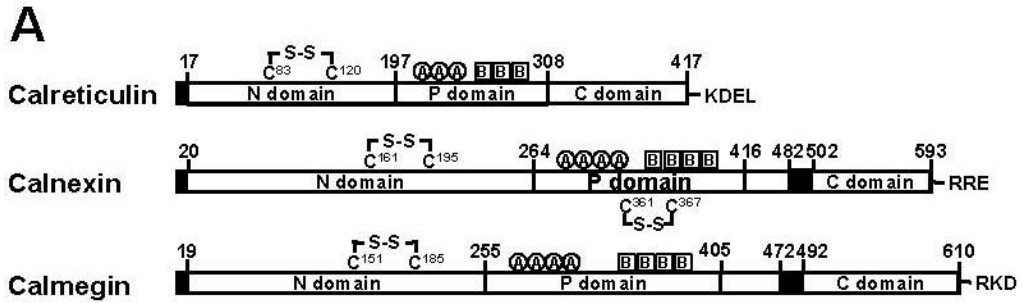


Figure 26. (A) Domain structures for calreticulin, calnexin, and calmegin. Disulfide bonds are indicated by -S-S-. Amino acid sequences at the C-termini represent ER retention sequences. Circles labeled A represent the acidic repeat sequence PXXIXDPDAXKPEDWDE. Squares labeled B represent the proline-rich repeat sequence GXWXPPXIXNPXYX. (B) Crystal structure of the N and P domains of calnexin (PDB entry 1jhn [430]). The P domain is shown in red, the N domain in blue, and amino acid residues involved in carbohydrate binding in white.

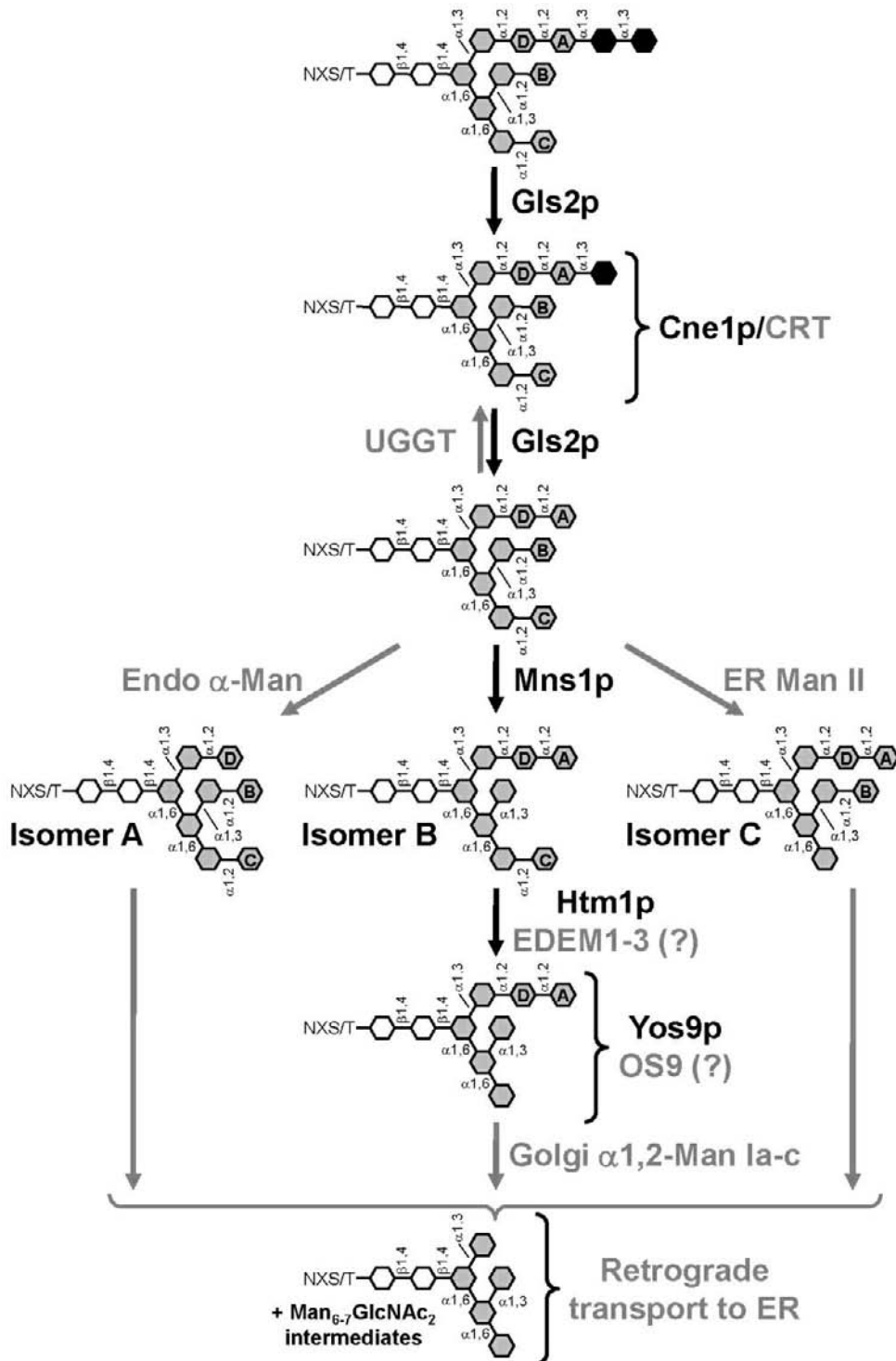


Figure 27. Modification of N-linked oligosaccharides in the calnexin (CNX)/calreticulin (CRT) cycle leading to recognition of unfolded proteins by Yos9p and targeting of unfolded protein to the proteasome. Pathways common to *S. cerevisiae* and mammals are shown in black, other pathways predominantly present in mammals are in grey.

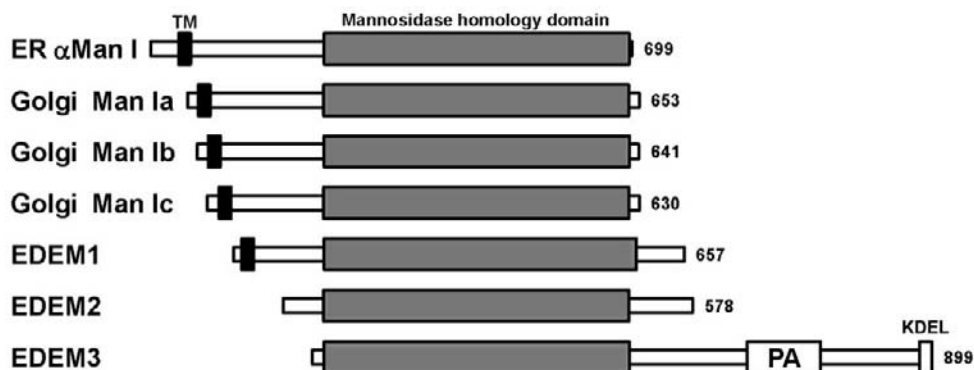


Figure 28. Domain structure of the GH47 mannosidase family members ER α (1-2) mannosidase I, Golgi mannosidase Ia (Golgi Man Ia), Golgi mannosidase Ib (Golgi Man Ib), Golgi mannosidase Ic (Golgi Man Ic), and EDEM1-3. The mannosidase homology domain is shown in grey, transmembrane domains in black, a KDEL ER retrieval sequence in white, and a protease-associated domain is represented by a box labeled 'PA'. Size of all polypeptide chains are indicated at their C-terminus. Little or no sequence conservation exists outside of the mannosidase homology domain between family members.

α -Glucosidase II

Entry of substrates into the calnexin/calreticulin cycle is controlled by α -glucosidase II, a soluble, heterodimeric protein of the ER. The α -subunit carries the catalytic activity and is retained in the ER through interaction with the β -subunit ending in a KDEL ER retention sequence [450-452]. The β -subunit is characterized by stretches with high homology to the mannose binding domain of the mannose-6-phosphate receptor [453], suggesting that it may contribute to substrate recognition. Cleavage of the two D-glucose moieties from the oligosaccharide by α -glucosidase II is sequential and may involve release of the substrate from the enzyme after the first hydrolytic event and reassociation of the substrate with the enzyme before the D-glucose moiety is released by α -glucosidase II [454], because of different orientations of the two α -glycosidic bonds in space. This transient release of α -glucosidase II from its substrate may allow calnexin/calreticulin to bind to the monoglucosylated form. In this model the affinities and association kinetics of α -glucosidase II, calnexin, and calreticulin determine how monoglucosylated substrates are partitioned between deglycosylation and entry into the calnexin/calreticulin cycle at the ER luminal side of the ER membrane or in the lumen of the ER. α -Glucosidase II displays 20% activity with GlcMan₈GlcNAc₂ and <10% activity with GlcMan₅₋₇GlcNAc₂ [455] compared to its preferred substrate GlcMan₉GlcNAc₂. Removal of D-mannose residues also decreases the affinity of calreticulin for monoglucosylated oligosaccharides [206, 456]. However, calreticulin retains ~60% of its affinity for GlcMan₅₋₈GlcNAc₂ compared to GlcMan₉GlcNAc₂ [456], suggesting that demannosylation overall prolongs the interaction of substrates with calnexin/calreticulin. The β -subunit is not required for enzymatic activity of the α -subunit [450, 452, 457], but is required for localization of the α -subunit to the ER in *Schizosaccharomyces pombe* [457], but not in *S. cerevisiae*. Further, deletion of the β -subunit

in *S. cerevisiae* yielded monoglucosylated oligosaccharides [452], suggesting that the β -subunit is involved in recognition of monoglucosylated substrates.

UGGT

UGGT preferentially recognizes partially unfolded protein structures, which can be close to or far away from the acceptor oligosaccharide [427, 458-462]. The D-glucose donor in the reglucosylation reaction catalyzed by UGGT is UDP-D-glucose. UDP-D-glucose is synthesized in the cytosol from α -D-glucose-1-phosphate and uridine triphosphate (UTP) by UDP-glucose pyrophosphorylase and imported into the ER by antiport with UMP [324]. UMP is formed by hydrolysis of UDP by the Mg^{2+}/Ca^{2+} -dependent apyrase UDPase in the ER [463]. Apyrases usually require millimolar Ca^{2+} concentrations to exhibit activity [463-465]. Thus, Ca^{2+} depletion of the mammalian ER may stall the calnexin cycle (Figure 10). In contrast to many other apyrases, ATP is only a poor substrate for ER-UDPase [463]. Regeneration of UTP from UMP in the cytosol consumes ATP. Thus, the calnexin cycle can be considered a chaperone foldase cycle that promotes the folding of its substrates in which hydrolysis of UDP-glucose instead of ATP provides the energy that drives protein folding in this cycle (Figure 10). However, how energy provision is kinetically coupled to protein folding or which conformational changes in UGGT, calnexin/calreticulin or other proteins participating in the calnexin/calreticulin cycle link hydrolysis of UDP-D-glucose to protein maturation is currently unknown. UGGT simultaneously recognizes two features in an unfolded protein: exposed hydrophobic sequences and the oligosaccharide moiety [466]. UGGT recognizes the innermost *N*-acetylglucosamine residue of an asparagine-linked oligosaccharide, which may only be accessible in a denatured conformation [426]. This residue interacts extensively with the polypeptide backbone of the protein [242]. The structural flexibility of this residue and neighboring amino acids may be a key determinant in recognition of unfolded proteins by UGGT [243]. UGGT preferentially glucosylates $Man_9GlcNAc_2$ and to a lesser degree $Man_8GlcNAc_2$ oligosaccharides, but displays little activity toward $Man_7GlcNAc_2$ oligosaccharides [466]. The exact nature of protein determinants recognized by UGGT is still unknown. UGGT binds to hydrophobic nonapeptides linked to sepharose 4B [426] or to phenyl-superoxe [459]. Completely denatured proteins are poor substrates for UGGT *in vivo* [458] suggesting that partially structured substrates are recognized by UGGT and that UGGT acts, on average, after the ER luminal HSP70 chaperones. Studies using *N*-terminal fragments of a 64 amino acid long, chemically glycosylated protein suggested that fragments possessing some, but not all, structural elements of the full-length protein were most efficiently glucosylated by UGGT [427]. Alternatively, shorter fragments may simply not have provided the minimum distance required between the glucosyl acceptor site and the protein determinant recognized by UGGT or did not contain an UGGT recognition determinant. The distance between these two contact points for UGGT in a partially folded protein is controversial. Depending on the model protein studied acceptor and recognition sites can be part of a local domain [460, 467] or up to 4 nm separated [462].

Exit from the Calnexin/Calreticulin Cycle

Folded proteins no longer display hydrophobic patches at their surfaces that are recognized by UGGT and thus are no longer a substrate for UGGT and exit the calnexin/calreticulin cycle. Slowly folding or folding-incompetent proteins continue to display such sites at their surface and will be retained in the calnexin/calreticulin cycle. To prevent poisoning of the calnexin/calreticulin chaperone machinery by these slowly folding proteins these also need to be extracted from the cycle and targeted for degradation. Demannosylation inhibits deglucosylation of monoglucosylated oligosaccharides by α -glucosidase II [455] and glucosylation of non-glucosylated oligosaccharides by UGGT [466], suggesting that unfolded proteins are extracted from the calnexin/calreticulin cycle by action of α -mannosidases (Figure 10). These demannosylation events are slow [468]. Removal of two outermost mannose residues (mannoses B and C, Figure 27) facilitates export to the Golgi complex via lectin receptors such as ERGIC-53, because it inhibits reglucosylation by UGGT and deglucosylation by α -glucosidase II and decreases the affinity of the *N*-linked oligosaccharides for calnexin and calreticulin [85, 469]. Removal of mannose A inhibits interaction with ERGIC-53 and possibly other ER export receptors and targets the protein toward ERAD [85]. Two types of proteins, α -mannosidases and α -mannosidase like proteins such as homologous to mannosidase I 1 protein (Htm1p) [470]/mannosidase-like 1 protein (Mnl1p) [471]/ER-degradation enhancing α -mannosidase-like protein (EDEM) [472-475] (Figure 28), participate in these demannosylation reactions and in recognition of demannosylated proteins. The major biochemical difference between these two proteins is that α -mannosidases are considered to have catalytic activity required to remove mannose residues from oligosaccharides whereas EDEM proteins may be lectins for which carbohydrate binding activity suffices to fulfill their biological roles. Slow demannosylation events superimposed over cycles of de- and reglucosylation catalyzed by α -glucosidase II and UGGT provide a time window in which a nascent, *N*-glycosylated polypeptide chain can fold into its native conformation. If the protein folds sufficiently fast, it is allowed to exit the ER and travel to the Golgi complex, if not, it is targeted for degradation by ERAD. The levels of UGGT and α (1,2)-mannosidase I activities are comparable [459, 476], indicating that a folding protein may require only a small number of deglucosylation-reglucosylation cycles to obtain its native conformation [477].

Several α -mannosidases may participate in modification of *N*-linked oligosaccharides while a protein is retained in the calnexin/calreticulin cycle. ER α (1,2)-mannosidase I is a membrane protein that converts $\text{Man}_9\text{GlcNAc}_2$ to $\text{Man}_8\text{GlcNAc}_2$ isomer B. At high concentrations as can be employed in *in vitro* experiments this enzyme can further degrade *N*-linked oligosaccharides [478]. Such degraded oligosaccharides have been observed on misfolded glycoproteins that are prohibited from exiting the ER [468, 476, 479]. Concentration of ER α (1,2)-mannosidase I together with glycoproteins at specific suborganellar sites has recently been reported [479, 480], suggesting that ER α (1,2)-mannosidase I may be involved in formation of these highly demannosylated oligosaccharides. Alternatively, trafficking of misfolded proteins between the ER and the *cis*-Golgi cisternae [481-486] may expose these proteins to α (1,2)-mannosidases of the *cis*-Golgi cisternae which are able to degrade $\text{Man}_9\text{GlcNAc}_2$ to $\text{Man}_5\text{GlcNAc}_2$ [487, 488] and to

endomannosidases of the ER-Golgi intermediate compartment (ERGIC) which can cleave the glycosidic bond between mannoses A and D in GlcMan₉GlcNAc₂ to yield Glc-Man and Man₈GlcNAc₂ isomer A (Figure 27). Finally, the α -mannosidase-like EDEM proteins may possess α -mannosidase activity after all. Overexpression of EDEM1 or EDEM3 gave rise to extensively demannosylated glycoproteins *in vivo* [489, 490]. EDEM proteins may function as lectins that interact with demannosylated proteins to target these proteins for retrograde translocation and degradation by the proteasome in a process called ER-associated protein degradation (ERAD) [81, 83]. Demannosylation inhibits reglucosylation and thus may increase the chances of an unfolded, demannosylated protein to interact with EDEM. In this model demannosylation to Man₇GlcNAc₂ is sufficient to induce a degradative fate, as reglucosylation of Man₇GlcNAc₂ structures is very inefficient [466]. Consistent with this view is that EDEM proteins have highest affinity for Man₈GlcNAc₂ [470, 471] oligosaccharides. Extensive demannosylation to Man₅₋₆GlcNAc₂ has been proposed to be a signal for ERAD [479, 491-494]. Exposed α (1,6)-glycosidically linked D-mannose residues are recognized by another lection protein in yeast Yos9p [495, 496], which is a component of a multiprotein complex containing the ubiquitin ligase Hrd1p [497-499]. Htm1p/EDEM removes the outer D-mannose residues to unmask the inner, α (1,6)-glycosidically bonded D-mannose residues for recognition by Yos9p (Figure 27) [495, 496]. The mammalian Yos9p ortholog OS9 may function in a similar way in targeting misfolded glycoproteins for degradation. Thus, successive demannosylation by ER α (1,2)-mannosidase I and Htm1p generates an ERAD substrate that is recognized by Yos9p through its mannose-trimmed oligosaccharide structure and Hrd3p via its polypeptide chain [495].

Conclusion

Three general chaperone machineries operate in the ER, the HSP70 chaperones BiP and Lhs1p, the HSP90 chaperone GRP94, and the lectin chaperones calreticulin, calnexin, and calmeglin. Research has provided considerable insight into the molecular function of these chaperone machineries, but still important questions remain to be answered. Little is known about the biological roles of the ER luminal DnaJ proteins and the biological roles of ER luminal nucleotide exchange factors for BiP. Research into potential functions of these proteins in protein folding in the ER may be guided by the more extensive knowledge on the function and regulation of the cytosolic HSP70 machineries. The same is true for GRP94, for which co-chaperones have not yet been identified. It remains to be seen whether GRP94 can indeed be a successful chaperone without co-chaperones. Co-chaperones that bridge of the ER luminal HSP70, HSP90, and lectin chaperone machineries have not been reported. Considering the toxicity of unshielded, free unfolded proteins, scenarios in which substrates are released from the HSP70 machinery to be detected *de novo* as unfolded by GRP94 or the lectin chaperones seem unlikely. An important difference to cytosolic protein folding is formation of disulfide bonds in newly synthesized proteins in the ER. How conformational folding of the polypeptide backbone is coordinated with oxidative folding is poorly understood. The existence of the J protein Erj5p, which in essence appears to be a PDI with redox potentials of its catalytic centers similar to those of thioredoxin plus a J domain

suggests that sophisticated mechanisms involving at least the HSP70 chaperones BiP and Lhs1p await discovery that couple oxidative folding to conformational folding of the polypeptide backbone.

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Chapter II

Type III Secretion Chaperones: A Molecular Toolkit for All Occasions

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Abstract

Common to many bacteria is the ability to establish a symbiotic relationship or to evade innate immune responses of an animal, plant, fish or insect host. Most often this capacity is mediated by a type III secretion system (T3SS). The function of these complex molecular machines is likened to a syringe-needle injection device that is dedicated to the translocation of effector proteins directly into target eukaryotic cells. Each translocated effector tends to possess a distinct enzymatic activity that aids in subverting host cell signaling for the benefit of the bacterium. Their translocation requires another class of secreted protein – the translocator – which form pores in the target eukaryotic cell plasma membrane through which the effectors may transit to gain entry into the cell interior. Most often, each secreted substrate requires a dedicated small, non-secreted cytoplasmic chaperone for their efficient secretion. Unlike traditional molecular chaperones, these specialized type III chaperones do not assist in protein folding and are not energized by ATP. Controversy still surrounds their primary role; as bodyguards to prevent premature aggregation or as pilots to direct substrate secretion through the correct T3SS. The latter is supported by recent evidence that these chaperones can dock directly to the cytoplasmic face of the T3S machinery, possibly serving as a recognition motif for substrate secretion. Added to this functional complexity is their important contribution to system regulation, which can ultimately confer temporal order to substrate secretion. Moreover, some chaperones display a bewildering propensity to interact with several additional T3S-associated proteins – the relevance of which remains

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uncertain. Structural data has now appeared for several important type III chaperones, either alone or in complex with their cognate substrate. This is proving a fillip in our attempts to understand the mercurial ways in which these versatile proteins operate in nature. It is hoped that this article will provide information on type III chaperone function, as well as highlighting key recent advances in the field. May it also be a testament to the value of continued intense effort in unravelling the mysteries of type III chaperone biology.

Introduction

Many bacteria at some point live their life in contact with vertebrate or invertebrate hosts. These associations may often be mutually beneficial. The host may provide a supply of growth nutrients or a safe haven in which bacteria are protected from harmful external environments. On the other hand, bacteria may breakdown complex bio-molecules that provide the host with usable by-products. This is exemplified by the soil bacterium *Rhizobia* that forms root nodules on legume plants. In this symbiosis, the bacterium receives carbon made available by the plant, while the plant receives nitrogen fixed by the *rhizobia* [1]. On other occasions however, bacterial-host associations may be of a parasitic nature, whereby bacterial colonization of plants, animals, fish or insects can lead to the onset of disease that may even be lethal for the host [2]. In this regard, the emergence of bacterial pathogens could be an accidental consequence of mutualism gone astray. After all, their ability to induce a fatal disease in the host might also mark the end-of-the-line for the infecting bacteria. It follows therefore that regardless of the mutualistic or parasitic outcome, bacteria can use common molecular mechanisms to establish a frame-work for cross-talk with a host [3-8]. Among the large group of Gram-negative bacteria, a frequently used mechanism for communication with the host cell is the type III secretion system (T3SS) [9-11].

Type III Secretion Systems

Biogenesis of a Functional Nanomachine

Bacteria harbor several different protein secretion systems for the purpose of moving cargo from the cytoplasm into or beyond the bacterial envelope [12]. Some of these are general systems required to fulfill normal physiological needs. Others, such as the T3SS, are more specialized in their use for intimate and direct communication with eukaryotic cells, thereby helping to facilitate meaningful bacteria-host interactions [13]. T3SS's are complex nanomachines, being composed of ~25 proteins that in a step-wise manner assemble together into a hollow conduit spanning the bacterial envelope [14]. Several of these core protein constituents are first secreted into the bacterial envelope via the universal *sec*-dependent secretion pathway. Polymerization of an extracellular appendage, or 'needle', extending from the bacterial surface signals the final phase of the assembly process. Secretion of these needle constituents are dependent on a working T3SS, which is established only after correct system assembly in the bacterial envelope. While T3SSs are a feature of many Gram negative

animal-, plant-, insect- and fish-interacting bacteria, their morphology can have some striking differences. The most obvious difference can reside in the surface needle appendage. In the plant pathogens for example, the needle takes on the appearance of a much larger pilus-like structure. A conceptual illustration of some distinct T3SSs is given in Figure 1. This includes the protein secretion system for the biogenesis of flagella, which will be discussed later in this review.

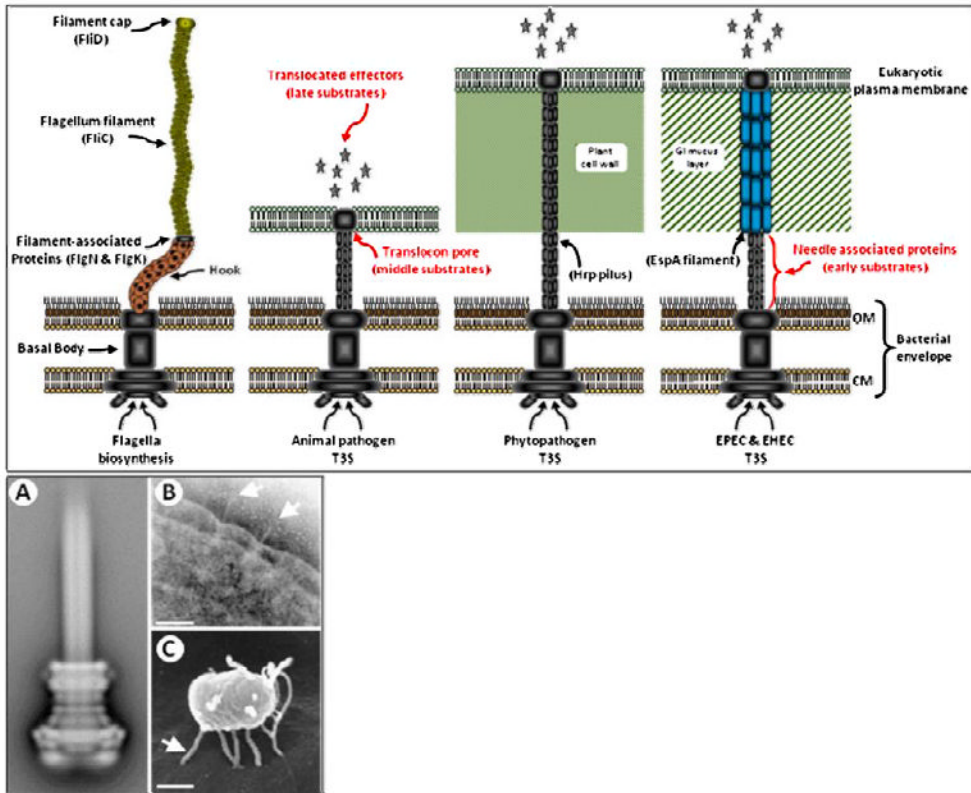


Figure 1. A schematic diagram of flagella and non-flagella T3SSs. The syringe-needle complex of non-flagella systems is reminiscent of the flagella hook-basal body structure. The typical non-flagella T3SS of an animal pathogen possess a short needle that connects with the eukaryotic host plasma membrane. Variants of this structure exist in plant interacting bacteria that possess an extended needle termed the Hrp-pilus for traversing the plant cell wall. In addition, pathogenic *E. coli* form an EspA filament on top of the needle that might be needed to penetrate through the mucous layer in the gastrointestinal tract (GI). Red highlight indicates the three substrate categories (early, middle and late) secreted by non-flagella T3SSs. This schematic was inspired from a review by He and colleagues[400]. Panel A: An electron micrograph refined by difference mapping of negatively stained T3SS syringe-needle complexes purified from wild type *S. flexneri*. The image is part of a study performed by Boekema and colleagues[289] and is reused with permission “Copyright (2007) Elsevier Publishers”. Panel B: An electron micrograph of negatively stained, osmotically shocked wild type *S. enterica* Typhimurium with the positions of the T3SS syringe-needle complexes in the bacterial envelope indicated by arrows. The image is part of a study by Galan and colleagues [401] and is reused with permission “Copyright (2000) National Academy of Sciences, U.S.A.” Panel C: Scanning electron micrograph of enteropathogenic *E. coli* adhered to red blood cells via the EspA filament (arrow) associated with a T3SS. The image is part of a study by Knutton and colleagues [402] and is reused with permission “Copyright (2001) Wiley-Blackwell Publishing Ltd.” OM – outer membrane, CM – cytoplasmic membrane.

During *in vitro* culturing of bacteria in defined laboratory media, these assembled T3SS nanomachines are competent for secretion of a diverse array of protein substrates into the extracellular milieu. Even though this offers a convenient means to study the biomechanics of type III secretion (T3S), this form of secretion is probably an *in vitro* artifact. The true role of T3SS is seen in response to target cell contact [15, 16]. Close contact is assumed to first trigger the secretion of a family of translocator substrates that assemble into a translocon pore in the eukaryotic cell membrane. Assembly of this pore is assisted by a ‘needle-cap’ protein that locates at the needle tip [17-22]. This T3S needle – translocon pore structure is believed to establish an uninterrupted connection between the bacterial cytosol and the host cell interior. Another set of substrates – the anti-host effectors, are then thought to travel through this conduit on their way to the cell interior. Although this dogma is widely accepted in the research community, ironically very limited experimental evidence is available to directly support it. To illustrate this, protein secretion through the needle conduit and release out through the distal tip has seldom ever been visualized [23-25]. Thus, despite intense effort, the precise mechanism of T3SS-dependent translocation of anti-host effectors into an infected eukaryotic target cell remains illusive.

Secreted Substrate Cargo

As stated, in response to target cell contact or growth in defined laboratory media, two major protein classes are secreted and released from the bacteria; the ‘middle’ and ‘late’ substrates. Late substrates constitute the anti-host effectors that are directly injected into target host cells through pores formed in the eukaryotic cell plasma membrane by the middle substrates – termed the translocators (Figure 1). Not surprisingly, all translocator proteins tend to harbor hydrophobic moieties promoting their integration into lipid membranes. However, how this translates into forming a functional translocon pore that can specifically recognize effectors and facilitate their translocation into the cell interior is not well understood [17, 26, 27]. Perhaps one or both translocator proteins interact with components of the host plasma membrane, such as cholesterol, which might facilitate their integration into the membrane to form the translocon pore [28, 29]. Pore formation also seems to be an active process because it is susceptible to feedback inhibition by the enzymatic activity of translocated effectors once they have reached a critical mass inside the eukaryotic cell [30-32]. This suggests direct interactions occur between the translocators and the anti-host effectors; either to guide their onward passage into the target cell interior or to establish feedback inhibition of pore formation[33].

In contrast, a considerable amount of functional data is available concerning the late substrates – the translocated bacterial effectors 34, 35. These anti-host molecules possess a modular domain structure demarcating areas responsible for secretion, translocation and effector activity. Any given T3SS can be responsible for the specific transport of several effectors, all with different enzymatic activities – numbers as high as 50 are routinely reported for some plant pathogenic bacteria [36]. Moreover, these molecules are often unique having no homology with each other. They were also acquired by bacteria separately from their cognate T3SS. Acquisition was probably through convergent evolution, pathoadaptation

or direct horizontal gene transfer, since their effector domains encode for enzymatic functions that mimic many of the signal transduction processes occurring inside the host cell [37]. The translocated effectors therefore alter the activities of host cell molecules, thereby disrupting normal host signal transduction pathways for the benefit of the infecting bacterium. Thus, through the use of T3S effectors, bacteria have acquired multiple ways to try and out-smart its host. Discussions of these are not in the scope of this title. The interested reader can learn much more by consulting one or more of the suggested reviews [34, 35, 38-43].

A miscellaneous group of ‘early’ secreted proteins, and therefore distinct from the translocator or effector families, are also targeted for T3S. Compared to the latter two substrate families discussed above, these are almost certainly the very first substrates secreted by a newly built system. However, several of them have undefined roles in the secretion process and are probably not translocated into the eukaryotic cell cytosol. As mentioned already in this chapter, the needle constituents are polymerized at the bacterial surface following their T3S [27]. Their secretion and polymerization into a needle structure must occur before most other substrates can be secreted. Therefore, these needle constituents represent a subset of ‘early’ secreted substrates (Figure 1). Other early type III secreted proteins fall into a heterogeneous group of regulatory anti-activators [44, 45]. Their secretion is triggered by inducing signals such as target cell contact. The signal sensor might even be a fully assembled needle complex [27]. Lowering of the anti-activator concentration in the bacterial cytoplasm through T3S serves to relieve system repression, which then stimulates elevated synthesis and secretion of middle and late T3SS substrates. This coordinates type III gene expression to various ‘quality control’ check-points along the path of appropriate T3SS biogenesis. Through necessity, this regulatory design restricts secretion substrate synthesis until a time when they are most needed, such as during the assembly process or as a translocation substrate for injection into eukaryotic cells [44, 45]. This ‘anti-activator’ concept will be revisited later in the article.

Quality Control of Type III Secretion: On the Role of Chaperones

A Molecular Chaperone Perspective

The Cytoplasmic Heat Shock Response

To safeguard against the dangers of protein misfolding and aggregation, all organisms produce various families of molecular chaperones and proteases that participate in essential protein folding and degradation control [46, 47]. This inevitably ensures that proteins fold correctly and therefore maintain functionality. When encountering extreme physiological stress, the roles of chaperones and proteases assume even greater importance [48]. Their production is elevated significantly in a phenomenon known as the heat shock response. Briefly, the bacterial cytoplasmic response to stress is controlled by the σ^{32} factor [48, 49]. The chaperones produced by bacteria grown in these conditions have variable size range (~10 kDa to ~100 kDa), but most function with the help of ATP hydrolysis to prevent aggregation and assist with protein folding or refolding in the bacterial cytoplasm. Apparently, unfolded

or misfolded proteins expose hydrophobic regions that act as the catalyst for chaperone intervention [48]. Even though the majority of newly synthesized proteins tend to fold correctly with only minimal chaperone input, the remainder (perhaps fewer than 20%) are dependent on chaperone activity. Misfolded substrates not amenable to refolding are targeted by proteases for degradation [46, 47, 50]. Coincidentally, the heat shock response is known to affect expression levels of T3SS genes encoded by various bacteria. This control of T3SS gene expression is often mediated by Lon- or ClpXP-dependent proteolysis of important regulators specifically involved in the control of T3SS synthesis [51-56].

The Extracytoplasmic Stress Response

Quality control of protein folding beyond the cytoplasm – in the bacterial envelope – can also require input from protein folding and degradation factors. This is especially needed when under assault from so-called extracytoplasmic stresses that can compromise bacterial envelope integrity and protein folding in the periplasm. Such periplasmic-located quality control factors function in an ATP-exclusion vacuum, which necessitates different molecular mechanisms to overcome issues of protein folding and refolding in the periplasm. Their production is under the control of two extracytoplasmic stress responsive pathways; the σ^E factor and the Cpx two-component phosphorelay system [48, 57]. Interestingly, T3SS gene expression and/or subsequent component assembly in the bacterial envelope also requires input from the σ^E and Cpx pathways [Liu and Francis, unpublished data] [58-65]. However, it is not yet clear whether this involvement requires a direct interaction of σ^E or the Cpx regulator with T3S gene promoters. An indirect association is also plausible through the production of protein folding and degradation factors that could function to ensure the correct assembly of T3SS components in the periplasm. On this point, several studies support a role for protein folding factors, such as the periplasmic disulphide oxidoreductase DsbA, in T3SS assembly in the bacterial envelope [Francis, unpublished data] [66-71]. In addition, alteration of the stoichiometry of bacterial outer membrane components, such as LPS, lipoproteins, or integral outer membrane proteins, can also have profound effects on T3S [72-77].

Sec-Dependent Secretion and the Phage Shock Response

Many bacterial proteins do not function in the cytoplasm, but must first be exported through the cytoplasmic membrane to take up residence in the bacterial envelope. On most occasions, protein precursors predestined for export across the cytoplasmic membrane must remain in an unfolded state. This export competence is achieved through the interaction of newly synthesized polypeptides with a distinctive molecular chaperone, termed SecB [78, 79]. Among the large family of molecular chaperones, SecB is unique in its ability to carry-out two specialized functions. It functions as a chaperone maintaining a large and heterogeneous group of proteins in an unstructured form necessary for export. It also functions as an ‘export pilot’ where the unstructured proteins are delivered to the membrane-located Sec-translocon for export of substrates through the cytoplasmic membrane. Substrate delivery occurs because each SecB-substrate complex possesses exquisite binding affinity for the SecA ATPase, a cytoplasmic protein that provides some of the energy needed for export of each substrate through the integral inner membrane Sec-translocon [80]. So important is this *sec*-dependent secretion system to bacteria that many of its components are essential.

Defects in *sec*-dependent secretion across the cytoplasmic (inner) membrane also activate another extracytoplasmic stress response pathway called the phage shock response (PSR) [81]. The actual role of the PSR pathway is still not well defined, but it is considered important for maintenance of the protein motive force established across the cytoplasmic membrane [82, 83]. As briefly stated, a functional *sec*-dependent secretion system is a prerequisite for the early stages of T3SS component assembly in the bacterial inner and outer membranes. It therefore follows that an intact PSR is also necessary for optimal T3S [84].

Thus, with this brief overview one feature is for certain; T3SS synthesis and assembly is inextricably linked to several important physiological stress response mechanisms of Gram negative bacteria principally involving important functions of molecular chaperones. Clearly multiple layers of input are required to maintain quality control during the synthesis and assembly of such an inherently complex multi-component nanomachine.

Type III Chaperones – A Brief Introduction

The remainder of this article will detail a unique feature of T3SSs – their use of cytosolic chaperones in quality control. Since being first discovered in the laboratory of Guy Cornelis [85], T3S chaperones have been the focus of extensive research that has and enlightened our view of the T3S process [86-90]. In the early stages it was tempting to draw parallels with the molecular chaperones – particularly SecB of the *sec*-dependent secretion system. However, they have since proven to be structurally and functionally distinct molecules. T3S chaperones do not contribute to protein folding or refolding, nor do their functions depend on ATP binding or hydrolytic activity. They are small (less than 20 kDa) proteins with an acidic isoelectric point. Their function is to form highly specific transient substrate interactions to promote efficient type III-dependent substrate secretion and translocation. The specificity of each interaction is acute with T3S chaperones tending to target only one, or at most a few, cognate type III secreted substrates. In addition, their gene location is often near that of their target substrate(s), linking chaperone and substrate expression in the bacterial cytoplasm. For convenience, chaperones associated with target-cell contact inducible T3SSs (also referred to the non-flagella T3SSs) have been grouped into four general classes based on their structural similarities and the known functions of their cognate substrate(s) [88]. A fifth class represents chaperones of the flagella T3SS. Despite these commonalities, amino acid sequence similarity among individual chaperones within and between classes is usually quite low. A list of currently recognized T3S chaperones and their corresponding substrates is summarized in Table 1.

Table 1. T3S chaperones and their cognate targets

Organism	Class	Chaperone	Crystal structure	Suggested function(s)	Known secretion substrate(s)	Other binding partner(s)	References
<i>Yersinia</i> spp. (plasmid)	Ia	SycE (YerA)	Yes (alone and in complex with YopE)	Masking MLD (Stabilizer)	YopE effector	YscM2(LcrQ), YscE	[85, 95, 96, 101, 137, 139, 159, 177, 213, 349]
	Ia	SycH	Yes (complexed with YscM2)	Hierarchal Secretion	YopH effector & LcrQ (YscM1 & YscM2) negative regulator		[100, 137, 236, 238, 239, 349]
	Ia	SycO	No	Masking MLD (Stabilizer) System regulation	YopO(YpkA) effector	YscM1(LcrQ)	[173, 24]2
	Ia	SycT	Yes (alone)		YopT effector		[92, 93, 350]
	II	SycD (LcrH)	Yes (alone)	Stabilizer (Partitioning factor) System regulation	YopB & YopD translocators	YscY, YscM2, YscE & TyeA YscO inner membrane component	[104, 108, 111, 113, 142, 143, 155, 158-161, 240, 349]
	Ia ³	SycN and YscB	Yes (complexed with YopN)		YopN regulator		[102, 103, 351-353]
	V	YscE and YscG	Yes (complexed with YscF)	Anti-polymerization	YscF needle	YscE with YscG, TyeA, SycE & SycD	[123, 354, 355]
	nd	LcrG	No	System regulation	LcrV needle tip protein		[124, 125]

	nd	YscY	No		YscX	LcrH	[158, 160, 356, 357]
<i>Yersinia</i> spp. (chromosome)	Ia	SycP	No		YspP effector		[186]
	II	SycB	No	System regulation	YspB & YspC translocators	YsaE activator	[166, 232]
<i>Pseudomonas aeruginosa</i>	Ia	SpcS (Orf1)	No		ExoS & ExoT effectors		[358]
	Ia	SpcU	No		ExoU effector		[359]
	II	PcrH	No	Stabilizer (Partitioning factor)	PopB & PopD translocators		[147, 154, 162, 360, 361]
Organism	Class	Chaperone	Crystal structure	Suggested function(s)	Known secretion substrate(s)	Other binding partner(s)	References
	Ia ³	Pcr2 (PycN) & PscB	No		PopN		[362]
	V	PscE and PscG	Yes (complexed with PscF)	Anti-polymerization	Each other & the PscF needle	Each other	[120, 122, 159]
	nd	ExsC	No	System regulation	ExsE negative regulator	ExsC inhibitor	[223-225]
<i>Shigella flexneri</i>	Ia	IpgE	No		IpgD		[363]
	Ia	IpgA	No		IscB		[364]
	Ib	Spa15	Yes (alone)	System regulation	IpaA, IpgB1, IpgB2, OspC3, OspB effectors	OspD1 anti-activator	[91, 168, 233, 365]

Table 1. Continued

Organism	Class	Chaperone	Crystal structure	Suggested function(s)	Known secretion substrate(s)	Other binding partner(s)	References
	II	IpgC	No	Stabilizer (Partitioning factor) System regulation Hierarchical secretion	IpaB & IpaC translocators	MxiE activator	[109, 145, 157, 229-231, 366, 367]
<i>Salmonella</i> spp. (SPI-1)	Ia	SicP	Yes (complexed with SptP)	Secretion pilot	SptP effector	InvC ATPase	[99, 169, 216]
	Ia	SigE	Yes (alone)		SigD(SopB) effector		[94, 141, 368]
	Ib	InvB	Yes (complexed with SipA)	Secretion pilot	SipA(SspA), SopA(SipF), SopE, SopE2 effectors		[98, 191, 369-373]
	II	SicA	No	Stabilizer (Partitioning factor) System regulation Hierarchical secretion	SipB & SipC translocators	InvF activator InvI inner membrane component	[112, 161, 163, 228]
<i>Salmonella</i> spp. (SPI-2)	Ia	SscB	No		SseF effector		[374]
	IV ⁴	SseA	No		SseB pilus, SseD translocator		[153, 164, 165]

	V ⁵	SseE	No		SseB pilus	SsaN ATPase	[375]
EPEC and EHEC ¹	Ia	CesF	No		EspF effector		[376]
	Ib	CesT	Yes (alone)	Hierarchal secretion	Tir, Map, EspF, EspG, EspZ, NleA, NleG, NleH, NleH2 & NleI effectors	EscN ATPase	[94, 138, 214, 215, 377-380]
		CesD	No		EspB & EspD translocators		[118]
		CesD2	No		EspD translocator		[117]
	IV	CesAB	Yes (complexed with EspA)	Anti-polymerization	EspA pilus, EspB translocator		[114, 115]
	nd	CesA2	No		EspA pilus		[116]
<i>Chlamydia</i> spp.	V	CdsE & CdsG	No		CdsF needle	Each other	[381]
		Scs2 & Scs3	No		Putative CopB translocator		[382]
		LcrH-2	No		LcrE(CopN)		[383]
<i>Aeromonas</i> spp.	II	AcrH	No		AopB & AopD translocators		[110]
	V	AscE & AscG	Yes (AscE)	Anti-polymerization	AscF needle	Each other	[121]
<i>Bordetella</i> spp.	Ia	BtcA	No		BteA effector		[384]
<i>Edwardsiella tarda</i>	II	EscC	No		EseB & EseD translocators		[385]
	nd	EscA	No		EseC translocator		[386]

Table 1. Continued

Organism	Class	Chaperone	Crystal structure	Suggested function(s)	Known secretion substrate(s)	Other binding partner(s)	References
<i>Vibrio parahaemolyticus</i> (T3SS-1)	Ia	VecA	No		VepA effector		[387]
<i>Erwinia amylovora</i>	Ia	DspB/F	No		DspA/E		[388, 389]
<i>Pseudomonas syringae</i> ²	Ia	ShcA	No		HopPsyA		[390]
	Ia	ShcB1	No		HopPsyB1		[170, 391]
	Ia	AvrF	No		AvrE		[392]
Organism	Class	Chaperone	Crystal structure	Suggested function(s)	Known secretion substrate(s)	Other binding partner(s)	References
	Ia	ShcF/AvrPphF/ORF1	Yes (ORF1 alone)		HopF/AvrPphF/ORF2		[97, 393]
	Ia	ShcV	No		HopPtoV		[170, 394]
	Ia	ShcM	No		HopPtoM		[170, 395]
	Ia ⁶	ShcS1	No		HopS1, HopO1-1 & HopO1-2		[396, 397]
	Ia ⁶	ShcS2	No		HopS1, HopS2, HopO1-1 & HopO1-2		[397]
	Ia ⁶	ShcO1	No		HopS1, HopS2, HopO1-1 & HopO1-2		[396, 397]
	nd	HrpG	No	System regulation	HrpV		[398]

<i>Xanthomonas campestris</i>	Ib	HpaB	No	System regulation	AvrBs1, AvrBs3, AvrBsT, XopC, XopF1, XopJ & HpaA effectors	HpaA regulator HpaC and HrcV structural proteins	[134, 135, 399]
<i>E.coli</i> / <i>Salmonella</i> (Flagella biosynthesis)	III	FlgN	No	Anti-polymerization System regulation	FlgK & FlgL hook-associated proteins	FliJ inner membrane component FliI ATPase	[294, 295, 297, 298, 304, 305]
	III	FliT	No	Anti-polymerization System regulation	FliD filament cap	FlhC regulator FliI ATPase	[294, 295, 298, 303]
	III	FliS	Yes (complexed with FliC)	Anti-polymerization	FliC filament		[291-293, 296]
	III	 ²⁸	No	System regulation	FlgM anti-  ²⁸ factor		[302]

¹ The stated chaperones are not necessarily all present in the one strain. Their distribution is often pathovar and/or strain specific.

² EPEC, enteropathogenic *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*

³ Two co-chaperones that form a heterodimer, which resembles class Ia structural classification

⁴ Shares some characteristics with the class IV CesAB chaperone [115, 153], but does not contain the tetratricopeptide repeats characteristic of the class II translocator chaperone family [104]

⁵ Contains structural similarity to class V T3S chaperones despite interacting with a translocator-like protein (SseB) [375]

⁶ Contain secondary structure more reminiscent of class Ia even though they target multiple effector substrates [133]

nd - not determined.

Distinct Classes of Type III Chaperones

Class I Chaperones

Small class I T3S chaperones (~10-15 kDa) target those anti-host effector substrates translocated into the host cell interior. They can be further classified based on their specificity for only one cognate effector substrate (class Ia) or several effector substrates (class Ib).

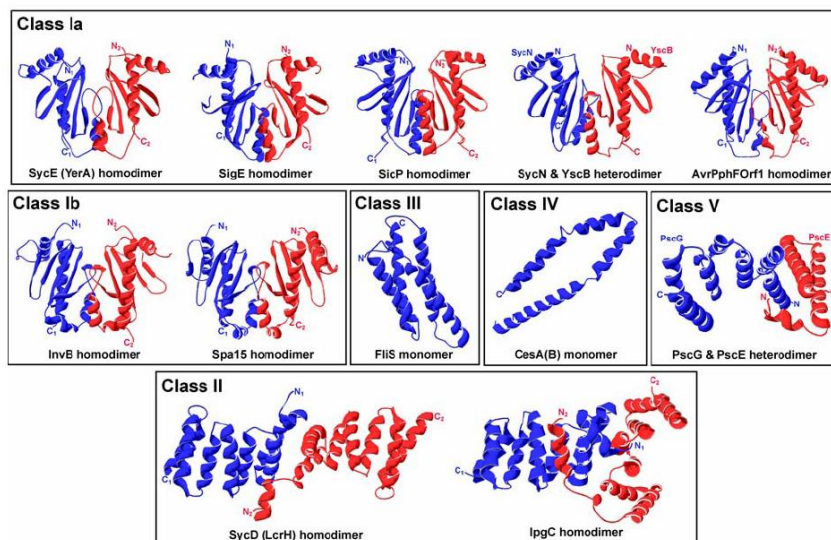


Figure 2. Ribbon models exemplifying structures of the various T3S chaperones associated with non-flagella and flagella mediated export through T3SSs. Models were drawn using the Swiss-PdbViewer [403] obtained from <http://www.expasy.org/spdbv/>. The monomers in the homodimer or heterodimer complexes are colored blue and red. Despite low sequence similarity, class Ia and class Ib T3S chaperones generally display remarkable structural conservation consisting of a tightly packed mix of β -sheets and α -helices. Significantly, these general structural characteristics remain conserved across diverse bacterial genera and are even maintained within the SycN-YscB heterodimer. However, other T3S chaperone classes are structurally distinct from each other and class I. For example, the analogous anti-polymerization function of class III and class IV T3S chaperones is not facilitated by similar structures and both function as a monomer; FliS adopts a compact four-helix bundle while CesA consists of a three helix hairpin. In addition, the class V T3S co-chaperone PscG that also plays an anti-polymerization role shares structural similarities with the class II T3S chaperone SycD possessing characteristic folds mediated by tetratricopeptide repeats. Class II T3S chaperones function as a homodimer although the two available crystal structures suggest the possibility of different orientations of the monomer pair – SycD is a head-to-head homodimer, while IpgC is an asymmetric homodimer. Examples shown are *Yersinia pseudotuberculosis* SycE (PDB identifier 1L2W; [139]), *Salmonella enterica* Typhimurium SigE (1K3S; [94]), *S. enterica* Typhimurium SicP (1JYO; [99]), *Y. pestis* SycN and YscB (1XKP; [103]), *Pseudomonas syringae* AvrPphF Orf1 (1S28; [97]), *S. enterica* Typhimurium InvB (2FM8; [98]), *Shigella flexneri* Spa15 (1RY9; [91]), *Aquifex aeolicus* FliS (1ORJ; [296]), enteropathogenic *Escherichia coli* CesA(B) (1XOU; [115]), *P. aeruginosa* PscG and PscE (2UWJ; [122]), *Y. enterocolitica* SycD (2VGX; [108]) and *S. flexneri* IpgC (3GYZ; [109]).

During the past few years, tertiary structures have emerged for several of these molecules, either alone [91-97] or in complex with cognate substrate [98-101]. Overall, these chaperones have a common mixed α/β fold. They function as homodimers, where a localized portion of a singular bound effector wraps around the chaperone surface in a non-globular extended conformation [98-101]. This gives a chaperone:substrate stoichiometry of 2:1. By and large, class Ia and Ib structures are remarkably similar despite their lack of amino acid sequence conservation (Figure 2). However, structural resolution of at least two class Ib chaperones, Spa15 and CesT, revealed a distinctive dimerization interface that generates different orientations of the monomer pairs [91, 94]. This exposes additional potential binding surfaces, which could be one reason why the class Ib chaperones (but not class Ia) display broad specificity for binding multiple substrates. Additionally, a heterodimeric complex composed of the monomeric co-chaperones SycN and YscB function together as a novel class Ia chaperone for the regulatory protein YopN of *Yersinia* [102]. A 1:1:1 tertiary structure of the YopN-SycN-YscB complex revealed the SycN-YscB heterodimer to be structurally similar to the homodimeric chaperone conformation (Figure 2). However, the obvious asymmetry generated by the SycN-YscB association means that the configuration of YopN wrapped around the chaperone heterodimer is distinct from other class I T3S chaperone-effector complexes [103].

Class II Chaperones

The slightly larger class II chaperones (~15-20 kDa) bind the two pore-forming translocator proteins that are essential for the translocation process. A striking structural feature of this class of chaperones is their triad of tetratricopeptide repeats (TPRs) [104]. Each TPR consists of two anti-parallel α -helices in which alternating small and large residues enable the opposing helices to interlock to form a versatile scaffold for mediating various protein-protein interactions [105-107]. Structural models based on solved structures of TPR-motif containing proteins gave the first indication that class II chaperones adopt an all α -helical structure utterly distinct from class I chaperones [104]. The modeled structure also indicated potential concave and convex surfaces available for substrate binding. This has since been confirmed via experimentally derived structures of SycD (LcrH) from *Yersinia* [108] and IpgC from *Shigella* [109] (Figure 2). Not only did these structures confirm the twisted arrangement of the TPR motifs that gave rise to the modeled concave and convex binding surfaces, but they also confirmed a homodimeric conformation that had been suggested earlier [110-113]. Notably, class II chaperone dimerization is an essential feature, because a stable monomer unable to dimerize failed to rescue the chaperone defective bacterial mutants [108, 109]. However, it is not yet clear how this dimer is actually arranged given that the two solved structures are different – SycD existed as a head-to-head homodimer, while the IpgC homodimer was distinctly asymmetrical (Figure 2). Perhaps both homodimer forms are possible in nature and are biologically relevant.

Miscellaneous Classes of Additional T3S Chaperones

The class IV and class V T3S chaperones (note that class III is reserved for chaperones of the flagella T3SS – see later), consists of a heterogeneous group of molecules that make up the remaining identified chaperones. At least conceptually, the class IV chaperone CesAB

might be functionally related to other T3S chaperones, even though the only physical property it shares to other chaperones is a small size [114]. Nevertheless, CesAB binds in a 1:1 ratio with the EspA filament protein, a surface extension of the T3SS needle produced by enteropathogenic *Escherichia coli*. However, the structure of CesAB is entirely distinct from other T3S chaperones (Figure 2) [115]. Adding to this intrigue, EspA polymerization at the bacterial surface also requires another T3S chaperone, CesA2, which is a peripheral inner membrane protein [116]. The extent of cooperation between CesAB and CesA2 is unknown. However, it looks comparable to the situation created by the highly aggregative translocator protein EspD that also requires two chaperones for secretion; the cytosolic CesD2 chaperone and the CesD chaperone, a peripheral inner membrane protein [117-119].

The PscE and PscG family of T3S co-chaperone heterodimers with specificity towards the T3S needle component, such as PscF from *Pseudomonas aeruginosa*, makeup the class V family [120]. Structural characterizations of members of this family revealed that all three proteins exist in a 1:1:1 ratio with the needle protein predominately in contact with PscG-like co-chaperone, while the function of the PscE-like co-chaperone is not obvious but it may stabilize PscG [121-123]. Interestingly, the PscG family also harbor TPRs with folds similar to the class II chaperone family (Figure 2) [108, 122, 123].

Finally, in the *Yersinia* research field, some have also proposed LcrG to be a chaperone for the needle tip protein LcrV. This is based on the formation of soluble LcrG-LcrV complexes and the observation that LcrG improves LcrV secretion levels [124]. This is not unanimously supported however, given that LcrG still possesses important regulatory roles in the absence of LcrV [125]. Nevertheless, this issue needs a resolution because homologues of both LcrG and LcrV exist in a sub-family of T3SSs encoded by a diverse group of important bacterial pathogens [10].

Analysis of various plant pathogens by whole genome and reporter-based screens has revealed many proteins with eukaryotic-like enzymatic motifs, all of which could be potential T3SS effector substrates [37, 42, 126]. Although a single strain may not encode every single identified effector, their repertoire can still reach over 50, which far exceeds the small effector substrate numbers associated with T3SSs belonging to pathogens of other hosts. Why plant pathogens need many more T3S effectors in comparison to other bacteria has been discussed in detail elsewhere. Those interested in the so-called ‘gene-for-gene hypothesis’ are encouraged to consult reviews on this topic, all which emphasize the fascinating and complex interplay between individual plant pathogens and their many susceptible and resistant plant hosts [37, 42, 127-132]. Probably, plant pathogens have developed extensive T3S chaperone networks to handle this vast amount of secreted cargo [10]. The occasional T3S chaperone has been discovered in plant pathogens that structurally resembles the class Ia variety; for example, avrPphF Orf1 from *Pseudomonas syringae* (Figure 2). However, some of these chaperones can differ from this usual paradigm in that they target multiple effectors that would normally classify them as class Ib T3S chaperones [97, 133] (Table 1). Another curiosity is the T3S chaperone, HpaB; to date the only known chaperone identified in *Xanthomonas campestris* [133]. Since HpaB supports the recruitment of several effectors to the secretion apparatus, it is considered to be a class Ib chaperone [134, 135]. However; in the absence of any other obvious T3S chaperone, an alternative possibility is that *X. campestris* uses HpaB as a global T3S chaperone for the export of all T3S substrates [135].

To develop this idea further, more must be learnt about HpaB biology. An obvious development would be solving the tertiary structure, either alone or in complex with various substrates, in order to make meaningful comparisons with other class I T3S chaperones.

Type III Chaperone-Substrate Recognition

Class I Chaperone – Substrate Interactions

Initial deletion mutagenesis experiments mapped the class I chaperone binding site to within the first ~100 amino acid residues of the cognate effector substrate [136, 137]. This has since been independently confirmed by various protease footprinting experiments whereby these chaperone binding domains (CBD) are protected from proteolytic digestion [98, 99, 103]. These findings are indicative of the CBD being one discrete modular domain at the N-terminus of effector proteins (Figure 3A). This has gained even further support from a study where CBD's were successfully exchanged between effector substrates with retention of function [138]. In a few cases, the CBD has been co-crystallized in a complex with the cognate class I T3S chaperone [98-100, 103, 139]. Within each of these structures, the CBD from a singular substrate wrapped around a chaperone dimer as an extended, non-globular polypeptide, interacting with hydrophobic patches on the chaperone surface (Figure 3B). Whether the bound CBD is actually unstructured has been recently questioned however – in the only structural study to use an entire substrate, the unfolded CBD of free substrate actually assumed an ordered state upon chaperone binding [140]. Hopefully these inconsistencies can be overcome with improved methods to co-purify and co-crystallize complexes of chaperone with full-length substrate. Despite this, close scrutiny of all known class I T3S chaperone-substrate co-crystal structures revealed a common binding motif. Within a large hydrophobic crevice spatially conserved in each chaperone, intermolecular contacts were made with each cognate substrate via the insertion of a few hydrophobic residues that comprise part of a conserved β -sheet motif [98]. Mutagenesis of these key hydrophobic residues within the β -sheet motif of the substrate confirmed the biological relevancy of this chaperone-substrate interface [98]. Independent mutagenesis of the hydrophobic residues that compose the large hydrophobic crevice of the chaperone also confirmed their importance to the interaction with substrate [141].

The structural conformation of class I T3S chaperone is constant. Even in the absence of substrate, all class I T3S chaperone structures highlight a homodimeric or heterodimeric state with similar structural folds (Figure 2) and a surface dominated by electronegative charges with sporadic patches of pronounced hydrophobicity (Figure 4A) [91-101, 103]. Through defined mutagenesis, the hydrophobic patches on the chaperone surface have proven to be critical for substrate binding [94, 141]. In contrast, electronegative residues do not necessarily affect substrate binding, so they might be more important for recognizing a component of the T3SS, such as the system ATPase (discussed later) [141]. These findings tend to suggest that subtle differences in the surface distribution of these hydrophobic patches are a determining factor in chaperone-substrate binding specificity.

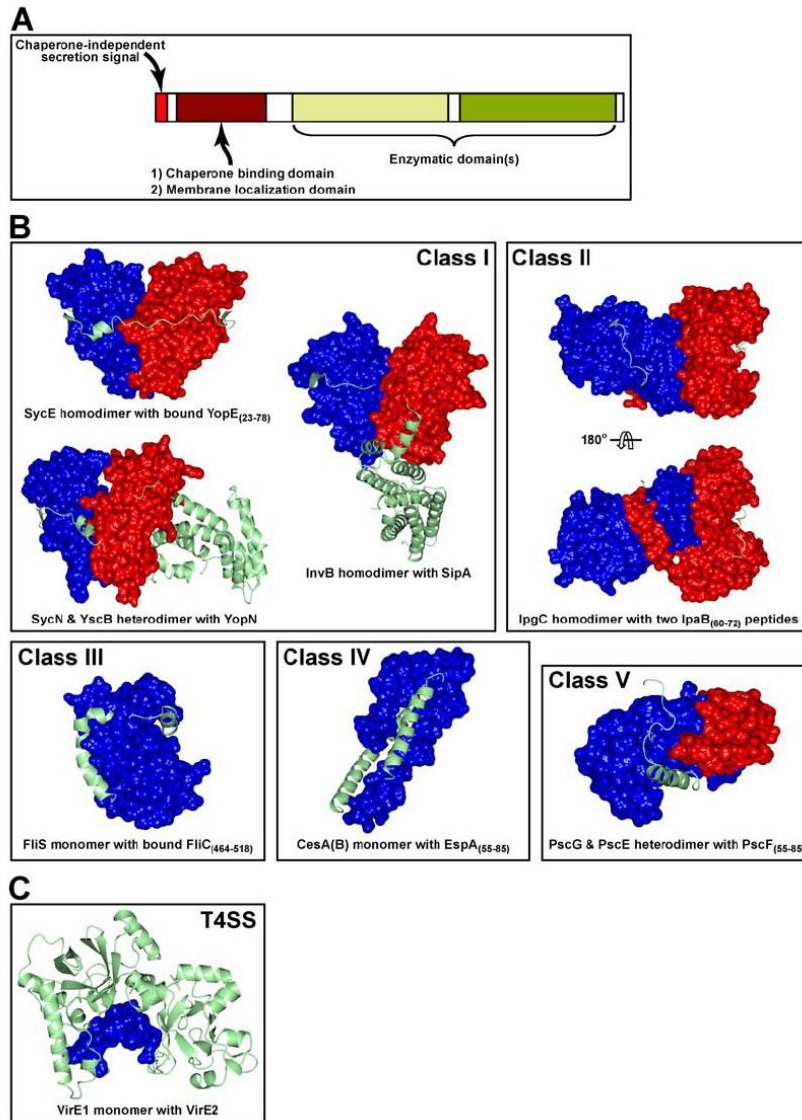


Figure 3. Structural characterization of T3S chaperone-substrate binding. (A) Translocated T3S effectors display a modular structure consisting of an N-terminal secretion signal, followed by the chaperone-binding domain (CBD) and one or more distinct effector domains that possess enzymatic activity when translocated inside eukaryotic cells. The CBD also overlaps with a membrane localization domain in some effectors that is involved in targeting the protein to the correct intracellular location. (B) An effector substrate – class I T3S chaperone interacts with a 1:2 stoichiometry. The effector monomer uses its CBD to bind to their class I T3S chaperone homodimer. This induces a localized partial unfolding of the CBD region; even in the presence of bound chaperone, downstream effector domains remain fully folded and enzymatically active. Translocator substrates bind to class II T3S chaperones in a ratio of 1:1. Each monomeric unit of the chaperone homodimer engages the CBD of a separate translocator substrate. In the example given, the IpaB peptide is seen transcending the concave binding groove of the chaperone monomer. As with the effector substrate CBD, the bound translocator substrate CBD exists in an extended conformation lacking any tertiary structure. While the structural context is quite distinct, a FliC filament also exists in a non-globular extended conformation when bound to the monomeric class III T3S chaperone FliS in a 1:1 configuration. In contrast, EspA

associates with the class IV T3S chaperone CesA through extensive coiled coil interactions, but also in a stoichiometry of 1:1. Binding of the PscF needle to the PscG class V T3S co-chaperone also represents a distinct interaction interface; the C-terminal PscF α -helix is engulfed by the concave binding groove formed by the tetratricopeptide repeat fold of PscG. It also appears that the third member of the 1:1:1 trimeric complex, PscE, does not directly engage the needle filament. (C) The structure of the T4SS chaperone VirE1 is a simple singular α -helix that is encased by a bi-lobed monomer of the VirE2 substrate. To some extent, this conformation is reminiscent of the FliS-FliC complex. Additionally, the manner in which EspA polymerization is inhibited by CesA could also explain how VirE1 prevents VirE2 polymerization [320]. Models were drawn using CCP4 MG (molecular graphics) software [404] obtained from <http://www.ytbl.york.ac.uk/~ccp4mg/> and with some helpful tips from Tobias Hainzl (KBC, Umeå University). Molecular surfaces of individual chaperone monomers are colored blue and red. The bound cognate substrate is depicted as a light green ribbon structure. Examples shown are already described in the legend to Figure 2, with the exception of *Agrobacterium tumefaciens* VirE1 and VirE2 (PDB identifier 3BTP; [320]) and *S. flexneri* IpgC and IpaB (3GY1; [109]).

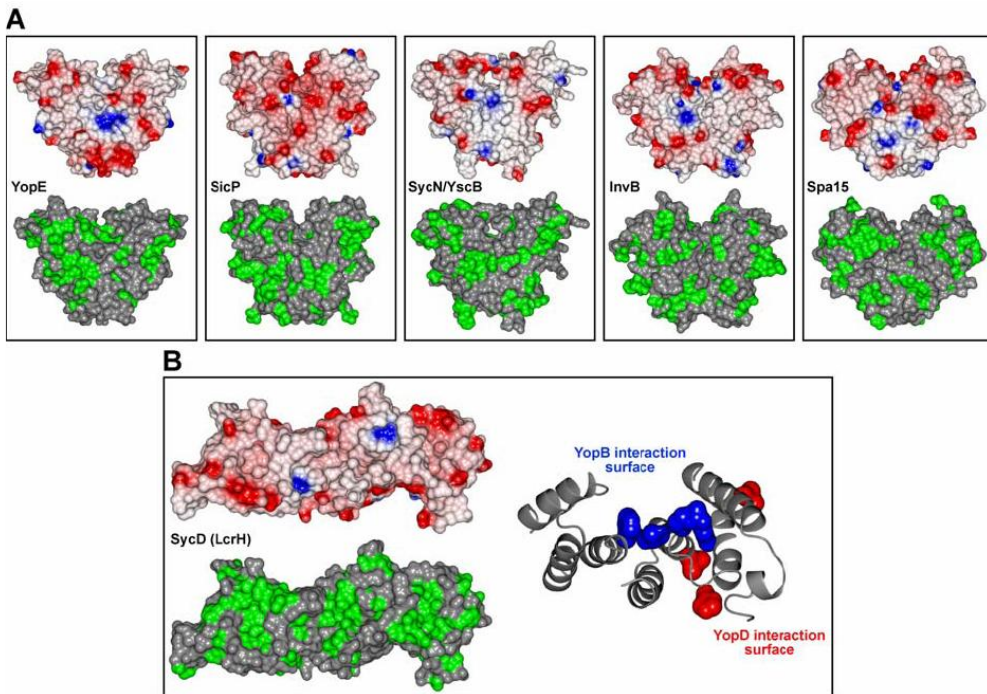


Figure 4. Determinants of the T3S chaperone-substrate interaction specificity. The molecular surfaces of representative class I (A) and class II (B) T3S chaperones have been colored according to their electrostatic (upper) and hydrophobic (lower) potential. An electronegative surface is in red, electropositive in blue and hydrophobic in green. Despite the structural orthology of T3S chaperones, each has a unique surface distribution of electronegative and hydrophobic patches. This key feature is considered to underpin chaperone-substrate specificity. Indeed, structure-based mutagenesis studies have confirmed the importance of hydrophobic and electrostatic residues in individual T3S chaperone function [154–156]. The distribution of these hydrophobic and electrostatic residues in the class II T3S chaperone LcrH may also contribute to directing the binding of one substrate YopB, to the concave surface and the second substrate, YopD, to the convex surface of the tetratricopeptide fold [155]. Models were drawn using CCP4 MG (molecular graphics) software [404] obtained from <http://www.ytbl.york.ac.uk/~ccp4mg/> and with some helpful tips from Tobias Hainzl (KBC, Umeå University). Examples shown are already described in the legend to Figure 2.

Class II Chaperone – Substrate Interactions

Mapping the class II T3S chaperone binding domains within the translocator substrates has proven to be more difficult. Each T3SS generally exhibits two primary translocator substrates. These do not display any sequence similarity, but do contain similar structural characteristics [26]. The first corresponds to the YopB family, which can consist of an N-terminal coiled-coil domain followed by two hydrophobic potentially membrane spanning domains. The second corresponds to the YopD family that possesses one (or sometimes two) hydrophobic transmembrane domains often followed by a C-terminal amphipathic α -helical domain. Arguably, interactions between the *Yersinia* LcrH (SycD in *Y. enterocolitica*) T3S chaperone and the YopB and YopD translocators have been studied in most detail. While deletion mutagenesis analysis failed to uncover a singular discrete chaperone binding domain in YopB [142], a similar strategy suggested a large N-terminal domain (including the putative transmembrane domain) and the C-terminal amphipathic α -helix of YopD contributed to chaperone binding [113, 143]. Moreover, hydrophobic residues within this amphipathic α -helix were critical for the chaperone interaction [143]. The amphipathic domain is essential for YopD function, possibly contributing to YopD oligomerization [Costa *et al.*, unpublished data] [113, 144]. Perhaps binding by LcrH therefore prevents putative YopD self-association inside bacteria. From recent work, it is apparent that the AcrH chaperone from *Aeromonas hydrophila* also binds to its cognate substrates in a similar 1:1 stoichiometry [110]. The entire N-terminal half of AopB (a YopB homologue) is protected from limited protease digestion by bound AcrH chaperone. In addition, AopD (analogous to YopD) also uses the N-terminus encompassing a transmembrane domain and the C-terminal amphipathic α -helical domain to bind chaperone. Moreover, in a watershed structural study visualizing the *Shigella flexneri* IpgC chaperone interaction with IpaB (analogous to YopB), a N-terminal CBD motif incorporating the six residue PELKAP peptide sequence was identified in this substrate [109]. Actually, two CBDs were crystalised; one associated with each of the concave binding grooves found within the two chaperone monomers of the homodimer unit implying a stoichiometry of 2:2 (Figure 3B). Reportedly, similar peptide motifs also exist in the N-terminus of other translocator class proteins. This is consistent with IpgC binding to IpaC [145] and the *Salmonella enterica* Typhimurium SicA chaperone binding to SipB [146] only at their respective N-termini.

The translocator substrates are naturally aggregative, highly hydrophobic pore-forming transmembrane proteins capable of causing membrane damage if not controlled. For instance, over-expression of either YopB or the *Pseudomonas aeruginosa* PopB protein in the absence of their cognate chaperone results in growth cessation and eventual lysis of host bacteria [Costa *et al.*, unpublished data] [142, 147]. This nicely illustrates that an important role of the class II T3S chaperones is to conceal the hydrophobic transmembrane domains of their cognate substrates in order for them to be maintained in a stable and soluble form. It may also be relevant that some translocators display multiple functions – SipC and IpaC (YopD-family members) have both pore-forming activity in biological membranes and also exhibit intracellular effector functions [148-152]. In addition, a portion of YopD is also translocated into the host cell cytosol, which may imply an additional role(s) inside target host cells [144]. This suggests that the translocator proteins may possess discrete binding domains for interactions with protein partners of different origin and function. Perhaps an extensive

coverage of the substrate by the chaperone is therefore needed to overlap with some of these possible binding sites. In this way, chaperone binding could minimize any premature intra- or intermolecular interactions from forming.

A few studies have investigated the molecular contributions made by the class II T3S chaperone in complex formation with substrate. The chaperone N-terminus has been suggested to contain information necessary for function, such as substrate binding or secretion [118, 153]. An *in silico* analysis of multiple sequences provided the first major breakthrough however, bringing about the discovery of a tandem array of TPRs in this class II chaperone family [104]. Using this structure as a scaffold for analysis of a large collection of mutagenesis data, our laboratory was able to confirm a role for these TPRs in chaperone stability, substrate binding and substrate secretion [104, 143, 154-156]. These data even revealed the possibility of simultaneous binding of chaperone to both substrates given the identification of two discrete substrate binding interfaces – YopB preferentially binds to the concave face while YopD attaches to the convex face of LcrH (Figure 4B). This possibility is supported by documented trimeric complexes of LcrH-YopB-YopD [142] and AcrH-AopB-AopD [110], although it appears unlikely to occur for IpgC, IpaB and IpaC [157]. Realistically, the only way to unravel these mysteries is to produce structural data of chaperone in complex with one or both substrates. This is a major technical challenge given the hydrophobic transmembrane properties of the translocators. However, a key advance in this direction has come with two recent studies. The crystal structure of LcrH/SycD was the first experimental structure of a class II chaperone specific for the translocators [108]. While this structure confirmed the presence of TPRs, it also demonstrated the functional relevance of SycD dimerization, formerly an underappreciated protein-protein interaction interface in T3S chaperone biology. The second study was able to co-crystallize the IpgC chaperone in complex with truncated IpaB (a YopB analogue) [109]. Not only did this study define the CBD within IpaB, but it also highlighted the extensive interactions that mediate chaperone substrate binding (Figure 3B). In particular, three substrate binding pockets were discovered in the concave binding groove on IpgC that are well conserved in class II T3S chaperones. These marry with the CBD contained within the IpaB translocator substrate. As such motifs are evident in other IpaB-related translocators, this is indicative of a conserved mechanism of substrate interaction for this class of T3S chaperones [109].

It is also notable that class II chaperones can possess other known interaction partners. For example, SycD/LcrH is also known to engage YscO, YscY, YscE and TyeA [111, 158-161], although the biological relevance of such interactions have not yet been sufficiently explored. Having the SycD crystal structure will now allow a directed exploration into these additional chaperone-substrate complexes as well. TPR arrays are highly versatile protein-protein interaction motifs able to contribute multiple binding sites for interactions [105-107]. Their conservation in class II T3S chaperones suggests that determining the full repertoire of chaperone binding partners and understanding the corresponding functional consequences of these interactions to T3S will be an area of fruitful investigation.

Other Chaperone – Substrate Interaction Classes

On the back of important structural studies, the molecular basis of binding between the class IV chaperone CesAB to the EspA pilus has been elaborated. Not only is the CesAB

structure distinct, but the CesAB::EspA interaction interface is quite different from the chaperone-substrate interactions discussed above. It is dominated by coiled-coils contributed by both the T3S chaperone and the substrate (Figure 3B) [115]. Another distinct interaction occurs between the class V T3S co-chaperone PscG/YscG family and the needle component PscF/YscF. In this complex, the C terminus of the needle protein is buried within the concave hydrophobic groove formed by the TPR-containing co-chaperone (Figure 3B) [121-123]. The function of the third member of this trimeric complex, PscE/YscE, is intriguing for it clearly associates with PscG, but not the needle.

Demarcating Type III Secretion Chaperone Function

One of the perplexing aspects of T3S chaperone biology is their apparent involvement in many T3S processes. Initially, T3S chaperones were broadly identified as bodyguards for their cognate substrates, ensuring their pre-secretory stabilization and efficient secretion. Such roles were initially likened to the function of the ‘traditional’ molecular chaperone SecB. During the last few years however, it has become apparent that T3S chaperones also ensure that their substrates are secreted through the correct T3S pathway, and perhaps even orchestrate a hierarchal secretion among the multiple substrates being secreted. Adding to this functional complexity is the clear evidence that some of these chaperones regulate gene expression and thereby create a molecular link between expression and secretion of T3S substrates. A summary of the major T3S chaperone functions are highlighted in Figure 5. Because most T3S chaperones tend to boast variations in their functional repertoires, there is some difficulty in pin-pointing their precise biological function. Such intrinsic functional diversity is probably a consequence of two issues; the unique physical and functional parameters displayed by the vast amounts of potential T3S substrates and the specific environmental niches where different bacteria employ their customized T3SSs. What follows below is an account of the most notable T3S chaperone functions. However, it is important to realize that these functions need not be mutually exclusive and that individual chaperones can exhibit multiple functions.

Preventing Premature Interactions

Partitioning Factors – Masking Substrate Interaction Domains

Arguably, one of the less contentious roles for T3S chaperones is that ascribed to the class II family – that of a partitioning factor to prevent premature association and subsequent degradation of the two translocator proteins prior to their secretion.

This was first described in an elegant study by Menard and colleagues [157] whereby the IpgC chaperone stabilizes both IpaB and IpaC by preventing a premature IpaB-IpaC interaction in the cytoplasm of *Shigella flexneri*.

Similar roles have subsequently been suggested for other chaperones of this translocator class [118, 142, 147, 153, 155, 162-166].

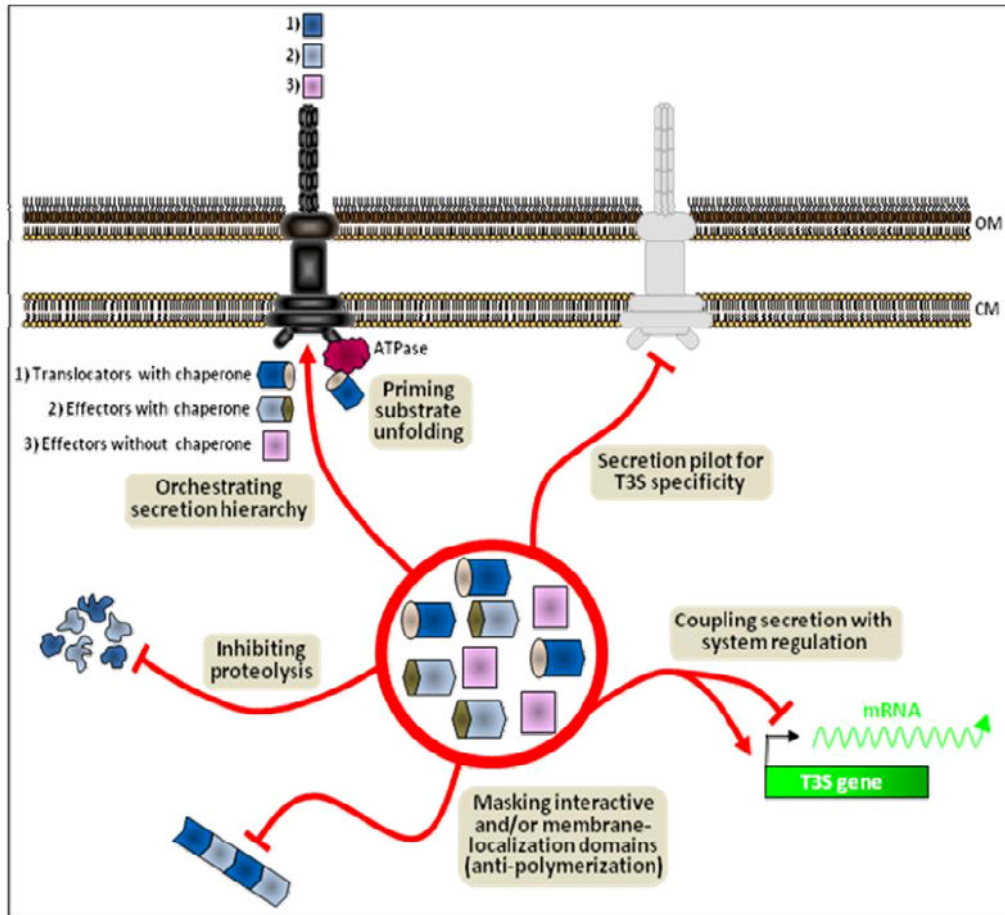


Figure 5. A schematic summary of the T3S chaperone molecular toolkit. At least six major functions have been ascribed to one or more T3S chaperones. Each function makes a significant contribution to efficient T3SS assembly and/or substrate secretion. The ratio of substrate bound to free chaperone is used to sense the status of T3SS activity that in turn influences the level of T3SS gene expression. Substrate bound by chaperone is also piloted to the cognate T3SS ensuring specific secretion through the correct secretion system. Bound chaperone can also be recognized by the system ATPase, aiding in localized substrate unfolding as a prerequisite for efficient substrate secretion. Specific recognition of different chaperone classes by a component of the T3SS probably also contributes to ordering substrate secretion to ensure that middle substrates (the translocators) are secreted before late substrates (the anti-host effectors). Finally, pre-made substrate pools are also stabilized in the bacterial cytoplasm through association with cognate chaperone. This prevents premature intra- or intermolecular interactions and confers resistance to endogenous proteolytic activity. OM – outer membrane, CM – cytoplasmic membrane.

It remains to be seen however if this partitioning is achieved by simultaneous binding of both substrates to the same chaperone molecular unit. Based on our own studies with class II T3S chaperones, this seems conceivable since the PcrH and LcrH chaperones appear to contain two distinct substrate binding sites – possibly one for each substrate [154, 155] (Figure 4B). One also wonders whether the chaperone interacts with structurally similar domain(s) within each translocator protein and whether these domains are also responsible for establishing the translocator-translocator interaction. If so, class II T3S chaperones would function to partition the translocators by shielding these interaction domains. This possibility has been endorsed by a recent structural characterization of the IpaC N-terminus in which the IpaB and IpgC binding domains are believed to partially overlap [145]. It is also consistent with a conserved N-terminal chaperone binding motif, originally identified in IpaB, present in a variety of other functionally related translocator proteins [109].

While the role of partitioning factor is clear, it is also worth remembering that some translocators are toxic to bacteria when expressed in isolation [Costa *et al.*, unpublished data] [142, 147]. Thus, by binding to the substrate N-terminus, class II T3S chaperones also mask the hydrophobic transmembrane domains that alleviates potential substrate toxicity. This means that this T3S chaperone family is essential for maintaining pre-made pools of the pore-forming translocator proteins in an innocuous form inside the bacterial cytoplasm.

In contrast, only some class I T3S chaperones are needed to stabilize pre-made substrate pools [167-170] used for rapid deployment following target cell contact [168, 171]. While it is appreciated that pre-made substrate pools can have instant impact on the outcome of a bacteria-host cell interaction, it is not immediately obvious why some substrates are unstable and degraded in the absence of their cognate chaperone while others remain stable or do not even require a chaperone at any stage of their production or secretion. Quite probably, proteins involved in premature intra- or inter-molecular interactions in the bacterial cytoplasm are targeted for proteolytic degradation. Presumably, these premature interactions would normally be prevented by bound chaperone, as is the case for substrates bound by class II T3S chaperones (described above). It is therefore intriguing that non secreted pools of effector substrates are susceptible to Lon protease digestion in the *Pseudomonas syringae* phytopathogen lacking the corresponding cognate chaperones, whereas substrate-chaperone complexes are resistant to Lon-dependent degradation [170]. It is not yet clear why some T3S effectors are specifically targeted for Lon-dependent degradation. Intuitively, these data highlight a potential universal mechanism for pre-secretory stabilization of selected T3S substrates in all T3SSs; that of protection from Lon-dependent proteolysis via the action of T3S chaperones. As previously mentioned however, not all T3S substrates are prone to degradation since some are stable in the bacterial cytoplasm even in the absence of a T3S chaperone [172]. In these cases, the cognate chaperone is more than likely essential for substrate targeting to the secretory apparatus (discussed in the next section).

Shielding the Membrane-Localization Domain of Effector Substrates

It has also recently surfaced that several effectors possess a membrane localization domain (MLD) within this first 100 amino acids just downstream of the small chaperone-independent secretion signal. Significantly, the MLD overlaps with the CBD (Figure 3A) [173]. MLD's target translocated effectors to host cell membrane compartments in a location

where they presumably engage their respective host molecular targets [173-176]. In the absence of a cognate chaperone, effector substrates harboring MLD's are prone to aggregate in the bacterial cytoplasm, while derivatives engineered to lack their MLD remain soluble even if they no longer bind to their cognate chaperone [173]. In fact, only in the presence of chaperone do MLD-containing proteins remain in a soluble form [139, 173, 177]. This work highlights another related function of some T3S chaperones; shielding their cognate substrate MLD's to avoid aggregation before they are successfully translocated to the eukaryotic cell interior.

Anti-Polymerization Factors

T3SSs are characterized by extracellular appendages that protrude from the outer membrane in much the same way as the bacterial flagella. In phytopathogens, this takes the form of a pilus-like structure, while enteropathogenic *E. coli* have a filament that extends out from the typical needle present on all other cell-contact inducible T3SSs (see Figure 1). These three types of T3S-associated structures consist of a highly aggregative major subunit that is prone to rapidly polymerize prematurely into proteinaceous extensions in the bacterial cytoplasm when over expressed alone [115, 120]. Not only is this potentially toxic for the bacteria, but it also prevents proper T3SS biogenesis as these polymers are simply too large for secretion. To solve this problem, T3S chaperones such as CesAB [114, 115] or the co-chaperone family of PscE and PscG [120-123] are employed to trap the cognate subunits in a monomeric state prior to secretion.

Structural modeling has been used to predict how these T3S chaperones may actually prevent this subunit polymerization. In particular, structural data from the non-polymerizable needle monomer derivatives PrgI (*S. enterica* Typhimurium) [178], MxiH (*Shigella flexneri*) [179] and BsaL (*Burkholderia pseudomallei*) [180] was merged with the structural data of PscF needle subunits in complex with the PscE-PscG co-chaperone family (see Figure 3B) [122, 123]. This showed that by binding to a helical domain located in the needle subunit C-terminus, the PscG co-chaperone could prevent this domain from contributing sites for extensive surface contacts between polymerizing subunits [27]. It is still too early to tell whether this anti-polymerization model is broadly applicable to all T3SSs, especially since cognate T3S chaperones are not yet identified for the needle proteins in most T3SSs. It is also important to establish the precise role for each of the co-chaperones. For example, is the PscG member solely responsible for anti-polymerization activity? Does the PscE derivative ensure co-chaperone stability and/or specific substrate piloting to the secretory apparatus? Whatever their individual roles, this model still nicely illustrates how T3S (co)chaperones can be used to quell the polymerization tendencies of highly aggregative proteins prior to transportation to their final assembly platform. In essence, this role is akin to a partitioning function ascribed to class II T3S chaperones.

Secretion Pilot for T3SS Specificity

T3S Promiscuity – A Conundrum

T3SSs are generally quite promiscuous in that a given substrate belonging to one system can readily be secreted by another heterologous system. This secretion promiscuity need not be restricted to genetically related systems. For example, effectors of animal pathogens are efficiently secreted by phytopathogens even though the latter produce a morphologically distinct T3SS [181, 182]. In addition, primordial T3SSs associated with the flagella biosynthesis pathway possess the ability to secrete, but not translocate, type III substrates associated with bacterial virulence [183-186]. This problem is compounded by the fact that a single genome can encode multiple independent T3SSs: those promoting motility associated with either polar flagella or lateral flagella, and those associated with eukaryotic cell contact (which can often exceed one system per genome) [10]. Because all these systems can be produced in the one bacterium, mechanisms must be in place to insulate each T3SS and ensure that individual substrates are secreted through the cognate system. One solution could have been to append common secretion signal sequences to all substrates of the same system; those with one particular signal are directed to one system, while those with another are directed to a different system and so on and so forth. This concept has been described previously for substrates of two different T3SSs produced by *S. enterica* Typhimurium (the SPI-1 and SPI-2 systems) [187], but in light of T3S promiscuity it has not gained wide acceptance.

On the other hand, in response to differing environmental cues it is clear that bacteria can isolate the expression of one system from all others. For example, *S. enterica* Typhimurium encodes two virulence associated T3SSs with distinct functions – one is encoded on the *Salmonella* pathogenicity island SPI-1 and is necessary for bacterial invasion into eukaryotic cells; the second on SPI-2 and functions in bacterial intracellular survival [188, 189]. Responding to complex, but distinct, extracellular and intracellular environmental cues, the expression of SPI-1 and SPI-2 components can be inversely coordinated by an interplay between various global and specific gene regulators [190]. This ensures that products promoting invasion are induced while products promoting intracellular survival are repressed and vice versa. In addition, these regulatory mechanisms also coordinate effector substrate production so that high levels are available for secretion at the same time as their cognate T3SS is active.

T3S Chaperones Confer Substrate Secretion-Pathway Specificity

However, another decisive means for ensuring substrate secretion-pathway specificity concerns an activity of T3S chaperones. As stated earlier, adjacent to the extreme N-terminal secretion signal of individual effector substrates lies about 100 amino acids that are essential for specific binding of their cognate chaperone – the CBD (Figure 3A). Mutagenesis within this CBD of *Salmonella* SPI-1 effectors, SptP and SopE, not only prevents respective SicP and InvB chaperone binding, but also leads to substrate secretion via the non-cognate T3SS of flagella [191, 192]. This demonstrates that the presence of CBDs in the effector N-terminal region and subsequent binding by the cognate chaperone is required to guarantee that

substrates are secreted through the correct T3SS. Importantly, this is not just a phenomenon of T3S chaperones of the effector class. Our own analysis revealed that in the absence of the LcrH chaperone, *Y. pseudotuberculosis* can secrete the YopB and YopD translocator proteins independent of a functional cognate Ysc-Yop T3SS; secretion probably occurs via another independent T3SS [Edqvist *et al.*, unpublished data]. Moreover, truncated versions of SipB translocator from *S. enterica* Typhimurium, which are unable to bind cognate chaperone, are also secreted via the flagella T3S, but not the SPI-1 T3SS [146]. T3S chaperones must therefore confer secretion pathway specificity, which prevents type III substrates associated with host-cell contact from being secreted by the flagella system, which might normally function as the default T3S pathway.

This suggests that the substrate-T3S chaperone complex must present a structural motif specifically recognized by the cognate T3SS. In the absence of either chaperone or the CBD within the substrate, secretion specificity is lost allowing secretion through the flagella pathway. Presumably, flagella-mediated secretion is supported by an extreme N-terminal secretion signal within the first 20 amino acids that represents a generalized canonical secretion signal used by all T3SS substrates – otherwise known as a chaperone-independent secretion signal (see below and Figure 3A). In contrast, the substrate-chaperone complex represents a second, unique secretion signal that aligns substrates for secretion via specific non-flagella T3SSs. As mentioned already in this chapter, several class I T3S chaperones have either been crystallized alone or in complex with their cognate substrate. Close scrutiny of multiple primary amino acid sequences and tertiary structures uncovered a conserved hydrophobic binding pocket in these chaperones that specifically accommodated a complementary short peptide of spatially conserved hydrophobic residues found within the chaperone binding domain of bound cognate substrates [98]. In light of this finding, it was suggested that many different T3SS substrates and cognate chaperones utilize this type of universal receptor-ligand interaction [98]. This structural commonality might well be the molecular mechanism governing secretion-pathway specificity that ensures the appropriate delivery of individual substrates to their cognate T3SS associated with bacteria-host cell contact. It is also otherwise known as a chaperone-dependent secretion signal. How this targeting mechanism may contribute to substrate secretion is discussed later.

Orchestrating Hierarchical Secretion

Prioritizing Early Substrate Secretion – The Specificity Switch

T3SSs are able to secrete three types of substrates; needle associated proteins and anti-activators (early substrates), translocator proteins (middle substrates) and effector proteins (late substrates). Following needle assembly completion (or the hook-basalbody in flagella biogenesis), a wealth of evidence supports a switching mechanism that triggers a change in substrate secretion from ‘early’ needle components (or flagella hook components) to the ‘middle’ translocator and ‘late’ effector proteins (or the flagella filament proteins). This is based on the crosstalk between the YscU-like, YscP-like and YscI-like proteins [193-199]. The inter-related sensing function of these protein families is universally conserved in both flagella and non-flagella T3SSs. The interested reader is referred to the following reviews for

details of the complex molecular mechanism [13, 200-203]. Despite this clarity, comparatively little is known about a secretion order that may exist among the middle and late secretion substrates. Current dogma stipulates however that en-route to the eukaryotic cell interior, effector toxins pass through translocon pores in the host-cell plasma membrane that are composed of the translocator proteins [17, 26, 27]. For assembly of a functional type III translocon, this therefore implies that translocators must be secreted before effectors.

Creating Secretion Order Among Middle and Late Substrates – T3S Chaperones and Beyond

Laboratory based secretion assays on fully induced T3SSs reveal no temporal differences between middle and late secretion substrates. Contributing to this is the asynchronous nature of T3SS assembly on the bacterial cell surface during in vitro culturing. At any time point, a bacterial cell will harbor both ‘immature’ T3SSs capable of secreting only early substrates and ‘mature’ T3SSs competent of secreting both middle and late substrates. This variable output thereby disguises any hint of ordered secretion. However, recent genetic evidence has provided the first indications that middle substrates are prioritized for secretion over late substrates. A heterogeneous family of proteins headlined by InvE of *S. enterica* Typhimurium influence T3SS substrate specificity by exhibiting a range of functions that essentially either promote the export of translocator (middle) substrates and/or inhibit the export of effector (late) substrates [204-212]. How these proteins function to distinguish translocators from effectors can vary. For example, InvE directly recognizes translocator-chaperone complexes that may prioritize their secretion [206]. Alternatively, the C-terminus of SepL may specifically bind effector substrates to stall their T3S from *E. coli* [204] or MxiC may bind the system ATPase creating a blockade that similarly inhibits effector secretion by *S. flexneri* [205]. No matter how it is achieved, these studies all represent further examples of built-in mechanisms for orchestrating hierarchical secretion among the T3S translocator and effector substrates.

T3S chaperones could also assist in establishing a secretion hierarchy. This idea was born from the initial discovery that only in the presence of other T3S substrates did the translocation of YopE into infected eukaryotic cells require both the cognate SycE chaperone and the internal CBD in YopE [213]. This suggested that SycE and the CBD conferred upon YopE a competitive advantage for secretion when in the presence of other substrates [213]. This has been reinforced by the proposal that substrates with two N-terminal secretion signals – the chaperone-independent and chaperone-dependent signals (see below) – might be prioritized for secretion over those substrates with no apparent chaperone-dependent signal [139]. As discussed in the following section, this is consistent with the perceived targeting role of chaperones on the back of their ability to bind to the ATPase, a core constituent of the secretory apparatus [98, 214-216]. In turn we have proffered that the obvious structural demarcation between class I and class II T3S chaperones could present two distinct recognition motifs to the ATPase as a mechanistic basis for establishing an order to secretion of the middle and late substrate categories [Amer *et al.*, unpublished data] [104]. However, a caveat in this concept is that so far no class II T3S chaperone is known to bind to the T3SS ATPase. Although this is not proof that an interaction does not occur, it is possible that recognition might be mediated through an additional component located at, or near, the portal

for substrate entry into the secretion nanomachine. For example, the InvE protein is known to physically interact with translocator-chaperone complexes to selectively promote their secretion prior to the late effector substrates [206]. In addition, the inner membrane proteins InvI (from *S. enterica* Typhimurium SPI-1) and YscO (from *Y. enterocolitica*) may contribute to selective secretion of middle ‘translocator’ substrates before late ‘effector’ substrates [161]. These proteins actively bind to free class II, but not class I T3S chaperones at the cytoplasmic membrane following secretion of their translocator substrates through the T3SS. The idea is then that InvI/YscO function to recycle these ‘empty’ chaperones back to new substrates waiting in the secretion cue advancing their secretion more rapidly [161].

It is also worth noting that T3S chaperones establish secretion hierarchy through control of gene transcription. However, a discussion of this function will immediately follow below. In addition, it is worthwhile to highlight how regulation by proteolysis may influence temporal substrate secretion. In the phytopathogen *P. syringe*, the Lon protease effectively degrades T3S effector substrates, but only in the absence of their cognate chaperone [170]. Since Lon activity can be suppressed by T3S chaperones, they could theoretically implement a secretion order among the effectors based upon the degree of this suppression [170]. One can imagine that a chaperone providing significant protection from Lon-mediated degradation will leave more substrate available for secretion and vice versa. Significantly, powerful advances in fluorescent tagging techniques combined with real-time imaging offer unique opportunities to analyze spatial and temporal dynamics of type III substrate secretion. Already, these assays have proven sufficiently sensitive to detect hierarchal effector translocation into infected eukaryotic cells [217-220]. Therefore, they should also enable further exploration into the role of proteolysis as a mechanism for generating type III substrate secretion hierarchies.

Coupling of Secretion with System Regulation

Type III gene transcription is often coordinated through the activity of AraC-like transcriptional activators [44, 45, 221]. In addition, T3SSs are frequently subject to feedback inhibition such that secretion competence is normally required for expression of type III genes. An active T3S is sensed through the cytoplasmic accumulation of free ‘co-activator’ and the concomitant depletion of a secreted ‘anti-coactivator’.

Such information is then conveyed to an AraC-like transcriptional activator that establishes a cascade of type III gene activation. With differing degrees of complexity, variations of this general theme are built into several T3SSs. In every case however, there is reliance upon the ability of a T3S chaperone ‘co-activator’ to target for secretion its cognate secreted substrate ‘anti-coactivator’. Examples of this T3S chaperone-dependent mode of system regulation are illustrated in Figure 6.

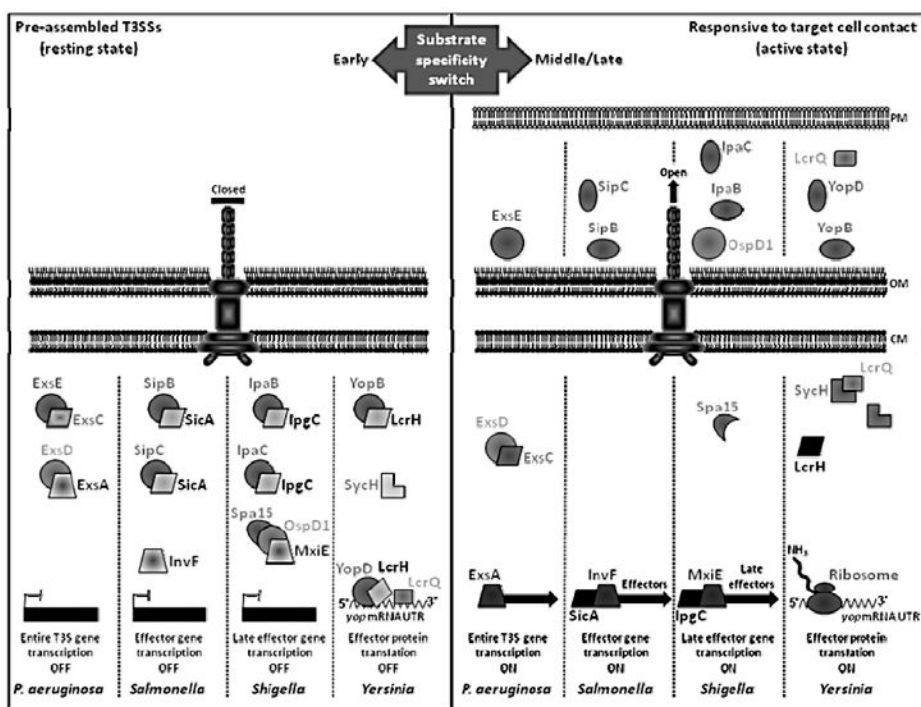


Figure 6. Schematic diagram summarizing the involvement of T3S chaperones in regulating T3SS activity in the animal pathogens *Pseudomonas aeruginosa*, *Salmonella enterica* spp. and *Yersinia* spp. Early substrates comprising needle associated proteins are secreted first and assembled into a needle complex. During which time, production of late secreted substrates is kept in check by the action of T3S chaperones. In *P. aeruginosa*, the anti-anti-activator function of the ExsC chaperone is guarded by non-secreted pools of cognate substrate (ExsE), while the anti-activator ExsD silences the transcriptional activator ExsA. In *S. enterica* and *Shigella*, the co-activator T3S chaperones SicA and IpgC are inhibited by bound substrate (SipB/C and IpaB/C respectively). In *Shigella*, the anti-activator complex of Spa15/OspD1 further refines transcriptional repression of gene expression by binding to the transcriptional activator MxiE. An alternative post-transcriptional inhibitory mechanism in *Yersinia* relies on the anti-activator function of the LcrH T3S chaperone, which acts in concert with cognate substrate YopD and another regulatory element LcrQ to bind untranslated T3S gene mRNA. Following induction via target cell contact, the middle/late substrates are actively secreted following formation of a syringe-needle complex. This frees up ExsC to titrate away ExsD from ExsA (*P. aeruginosa*) or SicA/IpgC to directly activate InvF/MxiE (*S. enterica/Shigella*) and induce T3S gene transcription. By comparison, destabilization of the LcrH-YopD-LcrQ complex via chaperone (LcrH and SycH)-mediated substrate secretion (YopD and LcrQ, respectively) permits ribosome access and mRNA translation. This schematic was adapted from the recent review by Brutinel and Yahr [44]. PM – plasma membrane (eukaryotic cell), OM – outer membrane, CM – cytoplasmic membrane.

Pseudomonas Aeruginosa T3SS

The most recent example of this concept comes from elegant work with the *P. aeruginosa* T3SS. In this system, transcription of all genes is under the transcriptional control of ExsA, an AraC-like transcriptional activator. ExsA activity is restricted by three additional

proteins with functions linked to the secretion competence of the T3SS. The first is an ‘anti-activator’ ExsD that binds to and inactivates ExsA when the T3SS is closed [222]. A closed T3SS also accumulates high cytoplasmic concentrations of ExsE enabling sequestration of an ‘anti-anti-activator’ ExsC [223, 224], a protein with class I chaperone characteristics [225]. However, opening of the T3SS channel through target cell contact first allows ExsC to promote the secretion and translocation of ExsE reducing these internal inhibitory pools [223, 224, 226]. It then allows ExsC to seize ExsD, which in turn releases ExsA to activate transcription of type III gene promoters [225, 227].

Salmonella SPI-1 and Shigella T3SS

In contrast to this *P. aeruginosa* scenario in which the entire T3SS is up-regulated in response to activated secretion, T3SS activation in both *Salmonella* and *Shigella* only concerns the induction of effector genes. This illustration is based on the function of the translocator class II chaperones IpgC of *Shigella* and SicA of *Salmonella*, in concert with their two translocator substrates IpaB/IpaC and SipB/SipC respectively. In the absence of substrate, free IpgC or SicA acts in cooperation with the AraC-like transcriptional activators, MxiE and InvF respectively, to specifically transcribe mRNA from effector gene promoters [112, 163, 228-231]. In contrast, gene transcription is not induced when IpgC or SicA are bound to their cognate substrates trapped in the cytoplasm – therefore denying complex formation with their respective transcriptional activators. Hence, only after the secretion of their translocator substrates (‘anti-coactivators’) through an opened T3SS, is the chaperone free to act as a ‘coactivator’. Notably, this regulatory coupling also doubles as a means to establish a secretion hierarchy because chaperone-activator complexes form and then trigger transcription of effector genes only after the cognate translocator substrates have been synthesized and secreted [229]. This mechanism, based on using a class II T3S chaperone as a coactivator, might be a common solution for creating a program to order type III substrate secretion by bacteria. For example, the chromosomal encoded T3SS of *Y. enterocolitica* depends upon a similar SycB chaperone/YsaE activator complex for transcriptional regulatory control, although in this case it is too early to say if this is coupled to secretion activity [232].

In *Shigella*, another layer of regulation is imposed to further refine this hierarchal secretion process. This is based upon an ‘anti-activator’ function to keep MxiE quiet, even in the presence of free coactivator IpgC. In this scenario, the anti-activator OspD1 (a secreted effector) would bind to MxiE shielding it from coactivation by IpgC. Only after secretion of OspD1, presumably mediated by the class Ib chaperone Spa15, will MxiE-IpgC complexes form to activate late effector genes that are under MxiE control [233]. This then assumes that fine-tuning of MxiE activation by the opposing anti-activator and co-activator regulatory pathways ensures late effectors are produced only after the translocators and early effectors (those not controlled by MxiE, such as OspD1) have been secreted through an active T3SS [233].

Yersinia T3SS

The previous examples describe systems which couple T3S activity to the control of gene expression at the transcriptional level. In contrast, *Yersinia* spp. combine activity of their

plasmid encoded T3SS with control of gene expression using a post-transcriptional mechanism. In this case, relief from T3SS feedback inhibition comes from the secretion of at least one negative regulatory element, LcrQ [16, 234-236]. In the cytoplasm, LcrQ is thought to bind to the 5'-untranslated region of messenger RNA [237]. Upon induction through target cell contact, an activated T3SS releases LcrQ with the assistance of the class I chaperone SycH [238]. Presumably, ribosomes then have access to translate each transcript, resulting in an upsurge of substrate synthesis and secretion [16, 236, 238, 239]. A complex of the YopD translocator and the LcrH class II chaperone is also a negative regulatory feature of T3S control in *Yersinia* [160, 240, 241]. It seems that YopD-LcrH complexes also bind to the 5'-untranslated region of mRNA collaborating with LcrQ in the post-transcriptional silencing of type III gene expression [Costa *et al.*, unpublished data] [237, 240, 241]. Although it is not yet apparent how a YopD-LcrH complex communicates with LcrQ, this model offers exciting possibilities for further investigation. It is important to clarify the actual targets of post-transcriptional control – is it restricted to the expression of genes encoding just the secreted substrates or does the regulon also include genes encoding the structural components? The current model also assumes that YopD is secreted along with LcrQ once the T3SS is activated. If so, this could free-up LcrH to initiate additional known contacts with other noted regulatory components (such as YscY, YscE and TyeA) [Francis, unpublished data] [158-160]. It remains to be seen how these other LcrH complexes fit into the regulatory scaffolds that control T3S gene expression in *Yersinia*.

LcrQ is a regulatory molecule unique to the *Yersinia* T3SS. It can reportedly interact with several T3S chaperones – most notably, the class I chaperones SycH and SycO and the class II chaperone LcrH [111, 159, 238, 242]. Intriguingly, another target of LcrQ was recently shown to be phosphoenol pyruvate carboxylase (PEPC) [243]. The consequence of this interaction was inhibition of PEPC activity, effectively reducing the biosynthesis of oxaloacetate derived amino acids. With these observations, LcrQ was suggested to be an important link between *Yersinia* T3SS and central metabolism; the dual ability of LcrQ to bind both T3S chaperones and PEPC, might ensure the availability of amino acids at times when the synthesis of T3S substrates are most needed – during target cell contact [243]. The model proposes that following T3S of premade pools of substrate, LcrQ is sequestered by free T3S chaperone. This liberates PEPC to re-establish amino acid pools to initiate a subsequent round of T3S substrate synthesis. To ensure their solubility and stability prior to secretion, these *de novo* synthesized substrates recruit their cognate T3S chaperone. This releases LcrQ to seek out and neutralize PEPC activity to reduce unnecessary synthesis of amino acids. Since earlier studies have hinted at a link between metabolic status and regulation of flagella and non-flagella T3SSs [244-250], this cross-talk model offers a step forward and therefore deserves to be rigorously tested. At the very least, it stands to provide important insight into the relevance of the varied LcrQ-T3S chaperone interactions in *Yersinia*.

Variations on the Same Theme

The regulatory mechanisms described herein are ingenious ways in which bacteria have coupled their secretory activity to the control of gene expression. The key mediators of these regulatory pathways are chaperones or chaperone-assisted secreted substrates. They sense the

operational ‘readiness’ of each T3SS and relay this information to transcriptional activators that sit at or near the top of this regulatory cascade. As already noted by others [44], a feature of these regulatory mechanisms are the inherent flexibility of the individual components. A good illustration of this is the varied functions of the class II T3S chaperones. The *Salmonella* and *Shigella* systems utilize their respective SicA and IpgC chaperones as cofactors for the transcriptional activator, while the *Yersinia* system employs the equivalent LcrH chaperone as a post-transcriptional negative regulator with no known connection to the transcriptional activator [Carlsson *et al.*, unpublished data]. On the other hand, the *P. aeruginosa* system does not engage the homologous PcrH in any apparent regulatory activity [158, 162]. Such resourcefulness probably reflects how related bacteria have utilized different environmental conditions to ensure type III genes are expressed only when needed (for example; during target cell contact within a unique infection niche), whereas energetically wasteful expression is kept to a minimum.

Mechanisms of Substrate Secretion

The means by which T3S substrates are delivered to the cognate T3SS and then subsequently secreted continues to be the focus of intense research in the T3S field. Insights into several inherently complex mechanisms have been revealed. While they all contribute towards efficient substrate secretion, the various molecular details are still poorly understood. Adding to this complexity is the knowledge that the portal through which the substrates are assumed to be secreted, the needle channel, is far too narrow to accommodate folded proteins [27, 251]. Hence, energetically favorable means to unfold substrates is also a prerequisite for secretion [252].

Chaperone-Independent N-Terminal Secretion Signals

It was realized early on that the translocated effector substrates of *Yersinia* contained information within their first 20 amino acids that is sufficient to confer upon signalless reporter proteins an ability to be secreted in a type III-dependent manner [253, 254]. Subsequent in depth analysis of effector substrate N-termini failed to identify any consensus amino acid sequence; although a contributing amphipathic secondary structure was proposed [171, 255]. Secretion of some effector substrates is also aided by information in the 5'-prime untranslated mRNA sequence [256, 257]. However, some other effector substrates apparently contain a N-terminal secretion signal devoid of any secondary structure in either amino acid or mRNA signals [258]. We and others have also tried to resolve the precise structural features of the translocator substrate N-terminal secretion signals. Although the molecular makeup is still uncertain, it seems unlikely that mRNA signals contribute to translocator secretion [Amer *et al.*, unpublished data] [145, 146, 259]. Nevertheless, evidence supporting the mRNA and protein sequence models has intriguing consequences, for each proposes a different mechanism of substrate recognition and subsequent secretion by the T3SS. The mRNA hypothesis implies that a mRNA structure is first recognized by the T3SS that then

allows coupling of translation directly to secretion (Figure 7A). On the other hand, the protein hypothesis implies recognition of pre-made pools of the N-terminal polypeptide (Figure 7B). It is clear that the N-terminal secretion-signal debate is not over. However, it may never really be resolved when most studies examine substrate secretion in artificial conditions that may not reflect the true inducing signal – target cell contact. More detailed information concerning the function of this so-called chaperone-independent N-terminal secretion signal can be obtained from several worthy reviews [13, 260-263].

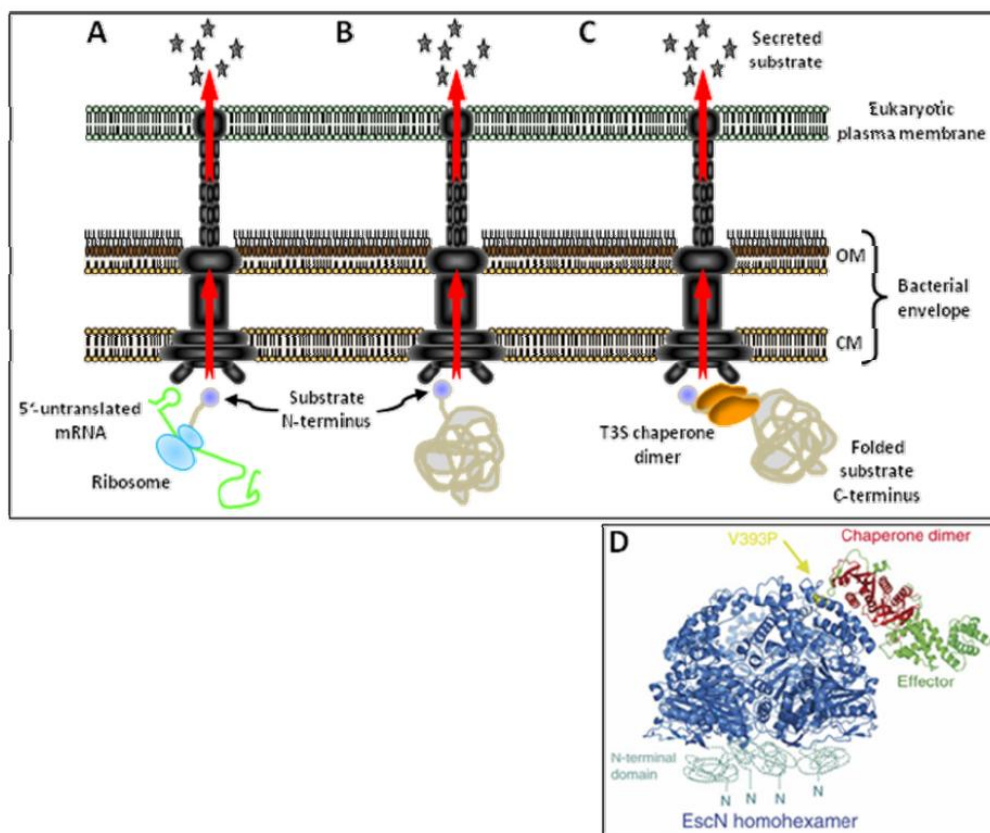


Figure 7. A schematic illustration of the possible mechanisms of type III substrate secretion. The T3SS may recognize the 5'-untranslated mRNA, with the implication that this recruits ribosomes to the inner face of the secretion apparatus and couples substrate translation to secretion (A). The amino acid sequence of the extreme N-terminus of individual substrates may represent a structural motif directly recognized by the T3SS (B). Information contained in the T3S chaperone bound to substrate may also constitute a secretion signal. It is the chaperone that interacts with the T3SS (most likely the system ATPase) and this primes the substrate for unfolding prior to secretion (C). This schematic was motivated by reviews from Aldridge and Hughes [405] and Cornelis [263]. The chaperone dimer (red) in complex with its cognate substrate (green) is proposed to dock at a specific site of the T3SS ATPase (blue) that encompasses the functionally important valine (V; marked in yellow) residue at position 393 (D). Panel D is part of a study by Strynadka and colleagues [273] and is reused with permission "Copyright (2007) Macmillan Publishers Ltd." OM – outer membrane, CM – cytoplasmic membrane. OM – outer membrane, CM – cytoplasmic membrane.

Chaperone-Dependent Secretion Signals

As discussed earlier, located directly adjacent to the N-terminal secretion signal are about 100 amino acids of substrate that are involved in direct binding to the cognate chaperone. It is now clear that chaperone bound to this region comprises a chaperone-dependent secretion signal [264]. This has consequences for prioritizing T3S when in competition with other substrates [213] and in ensuring specific secretion through the correct T3SS pathway [191, 192]. Secretion pathway specificity is conferred through association of chaperone-substrate complexes with an ATPase linked to the cytoplasmic side of the T3S machinery (Figure 7C). This was first examined in enteropathogenic *Escherichia coli* for the T3S substrate Tir and its chaperone CesT and their interactions with the T3SS ATPase EscN [214, 215]. Similarly, within the *S. enterica* Typhimurium SPI-1 T3SS, the InvC ATPase interacts with the SptP effector substrate in complex with its chaperone SicP [216]. Such findings endowed T3S chaperones with a new responsibility – targeting their substrate cargo to the cognate T3SS through specific associations with the core ATPase. This does not offset the role of the extreme N-terminal chaperone-independent secretion signal, because in some experimental setups, chaperone-free effector substrate could still associate with their respective system ATPase [214, 265]. Thus, chaperone-dependent and –independent substrate secretion signals probably act in concert to dock the substrate to the T3S apparatus. For reasons that are outlined below, the T3SS ATPase is an appealing target for docking of substrate at the inner face of the T3S machinery. However, it is prudent to remain open to other possible interactions given that studies in *Yersinia* suggest that the ATPase YscN does not engage free SycE chaperone or a SycE-YopE effector complex [140].

Substrate-Chaperone Recognition by the T3S Machinery

The T3SS ATPase is ubiquitous to all T3SSs. The family typically contains the two Walker boxes, A and B, which are associated with ATP binding and hydrolysis. In addition, all exhibit some similarity to the β -subunit of F_0F_1 proton-translocating ATPases. Consistent with these observations, T3SS ATPases hydrolyze ATP in line with their role as energizers for substrate secretion [266–271]. The ATPases form a hexameric ring-like structure that probably sits at the base of the T3S machinery peripherally associated with the inner membrane [272, 273]. Oligomerization is a known stimulator of ATPase activity and might be critical for driving substrate secretion [269, 270]. However, other T3SS components are presumed to interact with the ATPase, which might be important in regulating oligomeric formation or perhaps even in restricting ATPase activity to the process of T3S [268, 270, 271, 274, 275].

So what is the true function of these ATPases? The crystallization of various T3SS components revealed a puzzling conundrum – fully folded secreted substrates are far too large to be accommodated by the small opening of the T3S nanomachine [27, 251, 276]. For secretion to occur, substrates must therefore assume an unfolded state. As mentioned earlier, T3S chaperones cannot initiate substrate unfolding. While the region of each substrate contacting the chaperone is unfolded, the remainder of the substrate is usually still fully

folded [94, 103, 139, 173]. However, the findings that T3S chaperones dock their substrates at the T3SS ATPase proffered an attractive resolution – ATPase activity initiates substrate unfolding prior to transit through the T3S machine. In a seminal study of T3S in *S. enterica* Typhimurium, not only did the ATP hydrolyzing activity of the InvC ATPase disassociate the SptP effector from the SicP chaperone-bound state, but it also caused the effector to unfold [216]. In this context, T3S chaperones pilot their cargo to the system ATPase, which then triggers chaperone-substrate disassembly and substrate unfolding that facilitates substrate entry into the secretory apparatus. Structural determination of the enteropathogenic *E. coli* T3S ATPase EscN, revealed a surface cavity in the C-terminus that could serve as a substrate-chaperone binding site (Figure 7D) [273]. ATP hydrolysis could induce structural changes in this region that precipitates chaperone disassociation [273]. However, ATPase-dependent unfolding must also be assisted by unknown structural properties inherent to T3S substrates because when fused to tightly folded heterologous protein domains, substrate unfolding and secretion is severely impaired [216, 277-279]. Thus, only substrates permissive to ATPase-dependent unfolding are secretion competent [216].

The energy to drive the substrates through the T3S machinery remains an open question. Work from the Galán laboratory demonstrates the importance of ATP hydrolysis in substrate unfolding [216]. In addition, some observations suggest that the proton motive force may contribute to the passage of substrate through the secretion channel [201, 252, 280]. Significantly, aside from their secretion piloting role, T3S chaperones are also hypothesized to reduce the energy requirements needed for substrate secretion. The majority of structural evidence indicates that when in complex with chaperone, the CBDs of substrates are maintained in an extended, non-globular conformation (see Figure 3B) [94, 98-100, 103, 109, 139]. Maintenance of this localized unfolded state by a bound chaperone may act as a primer generating a more energetically favorable environment for further substrate unfolding by the ATPase during presentation of the substrate to the secretion apparatus [86, 98, 99, 279, 281]. In contrast to this model however, binding by the SycE chaperone to the full-length YopE effector actually induced a localized disordered-to-ordered structural transition in the substrate CBD [140]. While these findings may not agree with the chaperone ‘priming’ model, they reaffirm that chaperones probably contribute to a recognition signal that directs their substrate to the T3S secretory machine.

T3S Chaperones for Protein Export in Flagella Assembly

Bacterial swimming motility is conferred by the rotation of a complex nano-motor termed the flagellum. These filamentous organelles extend from the bacterial inner membrane to well beyond the bacterial surface. A flagellum is composed of three parts; the basal body entrenched in the bacterial envelope (the motor), followed by a hook (a connecting ‘universal joint’) and then a filament (the propeller) which protrudes outwards from the bacterial surface [201, 282, 283]. Remarkably, the structure of the flagellum hook-basal body closely resembles non-flagella T3SSs associated with host cell contact (Figure 1) [198, 251, 284-289]. This adds further impetus to the idea that T3SSs needed for interactions between

bacteria and eukaryotic cells are descendents of the protein export system linked to bacterial flagella assembly [290]. The flagella-associated T3SS exports a number of proteins needed for flagella assembly [282]. At least four of these are subunits of the flagellum filament, which assemble outside of the bacterium; FliC (major filament subunit), FliD (filament cap) and FlgK and FlgL (the hook-filament junction proteins). Export of each of these proteins requires assistance from a dedicated T3S chaperone; FliS (for FliC) [291-293], FliT (for FliD) [294, 295] and FlgN (for FlgK and FlgL) [294, 295].

Mediators of Anti-Polymerization

Despite physical similarities to class I T3S chaperones of host cell contact inducible T3SS – being small, predominantly α -helical with an acidic pI – flagella-specific T3S chaperones display notable differences warranting their division into a different T3S chaperone group, designated class III (Table 1) [88]. This is reinforced by a FliS crystal structure that is unrelated to any other T3S chaperone (Figure 2) [296]. Intriguingly however, a similar means of substrate recognition and localized substrate unfolding may still apply – the FliC substrate wraps around the outside of FliS in an extended conformation lacking any tertiary structure (Figure 3B) [296]. In addition, flagella class III T3S chaperones bind to the extreme C-terminus of their substrates [291-293, 295]. These C-terminal domains are thought to be the glue by which individual filament subunits polymerize together during assembly of a growing flagellum. The monomeric forms are therefore inclined to rapidly oligomerize unless the C-terminal interacting domains are stabilized by a bound chaperone. This has been experimentally demonstrated for the FliS chaperone [291-293] and the FlgN chaperone [294]; their respective binding to the major flagellum filament subunit FliC and the hook associated axial protein FlgK prevents premature polymerization. This anti-polymerization role is analogous to the class IV and class V T3S chaperones belonging to T3SSs associated with host cell contact [115].

Establishing Ordered Substrate Secretion

Preventing polymerization of cognate flagella substrates is arguably the foremost role of flagella-specific T3S chaperones. However, assembly of filament subunits into the growing flagellum is strictly sequential; the hook-filament junction proteins, FlgK and FlgL, must be assembled before the FliD filament cap, which in turn is needed for FliC flagellin filament incorporation [201, 282, 283]. This implies a strict secretion order, which may result if each chaperone-substrate complex interacts with a different affinity to a component of the flagella-specific T3SS. On top of this, competition for recognition of the FlgK, FlgL and FliD minor flagellum subunits by the T3SS would need to occur in an unfavorable environment replete with excessive amounts of FliC major flagellum subunit [282, 297]. Investigation into this problem by the Colin Hughes laboratory revealed two key observations. First, FlgN-FlgK and FlgN-FlgL chaperone-substrate complexes are recognized by the system ATPase FliI [298], a finding analogous to some class I T3S chaperones associated with virulence [214-216].

Extrapolating from the work of Jorge Galán and colleagues [216], the flagella-specific FliI ATPase probably disassociates the FlgN-FlgK and FlgN-FlgL complexes to promote FlgK and FlgL unfolding and secretion. However, what happens to the ‘free’ T3S chaperone post-substrate secretion? This was investigated in a second study of Hughes and colleagues. They described an event post-substrate secretion in which free chaperone is then selectively recycled to another substrate to promote rapid export [297]. Significantly, the protein involved in this chaperone recycling process, FliJ, interacts only with FlgN and FliT, chaperones of the minor filament subunits. FliJ does not interact with the major filament chaperone FliS. Thus, by specifically recruiting newly available free FlgN and FliT chaperone and then quickly making it available to the next cognate substrate in line for secretion, FliJ-chaperone complexes may therefore confer an advantage towards the secretion of minor filament subunits over the FliC major subunit [297]. Further credence for such a novel hierarchal secretion mechanism comes from a preliminary analysis of two FliJ orthologues from cell contact inducible T3SSs, namely InvI (from *S. enterica* Typhimurium SPI-1) and YscO (from *Y. enterocolitica*) – both seemed to preferentially bind to free class II T3S chaperones targeting the translocators compared to class I T3S chaperones targeting the effectors [161]. Hence, class II T3S chaperone complexes with InvI or YscO might be expected to prioritize translocator substrate secretion. This work with the FliJ protein family has highlighted a new mechanistic concept for orchestrating hierarchal secretion, which warrants further study to determine its biological relevance.

Coordinating Gene Expression with Flagella Assembly

The coordinated assembly of flagella is a complex process – a review of these regulatory mechanisms within the flagella regulon is beyond the scope of this article. However, interested readers are referred to a collection of recent reviews that give a thorough description of this topic [282, 299-301]. In very brief terms, structural genes are expressed in a three-tiered transcriptional hierarchy based on class I, II and III promoter activity that are coupled to built-in assembly checkpoints within the growing flagellum. This transcriptional coordination ensures the sequential expression of class I genes (encoding the master regulators FlhC and FlhD for class II gene expression) before class II genes (encoding hook and basal body components as well as the σ^{28} sigma factor required for class III gene expression) before class III genes (encoding late secreted substrates and chemotaxis components). Each gene product is therefore made only when needed during the assembly process.

In this context, it is significant that all late secretion substrates – i.e., those which are secreted only after hook-basal body assembly is completed – require a chaperone [302]. Nor is it a coincidence that most of these flagella-class T3S chaperones (with the exception of FliS) exhibit intricate regulatory functions (Figure 8). These dual functional T3S chaperones therefore create the links between transcriptional regulation and sensing of flagellum assembly status. For example, the substrate FliD sequesters its cognate FliT T3S chaperone in the cytoplasm prior to completion of a secretion competent hook-basal body [303]. Once completed however, FliD is escorted by FliT for secretion through the hook-basal body.

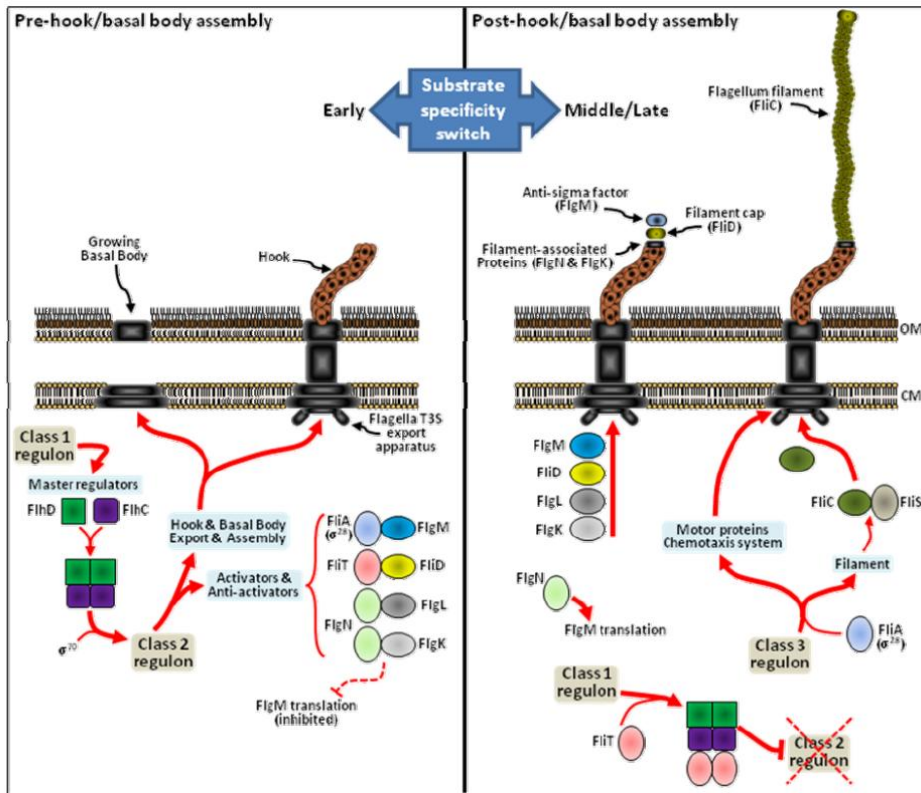


Figure 8. Use of chaperones to couple flagella gene expression with flagellum assembly. The initial stages of flagella biogenesis require the assembly in the bacterial envelope of a hook-basal body structure. Components of this structure are encoded on a series of σ^{70} -dependent operons within the Class II regulon, which is under the control of the master regulators (FlhC and FlhD) encoded within an operon of the Class I regulon. Late secretion substrates are also produced, but cannot be secreted until the hook-basal body is secreted. They are stabilized in the cytoplasm prior to secretion by bound T3S chaperones. Completion of the hook-basal body signals a switch in substrate specificity allowing for secretion of late substrates. Secretion of the anti-sigma factor FlgM frees-up the cognate chaperone, FliA (σ^{28}), to initiate expression of genes within the Class III regulon. This includes the major filament subunit FliC and its cognate chaperone FliS, which allows assembly of the external flagellum. In addition, when freed by the secretion of FlgL and FlgK, the FlgN chaperone positively regulates FlgM production. Moreover, free FliT can engage with FlhC of the master regulator complex inhibiting expression of genes from the Class II regulon. This schematic was inspired by the reviews of Chevance and Hughes [282] and Brutinel and Yahr [44] as well as primary studies from the Hughes [304] and Kutsukake [303] laboratories. OM – outer membrane, CM – cytoplasmic membrane.

The FliT chaperone is then free to complex with the FlhC subunit of the FlhC-FlhD master regulator complex inhibiting its binding to class II promoter DNA [303]. This negative-regulatory loop is anticipated to fine tune class II gene expression, reducing the production of hook and basal body components following their assembly into a functionally complete structure. It also seems apparent that the FlgN T3S chaperone similarly contributes to the controlled translation of *flgM*, a class III gene encoding the anti- σ^{28} factor [304, 305]. In this scenario, the substrates FlgK and FlgL sequester FlgN in the cytoplasm before the hook-basal body is completed [304]. When completed however, FlgK and FlgL are secreted

through the hook-basal body freeing the FlgN chaperone to positively regulate *flgM* translation. These subtle alterations in the levels of FlgM are expected to have significant effects on σ^{28} -dependent transcription of class III transcripts. The anti- σ^{28} factor FlgM also needs to be secreted following assembly of the hook-basal body structure. This is needed in order to free up the σ^{28} sigma factor to direct the RNA polymerase to class III promoters upstream of genes encoding late flagellar components [306]. Interestingly, the σ^{28} sigma factor is the T3S chaperone for its own anti- σ^{28} factor FlgM [302].

While flagella and non-flagella T3SSs have different pursuits – one to propel bacterial movement toward or away from a chemical stimulus, the other to establish cross-talk with eukaryotic cells – fundamental similarities between the two protein export systems exist. It is now evident that the role of T3S chaperones is universal among both flagella and non-flagella T3SSs, and their influence is observed at multiple levels. Although their structural scaffolds and modes of action are sometimes different, their motives are always the same – to protect substrates from premature associations with self or other proteins; to guide substrates to the correct T3SS; to establish a secretion order among the substrates; and to couple gene transcription to the process of apparatus assembly (see Figure 5).

Chaperones of Other Specialized Host-Targeting Protein Secretion Systems

In recent years and aided by the whole genome sequencing explosion, two new secretion systems designated Type IV (T4SS) and Type VI (T6SS) have been added to the bacterial protein export arsenal. Analogous to T3SS, these additional systems are also believed to boast a capacity to directly transport proteins from the bacterial cytosol into host cell compartments.

Type IV Substrate Secretion

Just like T3SSs are ancestrally related to the bacterial flagella, T4SSs are related to the bacterial conjugation system that transfers DNA from donor to recipient cells [307-310]. As such, various T4SSs can be used to import DNA, or secrete DNA, protein or DNA-protein complexes free into the environment or directly into target eukaryotic cells. The latter is a process that resembles T3S effector translocation. The list of so-called effector molecules directed to the host cell by T4SS's is growing rapidly. However, in most situations it is poorly understood how these molecules are targeted to the secretory apparatus and prepared for orderly and efficient secretion. Secretion signal information usually exists in the C-terminus of most substrates, but this is not always sufficient for secretion [310, 311]. Moreover, in almost all T4SSs the substrate recognition, recruitment and export also relies upon an integral inner membrane located receptor termed a 'coupling factor' [312]. Finally, some researchers propose that T4SSs incorporate chaperone-like molecules to perform functions akin to the T3S chaperones – preventing premature interactions of substrate with

self or other proteins, maintaining substrates in a secretion competent state or delivering the substrates to the coupling protein [311].

With respect to the T4SS encoded by the plant pathogen *Agrobacterium tumefaciens*, one of the transported substrates VirE2 is stabilized by the VirE1 chaperone [313-317]. However, this interaction is not needed for transfer of substrate to the coupling protein and subsequent export [318, 319]. The chaperone therefore likely restricts premature VirE2 polymerization and binding to single stranded T-DNA in the bacterial cytoplasm. In this regard, it is interesting that although unique, the VirE2-VirE1 crystal structure does evoke some resemblance to the anti-polymerization complexes formed by the T3S chaperones FliS and CesAB with their respective substrates FliC and EspA (Figure 3C) [115, 296, 320].

The intracellular pathogen *Legionella pneumophila* also utilizes a T4SS for translocation of vast numbers of effectors into the host cell cytosol. The translocation of a subset of these effectors requires an interaction with the IcmS/IscW or IcmS/LvgA co-chaperone complexes in the bacterial cytoplasm [321-326]. However, how these co-chaperone complexes contribute to the substrate translocation process is poorly understood, but one role might be to present the C-terminal substrate secretion signal for recognition by the T4SS [321]. The *L. pneumophila* IcmR protein also exhibits chaperone activity for IcmQ [327, 328]. Not only does chaperone binding prevent IcmQ self-association, but it also prevents IcmQ pore formation in phospholipid membranes [329, 330]. Intriguingly, this chaperone-substrate complex is functionally reminiscent of the VirE1-VirE2 complex, where VirE2 also forms channels in planar lipid bilayers [331]. Parallels can also be drawn with the class II T3S chaperones that prevent the damaging effects of premature membrane insertion by the T3S translocator substrates [Costa *et al.*, unpublished data] [142, 147].

The last example of a putative chaperone-like molecule among components of T4SSs comes from the *cag* system encoded by the stomach pathogen *Helicobacter pylori*. CagA is the only known proteinaceous T4S substrate secreted by *H. pylori*. Secretion first requires an interaction with CagF that is primarily located in the cytoplasmic membrane suggesting that this initial binding might contribute to subsequent recognition of CagA by the type IV secretory apparatus [332, 333].

Collectively, these studies do suggest a universal need for unique chaperone-like molecules in coordinating T4S in diverse bacteria. However, indications are that at least some T4S chaperones have mechanistically diverged from their T3S chaperone counterparts – clearly a reflection of the different molecular constraints imposed upon by the two evolutionary distinct secretion systems within which each chaperone family are designed to operate.

Type VI Substrate Secretion

Far less is known about the T6SS since its widespread discovery has only occurred within the last couple of years. However, its discovery has prompted significant interest in the research community seeing as it may yet be another important strategy for bacterial survival in the presence of eukaryotic cells [334-337]. The system can consist of up to 25 proteins, most of which lack homology to any protein belonging to other independent

bacterial secretion systems. It follows that few secreted substrates have so far been reported. It is not clear how these substrates are secreted, or whether they require chaperone assistance for their stabilization and recognition by the secretory apparatus. The presence of dedicated chaperones unique to T6SSs has been speculated, but this is not supported by any direct experimental evidence [338, 339].

ClpV, a subfamily of the ClpB chaperone-like ATPases associated with maintenance of protein complexes, is most often associated T6SS [340]. However, its chaperone capacity is considered secondary to its ATP binding and hydrolysis activity, which may function as the general energizer for type VI substrate secretion [341]. None the less, the role of ClpV is not a transparent one. While recent data confirms that the ClpV ATPase activity is essential for T6S, it might preferentially be needed to disassemble a tubular-like structure composed of two proteins encoded by genes within the T6SS cluster [342]. However, the function of this tubular-like structure in T6S is not known, which brings into question the significance of its disruption by ClpV. Additional studies should eventually reveal the precise relationship between ClpV activity and T6SS. Hopefully further studies will also determine if other cytoplasmic chaperone-like accessory proteins are required to assist with type VI substrate secretion.

Conclusion

Use of the term ‘T3S chaperone’ has been met with some trepidation and confusion. This occurs particularly among those familiar with the diverse family of molecular chaperones that usually function to prevent non-functional protein complexes by promoting correct protein folding and to encourage the refolding of misfolded proteins [343]. In recent times however, new concepts in T3S chaperone biology have emerged courtesy of several recent structure-function studies. For example, some T3S chaperones might primarily be sensors of T3SS assembly, while others could be facilitators of T3S export [221, 300, 305]. Thus, while the term ‘chaperone’ may not be entirely consistent with the classical view of molecular chaperone function, it still admirably fits into the concept that T3S chaperones are critical for maintaining and orchestrating the biomechanics of a well-oiled nanomachine that in turn, enables multiple bacteria to establish interactions of significance with host organisms. From humble beginnings it has quickly become apparent that these small molecules pack an impressive array of functions in their small molecular scaffold. Continued research in this field promises to reveal further examples of bacterial engineering ingenuity through the sculpturing of T3S chaperones with impressive versatility – effectively offering the use of a compact small molecular toolkit for all occasions.

In closing, just like molecular chaperones are emerging as new targets for drug therapy of microbial virulence [344, 345], the importance of T3S chaperones in bacterial pathogenicity presents us with a golden opportunity to exploit their functions in the search for novel and effective anti-bacterial drugs. With the emergence of crystal structures of T3S chaperones in complex with substrates, this structural data could enable the design of small synthetic molecules that specifically block T3S chaperone-substrate interaction via competitive antagonism. Such an approach is already showing promise for the targeted

disruption of periplasmic chaperone binding to substrates that then assemble into surface pili appendages via the chaperone-usher protein secretion pathway [346]. Another independent approach could be to randomly identify small inhibitory molecules of T3S chaperone function through the high throughput screening of large chemical libraries. Indeed, high throughput screening procedures are successfully identifying synthetic chemicals with inhibitory effects against bacterial virulence determinants including T3SSs [347, 348].

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Chapter III

Advances in Heat Shock Proteomics: Towards a Better Understanding of the Physiology and Pathophysiology of Molecular Chaperones

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Abstract

Chaperones are a large group of unrelated protein families that stabilize unfolded proteins, unfold them for translocation across membranes or degradation, and assist in their correct folding and assembly. They represent one of the most ancient and evolutionarily conserved protective protein families found in nature. A fundamental group of molecular chaperones is the so-called heat shock proteins (HSPs), also known as stress proteins. Originally discovered as inducible molecules capable of maintaining cellular homeostasis against abrupt temperature changes, HSPs were later considered an adaptive physiological response that protects against a variety of different cellular proteotoxic stresses. Early in the study of these proteins, it was evident that these molecules also have physiological roles that facilitate the synthesis, folding, assembly, trafficking, and secretion of specific proteins in various cellular compartments in the absence of significant pathological processes. In summary, these proteins guard the cellular proteome against misfolding and inappropriate aggregation.

From a clinical point of view, modification of the chaperone proteome, mainly the induction of HSPs, has been observed in a wide spectrum of inflammatory and degenerative diseases, including cancer, infectious disease, autoimmune processes, neurodegenerative conditions, and prion disease. The involvement of HSPs in these

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diverse diseases highlights the importance of the chaperone machinery not only in cell biology, but also in pathophysiology. At the same time, the induction of HSPs in diseases suggests potential clinical applications for molecular chaperones, particularly HSPs, in the diagnosis, prognosis and, above all, therapy of different degenerative and inflammatory human diseases. On this basis, proteomic approaches represent a valuable method to study the roles, structural interrelationships, and intimate molecular mechanisms of the major chaperone families that have been insufficiently characterized, limiting their diagnostic and therapeutic potential.

Keywords: Cellular stress response, cancer, biomarkers, diagnostics, oncoproteomics, neurodegeneration, immunity.

Introduction

Molecular chaperones consist of several highly conserved families of proteins, of which the most important are the so-called heat shock proteins (HSPs), also known as stress proteins. Historically, the most important biological activity of HSPs is their ability to act as molecular chaperones. In this role, different HSPs facilitate the folding, assembly, trafficking, and eventual secretion of cellular proteins. They also influence, both directly and indirectly, protein degradation by the ubiquitin-proteasome pathway. Importantly, in metabolically active cells, HSPs prevent abnormal folding and incorrect aggregation not only among newly synthesized peptide chains, but also among mature proteins in the cytoplasm [1-6].

Initially, the role of HSPs in cellular physiology and pathophysiology was not well understood. Currently, it is known that HSPs are the main mediators of the cellular stress response. Importantly, however, they are not the only mediators of this peculiar cellular response: other molecules such as glucose-related proteins, various proteases, calreticulin, ubiquitin and, most recently, miRNAs have also been reported to be involved in the cellular stress response. Due to their multitude of functions, a better name for HSPs is “Stress Proteins,” but this term may be misleading because most HSPs are constitutively expressed, rather than expressed only in periods of stress [4-6].

A thorough review of the molecular and physiological aspects of the cellular stress response in general, and of stress proteins in particular, is beyond the aim of this work; these topics have been carefully analyzed in other articles [3-7]. Here, we stress some biological aspects of this intriguing family of proteins that are important for consideration of the “proteomics of HSPs.” In fact, proteomic studies, specifically cellular proteomics, allow for the study of dynamic modifications of molecular chaperone expression and the functional and structural relationships of chaperones with other biological molecules in the cell due to their intrinsic specificity and sensitivity.

Briefly, under stress conditions, cells from all organisms respond similarly by reducing the synthesis of most proteins and increasing the synthesis of stress proteins to promote cell survival. Hence, HSPs are an evolutionarily conserved family of proteins that play a key role in responding to a wide variety of environmental stresses (metabolic, hypoxic, inflammatory, toxic, etc.).

According to their approximate molecular weights, these proteins are generally classified into five major families: HSP100, HSP90, HSP70, HSP60, and the small HSPs [5, 7]. Moreover, these proteins generally function as components of complex machinery that includes other chaperones, co-chaperones, modulators of ATPase activity, and various accessory molecules [3-5].

Interestingly, HSPs with high molecular weights are characterized by an ATP-binding domain with intrinsic ATPase activity, while small HSPs and 40 kDa HSPs do not include this nucleotide-binding site. Small HSPs activity can also depend on the ATP concentration, however, as ATP modulates a variety of co-chaperone molecules. Moreover, the interactions of small HSPs (e.g., HSP27) with physiological ligands and/or partially denatured proteins depend upon their phosphorylation and oligomerization status [6].

In addition to their well-known molecular chaperone activity, accumulating data suggest that HSPs also have key functions in the regulation of cellular metabolism. The activities of an increasing number of proteins that regulate gene expression, including nuclear receptors, various kinases, and transcription factors, appear to be modulated by HSPs [2-6]. Hence, it is not surprising that HSPs are involved in multiple cellular processes, such as DNA replication, signal transduction, cell cycle regulation, cell differentiation, metastasis, senescence, and apoptosis [3, 8-13], through their participation in the post-translational regulation of signaling molecules, the assembly/disassembly of transcriptional complexes [8-11], and the processing of immunogenic peptides by the immune system [8, 12, 13]. This fascinating class of proteins, which plays important but previously unexpected roles in the development of several diseases, has attracted increased attention. In fact, interesting data show that altered chaperone function has been associated with the development of a wide spectrum of pathologies, including cancer, neurodegenerative disorders, ischemia, infection, and inflammation. HSPs have been classically considered intracellular molecules with housekeeping and cytoprotective functions [3, 6]; however, recent data indicate that they may also be secreted from cells in culture [14, 15], expressed on the cell surface [16], and found in the peripheral circulation of normal individuals [17-19]. These data suggest that HSPs play a role in the immunomodulatory response as well, as they are secreted into the extracellular fluid as inflammatory mediators [20].

Moreover, considering their biological activities, there is increasing interest in the pharmacological modulation of HSP expression for the treatment of cancer and other diseases [5, 6]. To this end, modulators of chaperone activity represent a new and emerging field of drug development [5, 18, 21]. Because of these emerging biological activities, HSPs have been the focus of numerous studies in various research areas, ranging from biophysics to molecular biology, structural biology to cell biology and, more recently, proteomics. However, despite the various studies that have been carried out diverse methodologies, the precise role of HSPs in cellular physiology and pathophysiology, along with their potential clinical applications, is not well understood. Importantly, considering the explosion of new HSP-modulating drugs that are currently undergoing preclinical and clinical evaluation, our poor understanding of the multiple roles of chaperones could be dangerous. This is particularly true for HSP-modulating drugs whose mechanism of action is questionable at the very least (e.g., drugs that induce HSP expression by promoting cellular stress or inhibiting the proteasome) [21]. Moreover, considering the growing physiologic and pathophysiologic

roles of cellular and secreted HSPs, not only in protein misfolding diseases, but also in immunity, immunopathology, infections, inflammation, and cancer, the urgent need to characterize the HSP proteome is clear [3-8, 22, 23].

Proteomic technologies now provide the opportunity to study HSP expression in terms of qualitative and quantitative changes, including post-translational modifications that are essential for explaining their activity. These studies will permit an investigation of the involvement and importance of HSPs in the etiology of specific diseases. As reported in recent reviews, HSPs are among the most repeatedly identified proteins in studies using proteomic approaches [24-27]. An understanding of the roles that HSPs play in the initiation and maintenance of various diseases (in particular, neurodegenerative diseases and cancer) may have important clinical implications for the diagnosis, prognosis, and, above all, treatment of disease.

Chaperone Proteomics: Molecular Physiology and Pathophysiology

Chaperones, Cancer and Proteomics

The peculiar pathophysiology of molecular chaperones in general, and of HSPs in particular, in cancer seems particularly promising for potential clinical applications. In fact, the complex intracellular and extracellular activities of these proteins justify an intense proteomic research program that can provide interesting results in term of pathogenesis, diagnosis, prognosis, and the therapy of cancer.

In cancer, HSPs seem to have fundamental tumor-promoting activities. First, HSP90 family members protect and maintain the functionality of aberrant conformations of point-mutated or grossly altered proteins with oncogenic functions (e.g., the products of chromosomal translocation, such as Bcr-Abl, or point mutations, such as variants of the epidermal growth factor receptor). Similarly, HSPs can facilitate genetic variation in cancer and facilitate clonal selection in response to microenvironmental modifications and/or pharmacotherapeutic pressure [28-30]. Moreover, by their pathophysiological activities, HSPs in general and the HSP90 family in particular stabilize the structure and thereby the function of different receptors (e.g., nuclear receptors), protein kinases (e.g., AKT, c-Src, and Raf-1) and transcription factors (e.g., MyoD, NF- κ B, and some nuclear steroid receptors) [10, 11, 31-32].

Another fundamental tumor-promoting activity of HSPs, which is mainly related to the HSP70 and -27 families, depends upon the strong inhibitory activities of these proteins against both mitochondrial and extra-mitochondrial programmed cell death (PCD or apoptosis) and cell senescence [6, 9, 31]. This very efficient PCD block not only synergizes with the other tumor-promoting activities of HSPs (specifically chaperone properties), but also has a significant role in the development of resistance to various cancer therapeutics [1, 3, 5, 18].

Importantly, the ability of HSPs to interact with various signal transduction pathways, their chaperone properties, and their anti-apoptotic effects render them facilitators of genetic

variation in cancer. These attributes of HSPs favorably influence the promotion of other cancer properties, including the tendency for local invasion, the promotion of angiogenesis and, although somewhat controversial, the induction of metastasis [6, 14, 21, 33-35].

From an immunologic perspective, the prevalent and accepted role for endogenous HSPs is immunomodulation. These molecules can act as chaperones for tumor antigen presentation to antigen presenting cells (APCs). In the context of cancer, HSPs may thereby stimulate a T cell-mediated response against the cancer [36-38]. However, the immunoregulatory role of extracellular HSPs is highly complex as it involves pro-inflammatory actions and anti-inflammatory effects. The balance between these activities appears to be dependent on the particular immunopathological environment [39]. Interestingly, some therapeutic vaccines consisting of cancer-derived peptides linked to HSPs are in advanced clinical trials [12-14].

Numerous oncoproteomic studies have shown that some cancer cells have an increased level of various heat shock proteins compared to normal healthy cells. For example, Sosa et al. [40] demonstrated that an increased level of intracellular and extracellular HSP27 are characteristic of human melanoma cells. Interestingly, these HSP changes showed a significant correlation with the levels of secreted protein, acidic and rich in cysteines (SPARC) proteins, a family of secreted proteins associated with increased aggressiveness of different human cancers. Similarly, Sarto et al. [41] reported the overexpression of HSP27, with significant prognostic implications, in human renal cell carcinoma. Feng et al. [42] and Luk et al. [43] independently analyzed the human hepatocarcinoma proteome and showed that, in response to the stressful cancerous microenvironment, the tumor cells exhibited increased expression of chaperone proteins as a cytoprotective measure to enhance tumor growth and metastasis.

The overexpression of HSP27 seems to be correlated with the induction of chemoresistance in cancer cells, as was recently demonstrated for gemcitabine in pancreatic cancer cells [44], paclitaxel in human melanoma cells [45], and 5-fluorouracil in colorectal carcinoma cells [46]. In addition, suberoylanilide hydroxamic acid, an inhibitor of specific histone deacetylases, was shown to suppress the expression of various cancer-related proteins (e.g., reticulocalbin 1 precursor, annexin A3) as well as HSP27, while increasing the expression of aldose reductase, triosephosphate isomerase 1, and manganese superoxide dismutase [47]. Interestingly, HSP70 seems to play a similar pathophysiological role. In fact, various reports that utilized a proteomic approach showed similar changes in HSP27, HSP70, and HSP90 expression [8, 14, 21, 43] in different neoplasias. Importantly, pharmacological inhibition of HSP90 synthesis by geldanamycin or radicicol induces a compensatory induction in the expression of other HSPs, HSP70 in particular [5, 48]. This compensatory response supports at least partial redundancy of the chaperone activities of the different HSPs.

Different proteomic approaches have confirmed the correlation between HSP70 overexpression and the differentiation level and/or aggressiveness of various types of cancer, for example gastric adenocarcinoma [49], hepatocarcinoma [50], and esophageal cancer [51]. Mortalin, which is also known as mitochondrial HSP70 (mitHSP70), is known to be involved in the pathways that regulate the cell cycle, cellular senescence, and immortalization. Interestingly, mortalin overexpression in colorectal adenocarcinoma has been correlated with poor prognosis [52].

From a therapeutic point of view, oncoproteomics of HSPs in general, and HSP70 in particular, suggest multiple potential clinical applications. In fact, numerous proteomic studies have clearly correlated HSP70 overexpression with therapeutic resistance [53-59]; this finding has fundamental implications in terms of biochemical and clinical oncopharmacology. For example, Pocaly et al. [60] used a proteomic approach to analyze the molecular basis of resistance to imatinib, a tyrosine kinase inhibitor that is used as a front-line therapy in chronic myeloid leukemia. By a comparative proteomic analysis of an imatinib-resistant cell line generated from the erythroblastic cell line K562 (K562-r), the authors identified only 24 proteins that were differentially expressed with respect to the control. These identified proteins include scaffold proteins, metabolic enzymes, DNA translation and maturation, and chaperones. Importantly, for this latter family of proteins, only HSP70 and HSC (heat shock cognate) 70 were significantly overexpressed in K562-r. This finding suggests roles for these proteins in the development of imatinib resistance.

The HSP70 family of HSPs has been studied more carefully with proteomic approaches than other HSP families due to its intriguing and potentially innovative applications for cancer immunotherapy. It is well known that the secreted and/or membrane-anchored forms of HSP70 can chaperone tumor antigens to various APCs, thereby inducing an immune response to neoplasia that could have diagnostic, prognostic, and therapeutic value [12-14]. As a further example, for diagnostic purposes, Fujita et al. [61] aimed to identify novel tumor antigens in an esophageal squamous cell carcinoma (ESCC) cell line (TE-2) and related autoantibodies in serum from patients with ESCC using a proteomics-based approach. They found that the concentration of serum HSP70-specific autoantibodies was significantly higher for patients with ESCC than for patients with gastric or colon cancer or healthy individuals. Similar results have been obtained by Takashima et al. [62] using serum of patients with hepatocellular carcinoma. Specifically, these patients showed increased levels of autoantibodies against HSP70, peroxiredoxin, and Mn-SOD. The authors considered these three antibodies to be patient-specific in hepatocellular carcinoma and thus candidate diagnostic biomarkers.

HSP oncoproteomics has the potential to define the precise role of the cellular stress reaction in the pathophysiology of cancer-related immune responses. In fact, a better definition of the signal transduction pathways and cellular and serum proteomic patterns associated with an immune response against a tumor antigen, especially in terms of global patterns of HSPs (HSP70 in particular), could contribute to an understanding of tumor immunology with fundamental diagnostic, prognostic, and therapeutic implications (e.g., the development of tumor vaccines with enhanced efficacy) [3, 9, 12, 13].

In addition to the HSP70 family of HSPs, the HSP90 family has been shown to play a significant role in cancer. This family of HSPs is considered the classic chaperone family in cancer. In fact, as previously discussed, HSP90 can facilitate genetic variation in cancer by permitting the function of mutated and less stable proteins with tumor promoting activities. Moreover, it has additional extracellular properties that can promote local and distal cancer dissemination [9].

From a proteomic point of view, as with the other HSPs, HSP90 overexpression has been correlated to the prognosis and evolution of cancer. Of note, Hayashi et al. [63] analyzed murine fibrosarcoma cell lines with different levels of aggressiveness. The proteomic data

clearly showed that a progressive murine fibrosarcoma cell line was mainly characterized by the differential expression of nine proteins, seven of which (calreticulin precursor, tropomyosin 1 alpha chain, annexin A5, HSP90-alpha, HSP90-beta, PEBP, and Prx I) were over-expressed and two of which (Anp32e and HDGF) were down-regulated. Additionally, similar experimental oncoproteomic approaches showed HSP90 overexpression in multiple tumor cell lines (including human leukemia, ovarian cancer, and hepatocarcinoma cell lines) [9, 14, 62-66]. Importantly, HSP90 overexpression in different types of neoplasia, including mantle-cell lymphoma (MCL, an aggressive subtype of B-cell non-Hodgkin's lymphoma), Waldenstrom macroglobulinemia, multiple myeloma, breast cancer and gastrointestinal cancer has also been detected by clinical proteomic analysis [30, 67-72].

Moreover, the proteomics has provided further insight into some interesting molecular mechanisms related to HSPs in general and HSP90 in particular. Falsone et al. [73] analyzed the HSP90-dependent ubiquitinated proteome and confirmed that HSP90 has diverse functions beyond signal transduction and gene transcription, such as inhibition of ubiquitination of a significant number of metabolic enzymes.

Functional proteomics permits the study of the extracellular role of HSP90-alpha in cancer cell invasiveness. This approach has demonstrated that HSP90-alpha, which is an extracellular protein, interacts with and activates matrix metalloproteinase 2, thereby enhancing cancer invasiveness [8, 30]. The modulatory role of HSP90 in the pathophysiology of tissue remodeling is an important topic of proteomic research, namely the secretome and degradome, with potentially informative diagnostic, and, more importantly, therapeutic implications for oncology.

In fact, pharmacological inhibitors of HSP90 (geldanamycin and its derivatives) could hamper various client proteins that act as key players in different tumorigenesis pathways, representing an innovative approach for cancer therapy [8, 30]. Moreover, considering the role of HSPs, particularly HSP90, in the induction of cancer cell resistance to different therapeutic approaches, pharmacological HSP inhibition could be a useful therapeutic strategy to supplement conventional anticancer therapies. However, given the fundamental role of the so-called "stress proteins" in the physiology of the cell, it is necessary to accurately define the pharmacotoxicologic profile of HSP-modulating drugs, especially in terms of global cell proteome modification.

In addition, from a pathophysiological point of view, some intriguing questions should be resolved in order to permit better definition of the potential clinical applications, including the development of therapeutics and diagnostic and prognostic biomarkers:

- i. Given their roles as physiological chaperone molecules, does the observed overexpression of different HSPs in cancer depend simply on increased protein synthesis to sustain cancer cell proliferation?
- ii. Is cancer-related HSP overexpression qualitatively and quantitatively distinguishable from the expression characteristic of non-neoplastic cell proliferation?
- iii. Is the extracellular HSPs proteome profile useful in clinical oncology?
- iv. Can proteomic analyses of the immune response to cancer be used to enhance the efficacy of cancer vaccines that modulate HSP activity?

Molecular Chaperones, Cardiovascular Disease and Proteomics

Cardiovascular disease refers to a wide range of heart and blood vessel problems, including coronary heart disease, hypertension, peripheral artery disease, rheumatic heart disease, congenital heart disease, and heart failure, but it is used most often used to describe damage caused to the heart or blood vessels by atherosclerosis, a multi-factor disease that affects the arterial vessels. Evidence suggests that inflammatory and immunological processes play an important role in the development of atherosclerosis, which causes the formation of plaques whose rupture and thrombosis can result in coronary heart disease and stroke.

Currently, several circulating biomarkers are used in clinical practice as diagnostic markers of cardiovascular disease with relatively low sensitivity and specificity, and several potential screening biomarkers are in the clinical evaluation phase. The real value and appropriate use of these markers remains a source of debate [74]. In spite of the considerable progress that has been made in reducing classic risk factors, such as high serum cholesterol, diabetes, hypertension, smoking, and infections, this disease remains one of the primary causes of death in developed countries [75].

In recent years, HSPs have attracted increasing attention in cardiovascular research in general and in atherosclerosis in particular, not only because of their involvement in the response of cells of the vessel wall to various types of stress, a major etiologic factor in cardiovascular disease, but also because of their interesting role in the immune reactions that occur during development of the vascular inflammatory process [76].

Recent investigations have implicated some molecular chaperones families, including the HSP60 family and the stress-inducible HSP72 in the atherosclerotic process [77] and ischemic myocardial disease [78]. HSP60 is expressed on the endothelial cell surface and in myocytes in response to biochemical and infectious insults [79, 80], and seems to play a significant role in triggering the inflammatory process, which causes early atherogenesis and destabilization of the atherosclerotic plaque [78] by activating the autoimmune response. Both vascular and myocardial HSP60 may elicit autoimmune reactions that can cause further vascular and myocardial damage. On the other hand, a high tissue level of HSP72, an inducible myocyte protein, has been documented in response to brief periods of ischemia or mechanical stress [79] and in myocardial adaptive processes to chronic or repetitive ischemia, known as “hibernation” [79, 81-83].

The mechanism of adaptation to stress remains unclear. Following observations of HSP70 upregulation in rat myocardium under stress, Zhao et al. [84] reported a similar increase in the level of Fas expression, suggesting that HSP70 is capable of protecting cardiomyocytes from stress-induced injury through inhibition of Fas-mediated apoptosis. HSP70, therefore, should be investigated as a potential therapy against cardiovascular injury caused by stress. Moreover, the levels of circulating HSP72, HPS60, and anti-HSP60 antibodies have been significantly correlated with the level of brain natriuretic peptide and systemic markers of inflammation in serum of patients with left ventricular dysfunction due to non-atherosclerotic cardiac disease [85]. The high level of HSP60 and HSP72 correlated with the extent of cardiac and microvascular dysfunction in patients with angiographically normal coronary arteries. This suggests that HSP activation most likely reflected early

pathophysiological mechanisms involved in microvascular and myocardial dysfunction, even in the absence of evident coronary artery disease.

As demonstrated by the large number of studies that have focused on atherosclerosis, this pathology has received substantial interest from the biomedical research community, as well as from the proteomics field. In fact, several interesting proteomic approaches have been applied in the search for a protein expression profile that could provide useful information regarding the formation of atherosclerotic lesions. Consistent changes in the expression of HSPs have been reported in a variety of proteomic experiments performed on biological samples, including cardiovascular samples (tissues or cells) and blood compartments (circulating cells and plasma). Several studies have been carried out by monitoring the protein profile of cardiac muscle and other components of the vascular system, including smooth muscle and endothelial cells under pathological conditions, and atherosclerotic plaques compared to healthy cells or tissue. In these studies, the level of HSPs was documented to vary significantly in many heart failure models [86-88]. These changes, which involved *de novo* protein synthesis and gene transcription, could be useful diagnostic signals and may facilitate an in-depth understanding of the molecular mechanisms involved in the development of cardiovascular disease.

To characterize the stress proteins and their possible role in cardiovascular disease, Portig et al. [89] used two-dimensional gel electrophoresis (2-DE) to isolate the major stress proteins from human endothelial cells. Ubiquitin, HSP27, HSP60, HSC70, GRP78, and GRP75 were found to be constitutively expressed in human endothelial cells, while HSP72 expression was induced exclusively in stressed cells.

In an attempt to analyze changes in the expression of different proteins in bovine vascular smooth muscle cells incubated in the absence and presence of 17β -oestradiol, Molero et al. [90] identified different proteins associated with smooth muscle cell proliferation (e.g., isoforms of HSP60 isoforms) whose expression appears to be reduced by treatment. Some data suggested that HSP60 was not only involved in mediating cell proliferation [91, 92], but also in modulating the atherogenesis process and triggering the onset of several autoimmune and chronic inflammatory diseases [91-93].

Pawlowska et al. [94] reported a detailed proteome of human umbilical endothelial cells following the transition from quiescence into proliferation and migration following induction by vascular endothelial growth factor (VEGF). In this process, which is considered to be a key event in the pathogenesis of atherosclerosis *in vivo*, the increased expression of all HSP family members (HSP27, HSP60, HSP70p5, HSP70p8, HSP90 and HSP96) was reported. The augmented expression of each HSP in VEGF-stimulated human endothelial cells was revealed by 2-DE and ESI-Q-TOF mass spectrometry and was verified at the mRNA level by RT-PCR.

Moreover, HSP90-beta, HSC71, HSP60, and HSP27 were reported in 2-DE reference maps and databases by Dupont et al. [95]. These authors focused on both intracellular and secreted proteins extracted from the primary culture of smooth muscle cells obtained from patients undergoing coronary artery bypass surgery. These proteins were separated by 2-DE, generating two protein patterns specific to the ASMC (arterial smooth muscle cell) proteome and secretome, which may prove to be very useful in future proteomic analyses. Furthermore, this report confirmed the involvement of HSPs in a wide range of biological functions and

provided potential markers for the characterization of smooth muscle cells during early and advanced phases of atheroma formation, which may be related to specialized functions and pathological disorders of blood vessels. Interestingly, a previous study has demonstrated that the HSP60 expression level correlated with the severity of atherosclerosis. In fact, a high level of circulating HSP60 and a high titer of anti-HSP60 auto-antibodies have been reported in patients with acute and chronic coronary artery disease [77, 81, 89]. Moreover, as suggested by Bason et al. [96], the production of anti-HSP60 in most atherosclerotic patients may be related to disease pathogenesis and the activation of human ASMC processes relevant to the development of atherosclerosis.

While the above-mentioned studies and others have provided considerable knowledge regarding cardiovascular disease and contributed to the creation of online 2-DE databases of human, dog, mouse, and rat myocardium [97-101], the proteome of human plasma is emerging as an active area of investigation. Proteomics has assumed an important role in the identification of cardiovascular disease-associated biomarkers [102]. It is well known that the proteomic analysis of plasma presents various problems, largely due to the presence of large quantities of specific proteins, such as albumin and immunoglobulins that can directly interfere with the discovery of pathology-related biomarkers by masking lower abundance proteins. These difficulties may explain why, despite the easy access to plasma and technological improvements in the depletion of highly abundant plasma proteins, proteomic approaches have yet to produce any clinically valid biomarkers [103]. However, it is reasonable to think that these technologies have the potential to advance our understanding in this medical research area.

One important advance was accomplished when a committed team of researchers undertook the HUPO Plasma Proteome Project in an attempt to create a plasma proteomic map that could serve as a starting point for further investigation. This project focused on cardiovascular research and involved distinct proteomic approaches that were performed in various laboratories worldwide. Of the 3,020 human proteins that were identified on the basis of their known functions and exhaustive literature searches, 345 were ascribed cardiovascular-related functions. These proteins were divided into eight different categories, and HSP70 p8 [104] was found in the signaling protein category. This finding is not surprising considering the number of studies that have shown the importance of stress as an important etiologic factor in cardiovascular disease.

Martin-Ventura et al. [105] applied an interesting proteomic approach based on the hypothesis that different patterns of protein secretion between carotid atherosclerosis plaques and normal end arteries could serve as potential markers for atherosclerosis. Among the differentially secreted proteins, the authors identified HSP27, which was initially detected by immunohistochemistry and found to be expressed by intact vascular cells of normal arteries and carotid plaques. The release of this protein from atherosclerotic plaques was dramatically decreased compared to the level released from control arteries. In confirmation of the results of the 2-DE-based secretome analysis, HSP27, as measured by ELISA, was markedly reduced (by about 20-fold) in the plasma of atherosclerotic patients relative to healthy subjects. Therefore, HSP27 could be negatively correlated with the atherosclerotic plaque complexity. On the basis of reported data, the authors proposed that a low level of plasma

HSP27 could serve as a potential marker of atherosclerosis, but further validation is needed in large patient cohorts.

Interestingly, Park et al. [106] performed comparative 2-DE analysis using carotid atherosclerotic endarterectomy samples and normal lesions and found that the total HSP27 and phosphorylated HSP27 level was significantly decreased in arterial plaques relative to the level in nearby normal lesions. They further confirmed these results by western blot. Interestingly, the level of phosphorylated HSP27 gradually decreased, with the lowest expression observed in the core plaque area. In the same study, the plasma level of HSP27 and HSP70, as detected by ELISA, was significantly higher in patients with acute coronary syndrome than in the healthy reference group, and the level of HSP27 significantly correlated with of the level of HSP70.

Evidence that oxidative stress is involved in the formation and progression of atherosclerotic plaques is provided by the proteomic analysis of Liao et al. [107]. In this study, which used chromatography and ESI-MS/Ms techniques to analyze the proteins secreted by vascular smooth muscle cells subjected to oxidative stress, the authors identified two factors, one of which was HSP90- α , and suggested that they played potentially important roles as physiologic mediators of the vessel wall response to reactive oxygen species (ROS).

Taken together, these results support the hypothesis that the stress response of the various vessel wall components is an important trigger of atherogenesis. This suggested relationship indicates an important role for HSPs in the earliest inflammatory stages of this pathogenic process. In contrast to the association between the plasma HSP60 concentration and coronary artery disease, which has been supported by multiple studies, the relationship between the plasma HSP70 level and the risk of coronary artery disease requires further investigation. Furthermore, as HSP70 is a very sensitive protein, the general inflammatory status may affect the serum level of HSP70.

Similarly, the cause and biological significance of changes in the expression of HSP27, whose possible role in atherosclerosis has been addressed only recently [108], remains to be determined. It is well known that this protein is involved in cytoskeletal organization, where it acts as a capping protein for actin. HSP27 can inhibit actin polymerization, thereby playing a crucial role in cardiac remodeling. The phosphorylation of HSP27 contributes to the regulation of microfilament dynamics following oxidative stress and may be involved in the adaptive response to ROS-generating agents, such as carcinogens, anticancer drugs, and other xenobiotics [109]. However, it is not known whether HSP27 has an atheroprotective role, such as the regulation of the mobility of smooth muscle cells and the coordination of actin activity or the inhibition of the activation of NF- κ B, which is widely involved in plaque instability and rupture, as indicated by previous studies [110, 111]. Alternatively, it could be that this function is a reflection of a pathological vascular remodeling process. This process is uncharacterized and requires in-depth study.

An altered level of HSPs, which have been documented by several proteomic studies, has been reported for multiple cardiovascular diseases. HSP isoform switching has been reported in a model of phenylephrine-induced cardiac hypertrophy [112], while a number of post-translational modifications, including reversible phosphorylation, have been observed in studies of dilated cardiomyopathy [86, 87, 97, 98]. The reversible phosphorylation of proteins

is one of the most important post-translational modifications, and the elucidation of the sites of phosphorylation is essential for understanding the regulation of key cellular processes, such as signal transduction.

In addition, Scheler et al. [86] described differential HSP27 species patterns in normal and failing human hearts using 2-DE in combination with immunostaining. They detected an elevated level of HSP27 in explanted hearts from patients with dilated cardiomyopathy or ischemic heart failure, supporting the involvement of HSP27 in cytoskeletal organization.

Additionally, proteomics was used to analyze right ventricular (RV) hypertrophy [113]. In this study, Faber et al. performed a differential proteomic profiling analysis of heart disease using an animal model of pulmonary artery banding in parallel with hemodynamic characterization. One particularly interesting finding from this study was the identification of three spots that were more intense in the cytoplasmic fraction of RV homogenates. These spots were identified as HSP27. A Western blot analysis and MS demonstrated that two of these spots were phosphorylated on Ser15. In accordance with data previously reported by others [102], which demonstrated altered HSP27 responses in cardiovascular disease, these results suggest an important role for such stress-related proteins in cardioprotection as well as heart failure. HSP27 was previously reported to be involved in smooth muscle dysfunction, as shown in the study of McGregor et al. [114], which analyzed changes in actin filament-associated protein expression in venous smooth muscle cells. A three-spot charge train corresponding to HSP27 showed a 1.6-fold increase in abundance, but with reduced representation of the phosphorylated Ser(82) and Ser(15)Ser(82) isoforms.

A further proteomic approach was carried out by Matt et al. [115], who performed 2-DE and mass spectrometry coupled with dephosphorylation and phosphostaining experiments to reveal protein alterations in aortic aneurysms of the bicuspid aortic valve (BAV) relative to those of the tricuspid aortic valve (TAV). Among the proteome alterations, which were confirmed by ELISA, levels of total HSP27 were significantly lower in aortic samples from patients with a BAV compared to TAV aneurysms. This may be due, at least in part, to a reduction in intracellular HSP27 phosphorylated at S78.

In an experimental proteomic approach used to study another cardiac disorder, Long QT syndrome (LQTS), which is characterized by alteration of the heart's electrical activity, Walker et al. [116] investigated the role of mutations in the *HERG* gene (*KCNH2*), which encodes the voltage-dependent delayed rectifier potassium channel. In the course of this work, they also identified known components of the cytosolic chaperone system, including HSC70, HSP90, Hdj-2, Hop (HSP-organizing protein), Bag-2 (BCL-associated athanogene 2), and several putative HERG-interacting proteins. The authors suggest that FKBP38, a membrane-integrated protein, acts as a co-chaperone for HERG, contributing to the HSC70/HSP90 chaperone system in the trafficking of wild-type and mutant HERG potassium channels.

In conclusion, HSP proteomics may clarify the underlying mechanisms of various and heterogeneous cardiovascular diseases, and this information could be useful for diagnostic, prognostic, and therapeutic purposes.

Molecular Chaperones, Immunopathology and Proteomics

As discussed above, HSPs play significant roles in immunopathology. Yet, while their pro-inflammatory and immunostimulant activities are well known and accepted, their anti-inflammatory and immunosuppressive effects have been less studied and are still debated [16, 17, 21]. Specifically, in addition to their housekeeping and cytoprotective functions, it is now known that HSPs and other stress proteins (e.g., the glucose-regulated proteins and calreticulin) can be released into the extracellular space from a variety of cell types, including neurons, monocytes, macrophages, B cells, and epithelial tumor cells [117-119]. Secreted HSPs can interact with adjacent cells or enter the bloodstream to act on target cells. In the late 1980s, when it was first reported that HSP70 and HSP110 were secreted from cultured mammalian cells, it was hypothesized this release may be implicated in a number of physiological or pathological events, suggesting that a cell may protect adjacent cells by this HSP secretion [120]. Further studies have confirmed that HSPs, particularly members of the HSP60, HSP70 and HSP90 families, are involved in autoimmune pathogenesis. It is commonly accepted that autoimmune diseases arise from an excessive immune response in the body that is directed against substances and tissues normally present in the organism. In regard to the role that HSPs play in immune system activation, the features that are important to consider are: i) the expression of HSP70 and HSP90 on the surface of tumor cells or virus-infected cells; ii) the ability of HSP-peptide complexes to generate a cytotoxic T lymphocyte response against cells producing these peptides; and iii) HSP-mediated cytokine production [121].

Interestingly, HSP70 surface expression has been observed in virus-infected cells and in cancer biopsies (colorectal, lung, neuronal, pancreas, oral dysphasia, and squamous cell carcinoma), but not in normal cells [122, 123]. As such, the cell surface expression of stress proteins may be relevant to immune surveillance. In fact, many studies have demonstrated that the surface expression of HSP70 can be correlated with natural killer cell-mediated cytotoxicity, which, as several investigations have shown, can be blocked by specific antibodies to HSP70 [124, 125]. Moreover, HSP70 has been reported to be a potential autoantigen in multiple sclerosis. In type 1 diabetes, the preferential expression of HSP70 by beta, but not alpha cells, in the islets of Langerhans seems to be an important event in the autoimmune process that ultimately leads to the destruction of beta cells [126].

In addition, an elevated HSP60 level has been observed in both the synovial tissue and plasma of patients with rheumatoid arthritis and juvenile chronic arthritis [127]. A role of HSP60 in type 1 diabetes (insulin-dependent diabetes mellitus, IDDM) was initially suggested following the observation that HSP60-specific T cells play an important role in the development of insulinitis and hyperglycemia in non-obese diabetic mice [128]. These results were confirmed by further studies demonstrating that the response to human HSP60 and HSP60 peptides by peripheral blood T cells from patients with type 1 diabetes was significantly greater than the normal blood donors [129]. These and other findings indicate a role for HSP60 in the development of the inflammatory processes observed in diabetes and in diabetes-associated complications. To this end, novel immunotherapeutic tools have been developed, such as an immunogenic peptide from HSP60 (p277; DiaPep277), which has been shown to arrest beta cell destruction in non-obese diabetic mice. Interestingly, a randomized,

double-blinded, phase Ib/II clinical trial of DiaPep277 was recently performed on recent-onset type 1 diabetes patients with remaining insulin production. The aim of this clinical trial was to study the immunological efficacy of this peptide therapy and its correlation with clinical outcome. Results showed that all treated patients demonstrated an altered immune response to DiaPep277, while the majority of placebo-treated patients remained non-responsive to treatment, indicating a 100% efficacy of immunization [130].

Relatively few proteomic studies have been published examining the changes in the HSP profile in diabetes. The few reported investigations were undertaken in an attempt to characterize the protein expression pattern in beta cells or in other tissues affected in diabetes. Recently, an interesting integrated proteomic and genomic approach of normal, healthy, and pancreatic mouse islets with type 2 diabetes has been performed. In this study, the link between insulin resistance and beta cell dysfunction was investigated in a mouse model with insulin resistance that progressively develops diabetes [131]. Among the differentially expressed proteins identified, a prominent group of proteins related to protein folding and stress, the HSP40 family including DNAJC3 and DNAJB11, was reported to be up-regulated in diabetic mouse islets. The up-regulation of DNAJC3 at the protein level was also shown in islets from patients with type 2 diabetes in a recently published study [132]. These findings, which revealed many important alterations in the level of stress proteins during beta cell dysfunction, led the authors to hypothesize that insulin resistance, through increasing the demand on the cell for insulin secretion, may lead to defective metabolic coupling and cell stress. As such, this situation would augment insulin secretion, but ultimately would cause a loss in glucose sensing and impaired insulin secretion, resulting in type 2 diabetes. In addition, a recent proteomic study on rat diabetic nephropathy, reported by Li et al. [133], used 2-D differential gel electrophoresis and mass spectrometry to investigate kidney protein profiles among control, untreated, or diabetic rats treated with grape seed proanthocyanidin extracts (GSPE). They reported the effective treatment of diabetic nephropathy using GSPE and identified significant alterations in the expression level of several proteins involved in oxidative stress as a consequence of diabetes. This HSP involvement in the response to elevated glucose levels also emerged in the proteomic study of Ahmed and Bergsten [134]. By comparing freshly isolated mouse islets with those cultured for 24 hours at high glucose concentrations, they demonstrated that exposure to a high glucose concentration generates oxidative stress. This study showed that the increased expression of antioxidant proteins and HSPs was, intriguingly, attributable to HSC74 and HSP40. A different investigative approach was undertaken by D'Hertog et al. [135] in an attempt to analyze differential protein expression profiles in insulin-producing INS-1E cells exposed to inflammatory cytokines *in vitro*. Using 2-D DIGE, they identified the differential expression of various molecular chaperones, including GRP78, ORP150, HSP70 protein 8 (HSPA8), and mortalin (HSPA9), among other identified proteins with roles in insulin biosynthesis, energy metabolism, cytoskeletal organization, RNA metabolism, and oxidative stress/defense. The altered expression of each of these proteins was apparent in more than one spot, suggesting that these proteins undergo post-translational modification. HSP-mediated cytokine production may play a crucial role in cellular defense mechanisms induced by the local inflammatory environment, leading to beta-cell dysfunction and a diabetic phenotype. This observation is supported by the recent intriguing observation that blockage

of IL-1 beta in type 2 diabetic patients partially restores beta cell function [136]. In conclusion, the expression profile of proteins whose expression is significantly modified in diabetes is similar in different proteomic studies using different experimental models [137]. Moreover, the stress proteins are important signals in the pathophysiology of autoimmune diseases. Oxidative stress plays a primary role in the pathogenesis of diabetes mellitus and its related complications. In conclusion, as a consequence of this increased oxidative pressure, an adaptive response occurs that requires functional chaperones, antioxidant production, and protein degradation.

An interesting proteomic study was undertaken to investigate the presence of autoantibodies in patients with another autoimmune disease, chronic hepatitis. Using the 2-DE separation of proteins extracted from HepG2 cells, Fukuda et al. [138] detected spots that reacted with serum from affected patients by immunoblotting and identified these spots by mass spectrometry. The identified protein spots included HSP70 and HSP60, suggesting that autoantibodies against the HSPs may modulate the autoimmune response and contribute to the pathogenesis of several diseases. In agreement with previous reports of the presence of autoantibodies to the constitutively expressed form of HSP70 in patients with autoimmune hepatitis and chronic hepatitis [139], a recently published proteomic study confirmed the detection of circulating anti-hepatocyte plasma membrane autoantibodies in autoimmune hepatitis [140]. Interestingly, among the proteins with known potential plasma membrane expression, the 60-, 70-, and 90-kDa HSPs were identified by ion-trap mass spectrometry and were confirmed using immunofluorescence and immunoelectron microscopy.

In general, antibodies against HSPs are found in the serum of patients with other autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis [17, 22]. Similar observations have been made in several experiments with animal models of inflammation, in which the induction of T cell reactivity to HSP60 and HSP70 inhibits arthritis by a mechanism that involves the production of regulatory cytokines (e.g., IL-4 and IL-10) by CD4+ T cells [141-144]. The significance of anti-HSP autoantibodies in these pathologies remains to be determined and requires further study.

Using 2-DE analysis, Yeo et al. [145] observed significant modifications of the HSP profile after *H. pylori* infection in RGM-1 cells. The authors focused on the role of HSPs in *H. pylori*-associated gastropathy and found that this infection, which causes inflammation in the gastric mucosa leading to gastritis, gastric ulcers, duodenal ulcer disease, and even gastric cancer, significantly attenuated the expression of HSP70. The authors proposed that the induction of HSP70 confers protection against *H. pylori* infection by inhibiting the expression of iNOS. These results, which provide information on the HSP expression profile in response to *H. pylori* infection, highlight the cytoprotective role of HSP70 in this type of infection.

In conclusion, all of these observations, and the fact that HSP60 and HSP70 are present in the peripheral circulation of normal individuals at a level capable of eliciting inflammatory responses in vitro, suggest that the inflammatory properties of these proteins must be carefully re-evaluated. In fact, a growing body of evidence supports an apparent contradictory set of both pro- and anti-inflammatory roles for this intriguing class of proteins [146]. Many studies support the general concept that an intracellular stress response may allow tolerance to environmental stresses through downregulation of inflammatory cytokines,

which in turn attenuates the inflammatory process. In contrast, many others studies expand this concept to address the importance of extracellular stress proteins that are released in response to certain stress stimuli. In this case, downstream events activate the inflammatory response, which promotes chronic inflammatory conditions such as arthritis, multiple sclerosis, diabetes, and cardiovascular disease. While these relevant aspects of HSP pathophysiology require further study, the use of promising proteomic technologies can certainly provide new opportunities for research, and has led to the speculation that HSPs can be either immunostimulatory or immunosuppressive, depending on the context. There is no doubt that HSPs are an important component of the response to environmental stress as well as the recruitment of regulatory T cells during the course of inflammation. These activities are essential to the maintenance of immunosurveillance homeostasis.

Molecular Chaperones, Neuropathology and Proteomics

Proteomic approaches have been widely adopted in the investigation of various neurological disorders, with the main goal of discovering meaningful biomarkers for diagnosis, prognosis, and therapeutic monitoring. Thus, brain, cerebrospinal fluid, and serum samples from patients with various vascular (e.g., focal and diffuse ischemia), inflammatory/immunopathological (e.g., prion diseases and multiple sclerosis), and degenerative diseases (e.g., Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis) have been analyzed by proteomics [4, 7, 20]. Proteomics has also been adopted for a large number of experimental neurology studies on cell lines and animal models of different neuropathies [147, 148]. The importance of brain proteomics was recently stressed at the Eighth Hupo Brain Proteome Project Workshop [149].

Furthermore, the role of stress proteins in neurology has been greatly expanded recently, in particular the pathophysiological role of chaperones in neurodegeneration [147, 150]. In fact, many neurodegenerative diseases share a common pathogenic mechanism: aggregation and deposition of abnormal proteins with the formation of plaques/inclusion bodies that co-localize with various chaperones and components of the ubiquitin-proteasome degradation system. Moreover, prion diseases have further highlighted the importance of the so-called disorders of protein conformation in neuropathology. Absolute and relative insufficiencies of stress proteins are likely to be involved in these puzzling pathogenic mechanisms.

Despite this fundamental role of chaperones in neurological disorders, brain proteomics has not yet focused on the proteome of stress proteins. Various studies have focused on the overexpression of a single HSP. For example, Fonte et al. [151, 152] used an experimental model of Alzheimer's disease (AD) to demonstrate that the constitutive expression of the small heat shock protein HSP16.2, which is homologous to vertebrate alphaB-crystallin, partially suppresses A β toxicity by interacting directly with the A β peptide and altering its oligomerization. However, other HSPs also seem to play roles in the pathogenesis of AD. In fact, Kakimura et al. [153] have demonstrated that through the induction of interleukin 6 and tumor necrosis factor alpha, HSP90, HSP70, and HSP32 increase the phagocytosis and clearance of A β peptides by microglia present in extracellular senile plaques. Similar results in terms of HSP overexpression have been obtained for Parkinson's disease, the

hallmark of which is the accumulation of α -synuclein, a protein in Lewy bodies. As such, chaperones certainly play a role in promoting the refolding and degradation of this protein. In fact, the overexpression of different HSP families has been demonstrated in various studies (Pridgeon et al.: HSP75 [154]; Xun et al., and Ahn et al.: HSP70 [155, 156]; Shen et al.: HSP90 [157]); and Ganadu et al.: AlphaB-crystallin [158]). In the prion field, proteomic studies were undertaken to evaluate the biochemical abnormalities underlying prion-induced neuronal death. These investigations have demonstrated a significant pathogenic role for various HSPs, especially in terms of protection against protein aggregation by the solubilization of prion proteins [159, 160].

A more organized proteomic approach for the analysis of HSP expression has been performed for amyotrophic lateral sclerosis. Specifically, the interaction between protein chaperones and the protein folding and degradation pathways, especially for the aggregation of mSOD-1 (superoxide dismutase-1) [161], was assessed from a pathophysiological perspective. Interestingly, these data suggest the inactivation of specific chaperones, such as HSP70 and its co-chaperones CHIP, HSP40, and HSP105. Other proteomic studies on this disease have demonstrated a simple overexpression of various HSPs, including alphaB crystallin, HSP40, HSP70, and HSP105 [162- 165].

Conclusion

Increasing attention is being paid to the importance of molecular chaperones, particularly HSPs, in a wide spectrum of inflammatory and degenerative diseases, as well as in cancer. Although proteomics certainly is a powerful technique for the investigation of the mechanism of the HSP response, which supports new therapeutic approaches for the treatment of these diseases, the overall impression following the analysis of scientific literature on “proteomics and heat shock proteins or cellular stress response” may be defined as a “missed opportunity,” at least for now. In fact, numerous proteomic studies have shown that in various diseases the overexpression of one or more HSPs can be observed without concomitant analysis of either the intimate pathogenic mechanisms that cause their induction or the consequences in terms of cellular pathophysiology. As a consequence, it is not possible to know the real or complete picture of the biological activities of a single HSP. This lapse has important negative repercussions, not only for the understanding of the pathophysiology of various degenerative and inflammatory diseases, but also for the clinical applications of HSPs as valid prognostic and diagnostic biomarkers.

Most importantly, pharmacological research has led to the development of a series of molecules capable of modulating HSP expression. Some of these new drugs have interesting therapeutic potential (e.g., the HSP90 inhibitor geldanamycin), while the pharmacotoxicological profiles of other molecules (e.g., proteasome inhibitors acting as HSP inducers) require careful evaluation. For all of these molecules, a proteomic approach could be useful to obtain a better understanding of some of the biological activities and redundancy mechanisms related to the induction of different HSP families.

In fact, understanding the functional interrelationships among different HSPs could clarify the role of each HSP with a prompt opportunity for clinical application. The

importance of this analysis is underscored by the growing discussion in proteomics of the so-called protein expression overlap. This overlap refers to the different pathogenic conditions (physical and chemical stress, inflammatory and degenerative diseases) that are associated with similar modifications of the cellular proteome, especially regarding HSPs and the proteins involved in oxidative stress and regulation of the cell cycle. Clearly, if this concept of “protein overlap” in terms of stress-induced modification of the cell proteome is confirmed, the potential utility of HSPs as diagnostic and prognostic biomarkers should be reconsidered.

Some intriguing peculiarities of HSPs have become evident as a result of proteomic studies. For example, the concomitant overexpression of Heme-oxygenase 1 and other HSPs strongly suggests an oxidative pathogenic role of HSPs in the cellular stress response [148]. Moreover, in neurodegenerative diseases, there is relatively constant overexpression of alphaB-crystallin and ubiquitin [147, 150] that, from a pathogenic point of view, must be clarified in terms of its intimate pathological role.

From a therapeutic point of view, proteomic studies have revealed a significant role for drug-induced overexpression of HSPs. This pharmacologically induced cellular stress response could likely play a promising therapeutic role in diseases that are characterized by abnormal protein conformations (e.g., neurodegenerative and prion diseases), and could also be used as a preventive measure in high risk patients. To this end, iatrogenic stimulated conditioning could also preserve cells and tissues from ischemic/anoxic damage (e.g., coronary heart disease and cerebrovascular disease). Last but not least, the appropriate modulation of HSP induction and release through the presentation of tumor-specific peptides to APCs could enhance the immune response against cancer.

In conclusion, proteomic studies should address the descriptive and functional aspect of molecular chaperone with the goal of better defining its physiology and pathophysiology. This understanding will certainly have fundamental pathogenic, diagnostic, prognostic, and therapeutic implications.

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Chapter IV

System Specific Chaperones for Membrane Redox Enzyme Maturation in Bacteria

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Abstract

A group of bacterial system specific chaperones are involved with the maturation pathway of redox enzymes that utilize the twin-arginine protein translocation (Tat) system. These chaperones are referred collectively as REMPs (Redox Enzyme Maturation Protein). They are proteins involved in the assembly of a complex redox enzyme which itself does not constitute part of the final holoenzyme. These proteins have been implicated in coordinating the folding, cofactor insertion, subunit assembly, protease protection and targeting of these complex enzymes to their sites of physiological function. The substrates of REMPs include respiratory enzymes such as N- and S-oxide oxidoreductases, nitrate reductases, and formate dehydrogenases, which contain at least one of a range of redox-active cofactors including molybdopterin (MoPt), iron sulfur [Fe-S] clusters, and b- and c-type haems. REMPS from *Escherichia coli* include TorD, DmsD, NarJ/W, NapD, FdhD/E, HyaE, HybE and the homologue YcdY. The biochemical, structural and functional information on these REMPs are reviewed in detail here.

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Introduction

Coordinating the folding, assembly, and targeting of complex enzymes to their sites of physiological function is an important feature of all biological systems. In bacteria, the generation of energy by respiratory electron transport chains involves the cytoplasmic membrane. The redox-active protein components of electron transport chains are often embedded in this membrane to interact with the quinone/menaquinone pool and significant numbers are located on the extra-cytoplasmic side. Extra-cytoplasmic respiratory enzymes such as N- and S-oxide oxidoreductases, periplasmic nitrate reductases, and formate dehydrogenases contain at least one of a range of redox-active cofactors including molybdopterin, [Fe-S] clusters, and b- and c-type haems. The assembly of such proteins is recognized as one of the most complex processes in the field of bioinorganic chemistry. With the notable exception of the c-type cytochromes, such cofactors are assembled into the protein in the cytoplasm prior to enzyme export from the cell.

The acquirement of molybdenum and the biosynthetic pathway for the molybdopterin cofactor (MoPt) or the more complex bis-(molybdopterin guanosine dinucleotide) (MGD) cofactor is a complex multi-step process requiring numerous enzymes (Schwarz, 2005). In bacteria, high-affinity molybdate transporters acquire the molybdate anion form of molybdenum. Molybdate is catalytically inactive within the cell until complexed with a tricyclic pterin cofactor (sometimes referred to as a molybdopterin, or molybdenum cofactor or Moco). Molybdopterin biosynthesis involves numerous enzymatic steps that can be abbreviated into 3 general steps: (1) Synthesis of precursor from guanosine-5'-triphosphate; (2) Incorporation of 2 sulfur atoms to form the tricyclic pyranopterin; and (3) Insertion of the molybdenum atom (each Mo atom can have one or two pyranopterin molecules associated) (Schwarz, 2005). Many bacterial molybdoenzymes utilize a fourth step in which a guanosine dinucleotide is added to the molybdenum-complexed molybdopterin (Schwarz, 2005). As the MoPt and MGD are very labile they must be protected in the protein at all times during the biosynthesis. This raises the question of their insertion into their final host proteins polypeptide chain, with consideration that this step occurs concurrent with protein folding during the conversion from apo to cofactor holo form.

The Twin Arginine Translocase (Tat) System

Studies of the model prokaryote *E. coli* have established that many of these cofactor-containing exported proteins are synthesized with amino-terminal leader signal peptides containing the distinctive SRRxFLK "twin-arginine" amino acid sequence motif (Berks, 1996). The leader contains 3 regions similar to Sec dependent leaders; the n, h, and c region. The twin-arginine motif is typically found between the n and h regions (Berks, 1996) (Figure 1). The "n-region" is a small charged region of the signal peptide that precedes the twin-arginine motif and is unique to each individual protein. The h-region is a hydrophobic stretch of 7-30 amino acids. The c-region contains a cleavage recognition site which is thought to be processed by leader peptidase I. Preproteins bearing these twin-arginine-leaders are secreted post-translationally and most interestingly post-folding, across the cytoplasmic membrane by

a unique protein translocase initially termed the membrane targeting and translocation (Mtt) system (Weiner et al., 1998) or the Δ pH translocase (Settles et al., 1997). The system is now most frequently referred to as the twin-arginine translocase (Tat) (Sargent et al., 1998; 2002; 2007a; Berks et al., 2000a; 2000b; 2003; Lee et al., 2006).

Table 1. Tat-dependent redox enzyme systems in *E. coli*.

Enzyme	Twin arginine leader containing subunit	Predicted REMP	Catalytic cofactor
Biotin sulfoxide reductase 1	BisC	YcdY ^a	MoPt
Biotin/TMAO reductase	BisZ/TorZ	YcdY/TorD ^a	MoPt
TMAO reductase	TorA	TorD	MoPt
DMSO reductase	DmsA	DmsD	MoPt
Putative DMSO reductase	YnfE	DmsD ^a	MoPt ^c
Putative DMSO reductase	YnfF	DmsD ^a	MoPt ^c
DMSO/TMAO reductase	YedY	DmsD/TorD	MoPt ^c
Formate dehydrogenase	FdnG	FdhD/FdhE	MoPt
Formate dehydrogenase	FdoG	FdhD/FdhE	MoPt
Hydrogenase 1	HyaA	HyaE	Ni
Hydrogenase 2	HybO	HybE	Ni
Nitrate reductase (periplasmic)	NapA	NapD	MoPt
Nitrate reductase (cytoplasmic)	NarG	NarJ	MoPt
Nitrate reductase (cytoplasmic)	NarZ	NarW ^a	MoPt
Hypothetical protein	YfhG	? ^b	None predicted
Hypothetical protein	YcdO	? ^b	Heme ?
Hypothetical Peroxidase (periplasmic)	YcdB	? ^b	Heme cofactor

^aPredicted REMP for enzyme in question based on predicted function/role of the enzyme and its relatedness to other enzymes with an appropriate REMP. However, no experimental evidence to support this.

^bNo predicted REMP for enzyme in question due to limited knowledge of its function.

^cPredicted catalytic cofactor of molybdopterin (MoPt) based on the presence of a signature domain in its sequence.

	N-	n-region	SRRxFLK	h-region	c-region	
DmsA	--	MKT	KIPDAVLAAEV	SRRGLVKT	---TAIG-G-LAMASSALTLPFSRI	42
FdnG	-----	MD	--V	SRRQFFK	-----ICAG-G--MAGTTVAALGFAPKQ	30
FdoG	-----	MQ	--V	SRRQFFK	-----ICAG-G--MAGTTAAALGFAPSV	30
HyaA		MNNEETFYQAMRRQGV	TRRSFLK	---YCSL---	AATSLGLGAGMAPKI	42
HybO		MTGDNTL	---IHSHGINRRDFMK	---LCAA---	LAATMGLSSK	34
NapA	-----	MK	--L	SRRSFMK	-----ANA--VAAAAAAGLSVPGV	28
TorA		MNNND	---LFQ	--ASRRRFLA	---QLG-GLTVAGMLGPSLLTPRR	36
NarG	-----	MSKFL	DRFRYFKQ	KGETFADGHGQLL	NTNRDWEDGYRQRWQHDKI	VRSTH 50
NarZ	-----	MSKLL	DRFRYFKQ	KGETFADGHGQVM	HSNRDWEDSYRQRWQFDKI	VRSTH 50

Figure 1. Twin-arginine leader sequences of Tat system substrates. Typical structure of leader peptides consists of a basic n-region (red), followed by the twin-arginine motif (purple), a variable length hydrophobic h-region (grey), and a short polar c-region (blue). The leader peptidase I cleavage site follows the c-region. The peptides of NarG and NarZ do not contain these structures as their proteins are not translocated and are attached from the cytoplasmic side of the membrane. Sequence alignment of all the peptides demonstrate the architecture of these structures and highlight the vestige motifs of NarG and NarZ.

A most remarkable feature of the Tat system is its substrates from both bacteria and plant chloroplasts are required to be fully folded before successful translocation can occur (e.g. DeLisa et al., 2003). Additionally, the system appears to translocate protein complexes where only one protein in the complex may contain a twin-arginine-leader (see reviews: Berks et al., 2003; Sargent 2007a; b). Biochemical studies have shown that the integral membrane proteins TatA, TatB, and TatC form the core components of the *E. coli* Tat system (reviewed by Berks et al., 2003). The TatBC unit is believed to form the signal recognition module, while TatA forms a very large oligomeric ring-structure presumed to be the protein-conducting channel itself (Berks et al., 2003).

The majority of work to date on the Tat system has been in the model bacterial system *Escherichia coli* which has has ~27 Tat targeted proteins (Tullman-Erick et al., 2007). The majority are cofactor-containing enzymes, most of which acquire their prosthetic groups in the cytoplasm prior to export. Additionally, assembly with signal-less partner subunits must occur prior to translocation and these partners must also have any prosthetic groups preloaded prior to translocation. Once the discovery of this Tat pathway was reported, the system became a focus of many excellent research groups (B. Berks, T. Palmer, C. Robinson, J. Weiner, L.-F. Wu, K. Cline, M. Müller and many newly inspired groups). Their focus has been on proteins that use the translocase, and the translocase itself, with some interest in comparing the twin-arginine leader to that of Sec-dependent leaders.

Discovery of REMPs

A key question was asked early on for this system; is there a twin-arginine leader binding protein for the Tat system? This was asked as the general secretory system utilizes at least two chaperones such as SecB and the signal recognition particle (SRP). The question was answered with the discovery of DmsD (Oresnik et al., 2001). This experiment utilized the twin-arginine signal peptide from the Dimethyl sulfoxide reductase catalytic subunit (DmsA)

fused to the N-terminus of glutathione-S-transferase and pre-bound to a Glutathione-sepharose resin over which anaerobic *E. coli* lysate was poured. Two proteins were found to bind to this peptide, the chaperone DnaK and a product from an uncharacterized reading frame YnfI, now labeled as DmsD. Subsequent studies described here identified other system specific chaperones. As the result of a bioinformatics study of the similar oxidoreductase systems, the collective term REMP (Redox Enzyme Maturation Protein) was proposed to refer to any protein involved in the assembly of a complex redox enzyme which itself does not constitute part of the final holoenzyme (Turner et al., 2004). Different REMPs in *E. coli* are listed in Table 1 but are also found in other organisms where similar redox enzymes are found. The need for these system specific chaperones in microbial physiology has become increasingly accepted in the past few years. Many potential roles for these systems specific REMPs have been suggested and include:

- Foldase chaperone – promoting assembly of the enzyme.
- Unfoldases – correcting mistakes in folding.
- Sec-avoidance chaperone – preventing incorrect targeting during assembly.
- Cofactor chaperone – maintaining the apoenzyme in a competent state for prosthetic group loading.
- Cofactor binding protein – binds the cofactor to transfer to the apoenzyme.
- Cofactor insertase – directly involved in prosthetic group insertion.
- Targeting protein – involved in specific targeting of their substrate to locations in the cell (e.g. prosthetic group biosynthesis, membrane, Tat translocase). This has also been referred to as an ‘escort protein’.
- Tat system Proofreading – suppressing transport until the assembly process is complete.
- Control “policing” protein – verification of ‘hitchhiker’ proteins that are associated prior to translocation. Hitchhiker proteins are co-translocated with twin-arginine-leader containing proteins, yet do not have such a signal sequence themselves. May or may not be a step in proof reading.
- Protection from proteases – preventing degradation during assembly.

Enzyme Systems and Their Cognate REMP Chaperones

Below, each enzyme system that requires a REMP system specific chaperone for maturation will be briefly introduced followed by a discussion of their system specific chaperone.

TMAO Reductase (TorD)

In *E. coli*, trimethylamine N-oxide (TMAO) reduction is important during anaerobic respiration. TMAO reduction is dependent on the gene products of the *torCAD* operon (Mejean *et al.*, 1994). The first gene product, TorC is a membrane anchored pentahemic c-

type cytochrome whose proposed role is to feed electrons to the catalytic subunit of the reductase, TorA (Mejean *et al.*, 1994; Gon *et al.*, 2001). TorA, encoded by the second gene of the operon, is a periplasmic molybdoenzyme which bares the characteristic twin-arginine motif and is thus a substrate of the Tat system (Voorduow, 2000). Characteristic to other Tat substrates in *E. coli*, TorA obtains its molybdopterin cofactor from the cytoplasm prior to transport (Voorduow, 2000). The third gene of the *tor* operon encodes for a 22kDa protein, TorD, the now established REMF for TorA. Homologues to the *tor* operon have also been identified in *Shewanella massilia* (*torECAD*) as well as *Rhodobacter capsulatus* (*dorCDA*) and *Rhodobacter sphaeroides* (*dmsCBA*). DorD and DmsB are the TorD homologues in the *Rhodobacter* species, and displays 26-27% sequence identity with *E. coli* TorD while *S. massilia* TorD shares 34% identity to *E. coli* TorD (Pommier *et al.*, 1998). Although TMAO reduction by TorC and TorA is well studied, the specific function of TorD has not been completely elucidated. However, of the chaperones involved in redox enzyme maturation, the most information is available on TorD. Multiple roles for TorD have been proposed, all of which are centered on its association with TorA to aid in the successful maturation of a fully functional protein.

Initially, it was thought that TorD was a membrane-associated protein, likely acting as another player in the electron transport pathway for TMAO reduction. Recognizing two hydrophobic sections in the amino and carboxy ends of the protein, it was proposed that these regions could anchor the protein to the membrane (Mejean *et al.*, 1994). However it was later shown that TorD is found primarily in the cytoplasm and interacts with an unfolded form of TorA reductase (Pommier *et al.*, 1998). TorD was then coined as a specific chaperone protein of TorA. There is some evidence that the fraction associated with the membrane may be the result of interactions with the Tat translocase itself (Papish *et al.*, 2003).

Proposed TorD functions

Over the past decade, many studies have suggested that TorD is involved in the maturation of TorA. This has been attributed to its protective role and its proposed ability to induce apoTorA into a cofactor competent state in a variety of conditions (Pommier *et al.*, 1998; Genest *et al.*, 2005; Genest *et al.*, 2006a, 2006b; Ilbert *et al.*, 2003; Shaw *et al.*, 1999). TorD was first implicated in a protective role for TorA in an *E. coli* strain deficient in molybdenum cofactor synthesis (Pommier *et al.*, 1998). In the absence of TorD, apoTorA levels were significantly lower than that of wild type levels, yet with the overexpression of TorD, apoTorA levels were restored. This was also observed during anaerobic growth in the presence of nitrate and TMAO creating a condition where the cofactor was limiting (Genest *et al.*, 2006). In these conditions, a strain devoid of TorD showed minimal levels of apoTorA. These data suggest that in the presence of TorD, apoTorA is either protected or stabilized against degradation. And, upon the availability of the MoPt cofactor, apoTorA protected by TorD could undergo maturation, suggesting that TorD may also be able to induce apoTorA into a cofactor accepting conformation.

TorD also appears to be capable of protecting apoTorA from temperature induced misfolding or degradation (Genest *et al.*, 2005). At high temperatures, the absence of TorD was accompanied by markedly decreased levels of TorA and substantially decreased TMAO reductase activity. However, in the presence of TorD, the maturation of TorA was unabated.

Similarly, in *R. capsulatus*, the absence of DorD (TorD homologue) caused a complete loss of dimethylsulfoxide (DMSO) activity, likely due to the degradation of DorA in the absence of its chaperone (Shaw *et al.*, 1999). The group also found data implicating DorD involvement in the insertion of the MoPt cofactor into apoDorA. This is complemented by data suggesting that TorD could modify the conformation of apoTorA in order to aid in cofactor insertion (Ilbert *et al.*, 2003). When TorD was preincubated with apoTorA, an increase in activation of apoTorA upon the availability of the MoPt cofactor is observed, suggesting that TorD acts on the apoprotein prior to cofactor insertion in order to induce apoTorA into a cofactor receptive state.

In a 2006 investigation (Genest *et al.*, 2006), TorD was shown to specifically protect the signal peptide from degradation. TorD co-purified with the apoTorA form that contained a preserved signal peptide. On the other hand, no co-purification was observed with a truncated form of apoTorA, missing regions of the signal peptide suggesting that TorD has high affinity to the signal peptide. In the absence of TorD, only a truncated form of TorA was seen corresponding to a loss of a 36 residue N-terminal region of the apoprotein. Even a cofactor loaded TorA had a truncated signal peptide in the absence of TorD. It is thus possible that the binding of TorD to the signal peptide protects a certain region that is a target for proteolysis.

The degradation of TorA likely begins at the N-terminal signal peptide and the data to date suggest that a role of TorD may be to bind and protect the twin-arginine-peptide of apoTorA. However, it is not clear if this is the primary function or simply a consequence of its primary role in maintaining the nascent polypeptide chain of TorD in a Moco competent state. It cannot be ruled out that other general chaperones are involved in TorA maturation, but it is convincing that TorD plays a more prominent role than any other protein alone.

TorD interactions with TorA

TorD is implicated in signal peptide binding (Genest *et al.*, 2006) and it is likely that TorD also binds the core region of apoTorA in order to aid in co-factor insertion. It is also proposed that the binding of a REMP to its substrate is a “proofreading event” to prevent the premature export of the protein before cofactor loading and/or dimerization in cases of multi-subunit enzyme complexes with only one binding partner containing the signal peptide. Here, the specifics of these binding events will be explored.

In a 2004 investigation (Jack *et al.*, 2004) it was demonstrated that REMPs recognize the twin-arginine signal peptide of their specific molybdoenzyme substrate. Using bacterial two-hybrid screening, TorD was shown to specifically bind the cleavable signal peptide bearing the twin arginine motif. Interactions were only found when the entire signal peptide was used. Furthermore, there was only a 1:1 ratio of TorD to the signal peptide of TorA, showing no evidence of TorD binding the signal peptide as a dimer or trimer. Despite the lack of interaction seen via bacterial two hybrid with a truncated form of apoTorA, a fusion protein experiment demonstrated that TorD may also bind regions in the core of TorA (Jack *et al.*, 2004) in order to produce a functional mature protein. When the signal peptide of TorA was replaced with that of another Tat substrate, TorD was still required for TorA maturation, although it could not bind the foreign signal peptide.

Specific regions where TorD binds the ssTorA was elucidated using isothermal calorimetry and synthetic regions of the TorA signal peptide (Hatzixanthis *et al.*, 2005). The hydrophobic h-

region as well as a positively charged n- and c-region is needed for binding. Interestingly, a mutation of the RR residues to KK in the n-region did not change the binding affinity of TorD to the peptide, thus suggesting that the twin-arginine motif is not the primary source for peptide recognition. Thus, the n-region containing the twin-arginine motif is not sufficient for binding but is necessarily required for efficient binding in combination with the core region of the peptide (Jack *et al.*, 2004, Hatzixanthis *et al.*, 2005). It is possible that TorD recognizes other residues in the n-region in order to distinguish from other Tat leader peptides.

Individual residues within a leucine rich portion of TorA signal peptide h-region was identified to be involved in TorD binding both *in vivo* and *in vitro* (Buchanan *et al.*, 2008). A TorD variant was also identified that displayed increased affinity for both wild type and variant TorA signal peptides. Residues within the leucine rich region were systematically mutated to glutamine residues, and subjected to bacterial two-hybrid screening to evaluate binding *in vivo*. Binding of TorD to L31Q mutant was undetectable, along with other leucine and glycine residues towards the c-terminus of the h-region. However, the L31Q mutation did not appear to have a physiological effect on TMAO reductase activity or periplasmic targeting of TorA, suggesting that binding to the core of apoTorA was uninhibited. Through the two-hybrid screen of a library of random mutants of TorD, the Q7L TorD mutant was identified with an increased affinity for wild type and the L31Q variant of the signal peptide. This mutant maintained its proofreading activity and could also complement a strain devoid of its endogenous *torD* allowing for the restoration of TMAO reductase activity. However, despite its increased binding, it was not co-transported by the Tat system.

Through random mutagenesis, two mutants with single amino acid mutations were identified in the fifth helix of TorD that were important for TorA maturation (Genest *et al.*, 2008). The residues identified were in less conserved regions of the REMP family, yet the protein still maintained the α -helical structure. These mutants also maintained their ability to bind the signal peptide with dissociation constants that were comparable to wild type TorD. On the other hand, when the interaction of the mutant TorD with apoTorA devoid of its signal sequence was probed via bacterial two-hybrid, no interaction between the mutant TorD proteins was seen with apoTorA. Additionally, TMAO reductase activity was significantly reduced in strains expressing the mutant TorD forms, suggesting that despite the ability to bind the signal peptide of TorA, cofactor insertion and thus TorA maturation was hindered. This supports the idea that twin-arginine-leader interactions with TorD are not sufficient for TorA maturation and that binding of TorD to the core of TorA is required and it is this role that takes precedence.

Other TorD substrates and binding partners

The binding event of TorD to the TorA signal peptide appears to be a primary event, which occurs with a K_D in the micromolar range. However, the events that trigger signal peptide release is not understood. It can be speculated that, like other general chaperones such as DnaK/DnaJ, GroEL/GroES as well as the Sec transport system, the binding and subsequent hydrolysis of nucleotides provides the triggering for binding and release of protein substrates. Thus, the nucleotide binding ability of TorD was investigated (Hatzixanthis *et al.*, 2005). Weak binding was seen with GTP using a tryptophan fluorescence based assay. Minimal changes to the dissociation constant were observed with GDP, GMP and cGMP, which suggest that the

guanosine group is what governs the binding. When TorA signal peptide was bound to TorD, the binding to GTP was stronger. Also, the Q7 equivalent in *S. typhimurium* DmsD was predicted to be involved in GTP binding which was shown to be associated with signal peptide binding as discussed above. With this data in mind, the binding of signal peptide could cause a conformational change in TorD so to induce and enhance the binding of GTP. Although weak binding was observed, hydrolysis of GTP was not detected with the assays used. This does not rule out the possibility that hydrolysis could occur, but other binding partners may be necessary if TorD lacks an intrinsic GTPase.

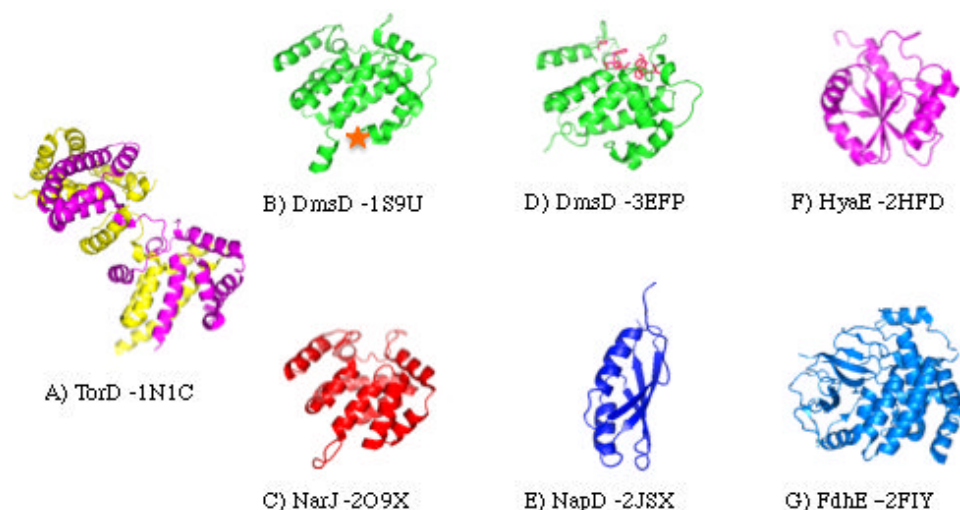


Figure 2. Structures of REMPs. A) *Shewanella massilia* X-ray structure (2.40Å). B) *Salmonella typhimurium* LT2 X-ray structure (1.38Å). A modeled site of GTP binding site is indicated as the star. C) *Archaeoglobus fulgidus* NarJ (3.40Å). D) *E. coli* DmsD (2.00Å). RR-leader binding site residues highlighted in red. E) *E. coli* NapD NMR structure. F) *E. coli* HyaE NMR structure. G) *Pseudomonas aeruginosa* FdhE X ray structure (2.10Å).

Since it has been shown that TorD interactions with the core of TorA is important for TorA maturation, it was important to investigate if TorD had the capabilities to bind components of the MoPt cofactor. Most recently, experimental data showed a 1:1 ratio for TorD binding with the cofactor intermediate Mo-molybdopterin (MoCo) as well as the final cofactor form, molybdopterin-guanine dinucleotide (MGD) (Genest *et al.*, 2008). These results coincide with the binding studies done with GTP (Hatzixanthos *et al.*, 2005) as it was proposed that the guanosine moiety was directly involved in binding. It is also likely that TorD could bind both GTP and Moco to help facilitate the synthesis of MGD. Finally it was found that MobA, the enzyme responsible for synthesizing MGD from Moco and GTP, associates with TorD. This interaction was shown by cross-linking as well as surface plasmon resonance (Genest *et al.*, 2008).

TorD structure

S. massilia TorD was seen to self associate into various oligomeric forms that retained the ability to bind TorA (Tranier *et al.*, 2002). The purified monomeric and dimeric species were stable and did not interconvert between the two forms unless they were exposed to pH

3.0 for a period of time. Binding capabilities of the monomeric and dimeric forms of TorD to TorA was explored via surface plasmon resonance. Both forms showed binding, however, the dimeric form bound TorA more efficiently than the monomeric form suggesting a physiological relevance for the dimeric form of this REMP. Stable trimer forms of TorD were also thought to occur. Furthermore, circular dichroism revealed that the secondary structure of the monomeric or dimeric species were very similar.

The solved crystal structure of *S. massilia* TorD (pdb:1N1C) has an N-terminal domain and a C-terminal domain that is attached via a hinge region, and when interacting as a dimer, domain swapping occurs such that the N domain from one monomer interacts with the C-domain of the other (Tranier *et al.*, 2003) (Figure 2A). A possibility exists for each domain to serve different roles when binding to TorA (Jack *et al.*, 2004). When the N and C domains were expressed separately in a *torD* mutant strain, the phenotype could not be rescued. However, when both domains were co-expressed, TMAO reductase activity was restored to near wild type levels. Thus, each individual domain is functional and allows for proper assembly and maturation of TorA when co-expressed, despite not being covalently linked. Furthermore, two conserved residues were identified, one each in the C and N domain that are important for Moco insertion into TorA. On the other hand, the separated TorD domains were incapable of Tat proofreading providing evidence that the hinge region is important for this potential function. Indeed, two residues identified in the hinge region were identified to be important for signal peptide recognition, while the two N and C domain residues were not required. It is then possible that the monomeric and dimeric forms are providing different roles when bound to TorA.

Concluding remarks for TorD

Taken altogether, TorD binds apoTorA in two regions, possibly in a cooperative manner to protect the molybdoenzyme from degradation as well as to help in Moco insertion and the successful maturation of TorA. Cooperativity may be why binding is not observed when the twin-arginine-leader signal peptide is lacking or mutated as this first binding event provides the catalyst for binding to the core of TorA. This is also consistent with the prediction that TorD first binds the signal peptide with high affinity to prevent the initiation of proteolysis of apo-TorA (Genest *et al.*, 2006). With the most current information on other binding partners of TorD, it is possible that TorD acts as a platform to connect all the necessary components for the final step of MGD synthesis in order to produce a fully functional TorA that is ready for transport by the Tat system.

DMSO Reductase (DmsD)

E. coli is able to grow anaerobically on dimethyl sulfoxide (DMSO) due to the presence of the membrane-localized DMSO reductase enzyme (Bilous and Weiner, 1985) encoded for by the *dmsABC* operon (Bilous et al., 1988). DMSO reductase (DmsABC) is a membrane-associated [Fe-S]-molybdoenzyme. During anaerobic respiration, electron flow proceeds from the electron donor menaquinol pool in the membrane bilayer through to DMSO, the terminal electron acceptor. The integral membrane protein, DmsC, is believed to be

composed of 8 transmembrane helices and contains a high-affinity binding site for menaquinol (Geijer and Weiner, 2004; Zhao and Weiner, 1998). DmsC serves to anchor the soluble DmsAB complex (Weiner et al. 1993). The DmsB subunit contains 4 [4Fe-4S] clusters; one of which interacts closely with the menaquinol oxidation site of DmsC (Rothery and Weiner, 1996; Zhao and Weiner, 1998). Electrons are passed to another [4Fe-4S] center in DmsB before moving to the molybdenum cofactor of the DmsA catalytic subunit (Rothery et al., 1999; Rothery and Weiner, 1996). DMSO is reduced to DMS (dimethyl sulfide) at the active site of DmsA which contains a bis-(pyranopterin guanosine dinucleotide) molybdenum cofactor (MGD). A predominantly negatively charged funnel-like active site entrance lined with aromatic residues may give DMSO reductase its more broad substrate specificity, accepting both S- and N-oxides (Kisker et al., 1997; McAlpine et al., 1998; Schindelin et al., 1996; Schneider et al., 1996), whereas the TorA active site is more specific for the N-oxide TMAO.

The active site of the DmsA enzyme contains a bis-(molybdopterin guanosine dinucleotide) cofactor (Schwarz, 2005). The depth to which the MGD cofactor is buried within the DmsA protein, would suggest MGD biosynthesis and insertion into DmsA involves a co-folding mechanism (Schindelin et al., 1996; Schneider et al., 1996). In addition to requiring the MGD association and complete folding prior to translocation by the Tat system, DmsA must also associate with folded DmsB, which must acquire four [4Fe-4S] centres. This hetero-dimeric complex is targeted to the Tat translocase via the twin-arginine leader on DmsA. The DmsA twin-arginine-leader is cleaved and DmsAB associates with the integral membrane protein DmsC. The chaperone DmsD plays a critical role in the maturation of the DMSO reductase enzyme.

DmsD interactions with DmsA

DmsD was originally identified by its ability to interact with the DmsA twin-arginine-signal peptide (Oresnik et al., 2001). Prior to this study the DmsD protein was uncharacterized in *E. coli* and known as YnfI, part of the *ynjEFGHI* operon. DmsD was shown to interact with the premature forms of both DmsA and TorA in an affinity chromatography technique in which a hexahistidine tagged DmsD was coupled to a Ni-NTA resin and exposed to anaerobically grown *E. coli* lysate (Oresnik et al., 2001). DmsD was shown to be required for biogenesis of DMSO reductase, likely mediated via the interaction with the DmsA twin-arginine leader (Oresnik et al., 2001; Ray et al., 2003). Further proof of the interaction between DmsD and the DmsA twin-arginine leader sequence (DmsAL) has been shown through an *in vitro* far-Western assay (Sarfo et al., 2004; Winstone et al., 2006) in which the DmsAL was fused at the N-terminus of GST. The dissociation constant (K_D) of the DmsD interaction with the DmsAL was determined to be 0.2 μ M using isothermal calorimetry (ITC) (Winstone et al., 2006). These ITC experiments used the same hexahistidine tagged DmsD and DmsAL:GST fusions mentioned above in which the first 43 (of a total 45) residues of the DmsAL were fused to the N-terminus of GST. Interestingly, a GST:DmsAL fusion protein (in which the DmsA signal peptide was fused to the C-terminus of GST) was unable to interact with DmsD *in vitro* suggesting that N-terminal display may be important for interaction (Winstone et al., 2006). DmsD binding to DmsAL:GST was also

characterized with SEC-FPLC in which monomeric DmsD bound DmsAL to form a 1:1 complex (Winstone et al., 2006).

Amino acid residues conserved in DmsD homologous proteins were subjected to site-directed mutagenesis, the variant proteins were expressed, purified and assayed for binding the DmsAL:GST fusion protein. More than 20 single amino acids in DmsD were substituted and 9 variant proteins exhibited reduced binding relative to the wild type protein (Chan et al., 2008). A DmsD structural model was made and the residues most affected (W72, L75, F76, P86, W87, P124, D126, H127) were found to cluster together on the surface of the DmsD model as a “hot pocket” (Chan et al., 2008) (Figure 3).

DmsD is capable of binding four different twin-arginine-leaders from different enzymes in *E. coli* (DmsA, TorA, YnfE and YnfF) (Chan et al., 2009). The specificity may originate from a four residue hydrophobic motif (-LAMA-) present downstream of the twin-arginine motif, that may be important for interactions with DmsD.

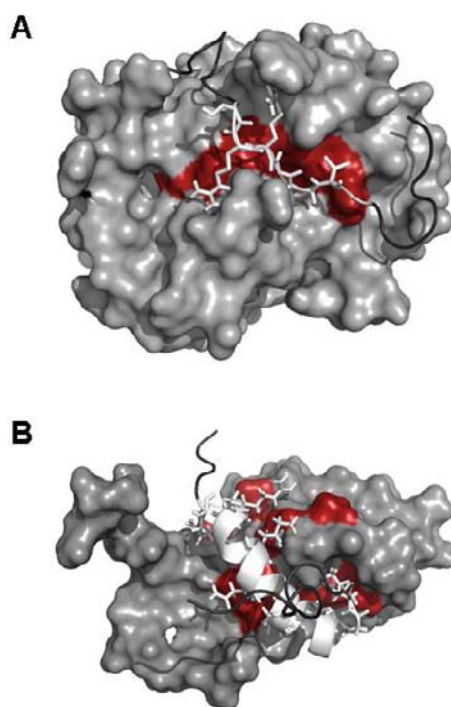


Figure 3. Twin-arginine signal peptide binding sites. A. *E. coli* DmsD and DmsA signal peptide residues 1-29 (3EFP.pdb; Stevens et al., 2009). B. EcNapD and NapA signal peptide residues 1-35 (2PQ4.pdb). DmsD and NapD surface representations are shown with residues implicated in binding colored red. DmsA and NapA peptides are colored dark grey or white (residues aligned with binding sites). The N-terminus of each peptide begins at the top and follows down to the C-terminus. The twin-arginine leader motif SRRxFLK is colored white in both structural representations.

DmsD interactions with the Tat system and other proteins

DmsD localization studies were performed in a variety of wild type and Tat system deletion *E. coli* strains. DmsD was shown to localize to the membrane in the presence of the Tat components (Papish et al., 2003). The membrane localization of DmsD in anaerobically

grown cells was shown to be dependant on the presence of the TatB and TatC subunits, suggesting that DmsD was capable of handing off the DmsA substrate to the translocase via an interaction with this TatBC receptor complex (Papish et al., 2003). Other studies investigated the localization of green fluorescent protein fused to DmsA and TorA leader peptides in *dmsD* deletion *E. coli* cells and showed that localization was similar to that of wild-type cells (Ray et al., 2003). The anaerobic growth of $\Delta dmsD$, $\Delta tatABCDE$ and wild-type *E. coli* supplemented with DMSO, showed that DmsD is essential for the biogenesis of the DMSO reductase enzyme (Ray et al., 2003). However, there remained a basal level of activity in $\Delta dmsD$. Together these observations suggest that the REMP is not absolutely required but is a critical helper in the biogenesis.

DmsD structure

DmsD has been shown to exist in multiple oligomeric and folded forms (Sarfo et al., 2004). Purification of *E. coli* hexahistidine tagged DmsD protein with an imidazole gradient has made it possible to separate monomers from dimers. Exposure to low pH induces a conformational change that is visualized as a 'ladder' on a native polyacrylamide gel suggesting that the protein may be aggregating or forming multimers (Sarfo et al., 2004). However, when the low-pH induced form of DmsD was characterized with SEC-FPLC the protein eluted as a single peak beyond the void volume of the column (Sarfo et al., 2004). This elution behavior suggests that the protein is present in solution as a single 'form' that was interacting with the column matrix. The secondary structure (alpha helical content) of the DmsD low pH form was almost identical to that of the native monomer and dimer when characterized by circular dichroism spectroscopy (Sarfo et al., 2004). The low pH induced form of the homologous protein *Shewanella masillia* TorD bound significant amounts of the ANS (1-anilino naphthalene-8-sulfonic acid) fluorophore while the native monomers, dimers and even guanidium hydrochloride (GdnHCl) denatured protein did not (Tranier et al., 2002). ANS binding is indicative of the presence of exposed hydrophobic surfaces (Semisotnov et al., 1991). Subsequently, Sarfo et al. (2004) found that if the low-pH induced form of *E. coli* DmsD was treated with 8 M urea (followed by its removal) the protein exhibited a similar native PAGE profile to that of the monomeric protein, suggesting re-folding to the native folded form. The significance of this low-pH folded form of DmsD is unclear; however, it should be noted that it is capable of binding the DmsA twin arginine leader peptide (Sarfo et al., 2004).

Intrinsic fluorescence spectroscopy has been used to characterize pH and denaturation profiles of *E. coli* DmsD. The protein has multiple pH dependant conformations (Sarfo et al., 2004). The nature of the significance and relevance of the different folding forms at different pHs has not been further explored by researchers in the field; however, as the folding transition occurs at pHs in the physiologically relevant range, such observations must be considered. Very little conformational change occurs when *E. coli* DmsD is exposed to urea concentrations at or below 5 M (Winstone et al., 2006). For significant conformational changes to occur greater than 7 M urea was necessary. GdnHCl denaturation of DmsD appeared to have 2 phases. These observations imply that this protein may have different folding forms.

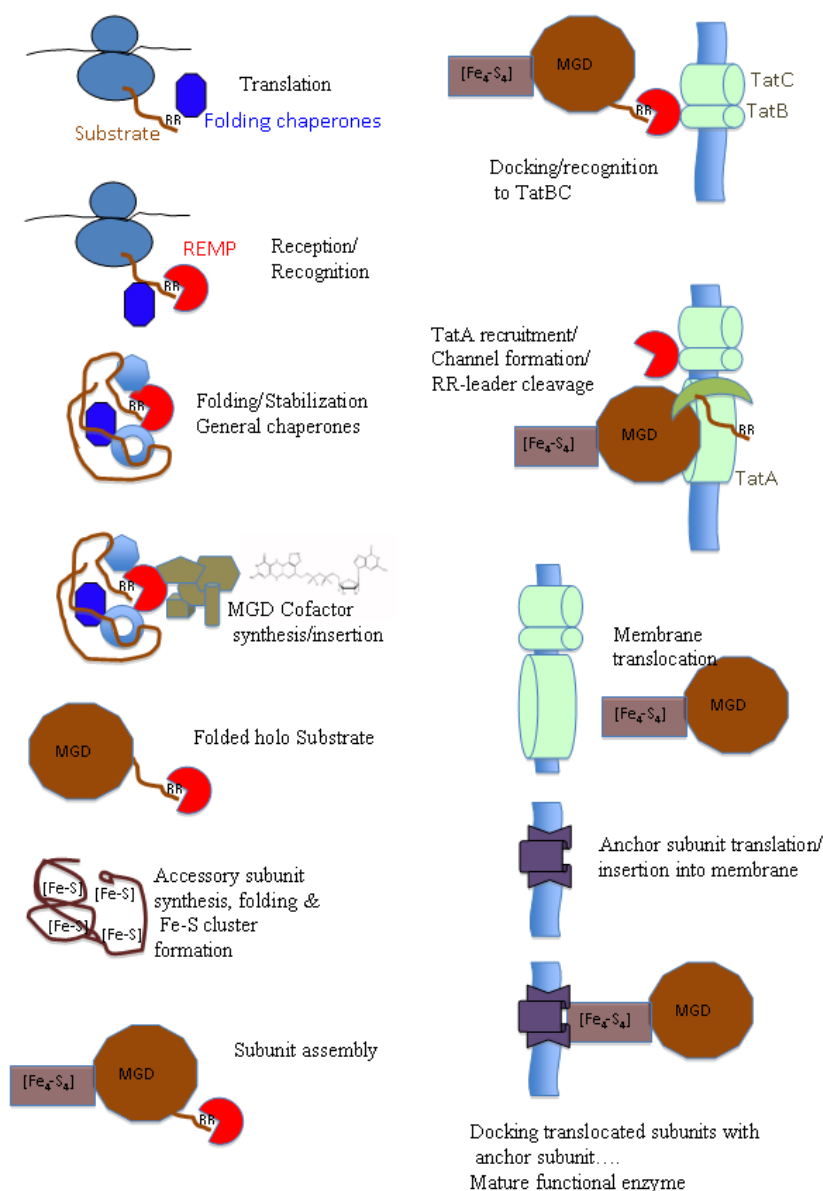


Figure 4. Model for an example maturation pathway (DMSO Reductase (DmsABC)). At the point of translation of the *dmsA* transcript, the system specific chaperone DmsD interacts to mediate the maturation pathway to target to general chaperones and molybdopterin cofactor biogenesis. Subsequent steps require the biogenesis of DmsB with iron-sulfur cluster formation and its interaction with the folded form of DmsA. The DmsAB complex must be targeted to the membrane and to the Tat system for twin arginine leader processing and translocation. The translocated DmsAB in the periplasm must then locate and dock to the membrane anchor subunit DmsC to interface with the quinone pool.

The protein structure of the DmsD homologue from *Salmonella typhimurium* homologue was solved (pdb:1S9U) (Qiu et al., 2008). The protein showed an all alpha-helical structure (12 helices) and similar organization to a monomeric unit of the previously solved *S. masillia* TorD homologue (pdb:1N1C) (Tranier et al., 2003). With the *E. coli* DmsD structure solved

to 2.0 Å resolution (pdb:3EFP) it was possible to perform docking and molecular dynamics simulations with a peptide composed of the 29 N-terminal residues of the DmsA twin-arginine leader peptide (Stevens et al., 2009) which showed support for the previously determined binding site (Chan et al., 2008) (Figure 3). Overall, the DmsD structures are very similar to each other.

Nitrate Reductases (NarJ, NarW, NapD)

Respiration using nitrate is performed by a set of redox enzymes in bacteria that catalyze the reduction of nitrate (NO_3^-) to nitrite (NO_2^-). Other enzymes also carry out further denitrification until NO_2^- is converted to NO, N_2O , and finally N_2 (summarized in Gonzalez et al., 2006). Three membrane-bound nitrate reductases have been identified in bacteria in addition to the soluble assimilatory nitrate reductases located in the cytoplasm, all of which coordinate a molybdenum-*bis*-molybdopterin guanine dinucleotide (MGD) cofactor in the catalytic site (Potter et al., 2001). *E. coli* contains two cytoplasmically-anchored enzymes commonly referred to as nitrate reductase A (NRA) and nitrate reductase Z (NRZ), or collectively as Nar. The last one is periplasmically localized and is commonly termed the periplasmic nitrate reductase, nitrate reductase P, or Nap. Several reviews provide extensive information into the structure, function, and mechanism of all three (Potter et al., 2001; González et al., 2006; Martinez-Espinosa et al., 2007).

Cytoplasmic nitrate reductases (NarJ and NarW)

All bacterial cytoplasmic nitrate reductases isolated to date are heterotrimeric enzymes anchored to the membrane through an integral membrane subunit (González et al., 2006; Martinez-Espinosa et al., 2007). Nitrate reductase A and nitrate reductase Z are no exception, consisting of NarGHI and NarZYV subunits, respectively. NarG is the catalytic subunit with MGD and 1 [4Fe-4S] cluster. NarH connects NarG to the membrane anchor NarI and contains 1 [3Fe-4S] and 3 [4Fe-4S] clusters. Finally, NarI is the membrane anchor subunit consisting of five transmembrane helices coordinating two *b*-type haems and connects to the menaquinone pool in the membrane. A structural overview of the NarGHI complex from *E. coli* is provided in Bertero et al. (2003).

Nitrate reductase Z was shown to exhibit similar physical and chemical characteristics to nitrate reductase A (Iobbi et al., 1987). Its gene products have high homology with the nitrate reductase A components (Blasco et al., 1990), where sequence comparisons identified conserved regions in NarZ similar to known molybdoproteins, NarY contained four cysteine clusters in the same order as in NarH, and NarV has five putative transmembrane segments and haem motifs as organized in NarI. These suggest that the three subunits have highly similar biochemical roles and functional organization as NarGHI. Unfortunately, few further studies have been performed on NarZYV.

Early studies demonstrated the absolute requirement of the accessory proteins NarJ and NarW for biogenesis of nitrate reductases A and Z (Sodergren et al., 1988; Blasco et al., 1992; Dubourdieu and DeMoss, 1992). NarJ was not found to be associated with the final holoenzyme of NarGHI, thus little understanding of its role in enzyme maturation was

understood at the time other than it was required for functional biogenesis of nitrate reductase A (Sodergren et al., 1988). Further studies demonstrated that NarJ is required for MGD cofactor assembly into NarG, a step that must occur prior to membrane attachment. This led Blasco and colleagues to propose that NarJ is a specific chaperone that binds to NarG to keep it in a cofactor-accepting conformation (Palmer et al., 1996; Blasco et al., 1998a). Additional experiments have also shown the requirement of NarJ for insertion of the [4Fe-4S] cluster into NarG and indirectly affecting haem insertion of NarI by preventing premature attachment of NarGH to NarI (Lanciano et al., 2007).

Functional characterization of NarJ reveals that it accumulates at lower stoichiometric levels than the other three subunits and co-purifies with NarGH or NarG by itself (Liu and DeMoss, 1997). Studies provide contradicting evidence on the cellular localization of NarJ (exclusive cytoplasmic versus exclusive membrane localization) that may be attributed to subtle experimental differences. However, it was evident that the exclusive membrane localization was dependent on the presence of the Tat complex (Chan et al., 2006). NarG from membrane fractions was isolated and identified using purified NarJ as bait. These observations implicate the previously accepted role that NarJ is a chaperone that only functions for nitrate reductase A biogenesis in the cytoplasm and suggests that nitrate reductase A targeting is dependent on the Tat system. Evidence further supporting this is indicated in recent studies demonstrating interactions of NarJ and NarW with the vestige Tat motif-containing N-terminal portion of NarG and NarZ and that the interaction with NarG *in vivo* has Tat-dependence (Chan et al., 2009; Li and Turner, 2009).

The homologous NarJ from *Thermus thermophilus* was found to be required for membrane attachment to the NarCI complex along with its requirement for the maturation of NarG (Zafra et al., 2005). In *E. coli*, NarJ did not appear to have an effect on membrane attachment of NarGH (Blasco et al., 1992; Vergnes et al., 2006). Its role appeared to be more important for maintaining the apoenzyme in a soluble and competent cofactor-accepting conformation while preventing premature membrane-anchoring to NarI (Vergnes et al., 2006). Recent studies have demonstrated that NarW cross-interacts with the NarG N-terminus that is important for membrane-attachment (Chan et al., 2009), an observation that was attributed to a potential rescue mechanism. Thus, NarJ may still be required for NarGH membrane attachment in *E. coli*, but its role may have been substituted by NarW. The two share 56% sequence identity and 71% sequence similarity, which was the highest amongst the ten *E. coli* REMP chaperones (Chan et al., 2009). Both NarJ and NarW have been identified as part of the DmsD family of chaperones including TorD and YcdY as members (Turner et al., 2004).

Periplasmic nitrate reductase (NapD)

The periplasmic nitrate reductase Nap was demonstrated to support anaerobic growth in a strain lacking *narG* and *narZ*, providing evidence that a third nitrate reductase exists in *E. coli* (Potter et al., 2000; Stewart et al., 2002). Nap appeared to be selectively preferred under low nitrate conditions (less than 0.1 mM in the medium), suggesting that it provides a selective advantage under limited nitrate conditions (Potter et al., 1999; Stewart et al., 2002). Nap consists of the NapABC subunits where NapA is the catalytic subunit with a twin-arginine motif and contains a MGD cofactor and [4Fe-4S] cluster (Thomas et al., 1999;

Brondijk et al., 2004). NapB contains two *c*-type haems and a conventional Sec-targeting signal (Thomas et al., 1999). NapC contains four *c*-type haems but unlike NarI or NarV which are multi-transmembrane integral membrane proteins, it contains a single transmembrane helix that inserts into the membrane from the periplasmic side (Roldan et al., 1998), hence the earlier description that Nap is membrane attached and not anchored.

Early studies established an essential role of NapD for nitrate reductase activity (Potter and Cole, 1999). NapD has been shown in two independent studies to interact with the twin-arginine motif-containing N-terminus of NapA. This interaction is very tight with a dissociation constant determined to be 7 nM (0.007 μ M) using the technique of ITC (Maillard et al., 2007; Chan et al., 2009). The tight association can even suppress transport by Tat, which may be a control mechanism for proper folding of NapA (Maillard et al., 2007). The structure of NapD was determined by NMR spectroscopy to have four β -strands and two α -helices, which are unique, compared to the other twin-arginine leader binding proteins with structural information currently available; DmsD and TorD exhibit all-alpha folds (Tranier et al., 2003; Maillard et al., 2007; Qiu et al., 2008) (Figure 2). A recent study also demonstrated a highly selective *in vivo* interaction between NapD and the signal peptide of HybO of the hydrogenase enzyme, which also bears a twin-arginine motif (Chan et al., 2009).

Formate Dehydrogenase (FdhD, FdhE)

E. coli has three formate dehydrogenase (FDH) membrane-bound isoenzymes that catalyze oxidation of formate (HCOO^-) to carbon dioxide (CO_2). Two FDH's are anchored to the cytoplasmic membrane from the periplasm while the other from the cytoplasm. The two periplasmic FDH's are transported across the cytoplasmic membrane via the Tat system (Stanley et al., 2002). The first periplasmic isoenzyme is commonly referred to FDH-N where the N-denotation stemmed from observations that its expression was induced by nitrate anaerobically. The isoenzyme, FDH-O or FDH-Z, is also induced by nitrate in the media but also in the presence of oxygen (Sawers et al., 1991; Abaibou et al., 1995).

FDH-N consists of the heterotrimeric FdnGHI subunits. FdnG is the MGD and [4Fe-4S] containing subunit that catalyzes the oxidation of formate. It contains a single selenocysteine residue (Berg et al., 1991). It is also the twin-arginine motif-containing subunit. FdnH is the electron conduit subunit that accepts two electrons from FdnG and shuttles them through its four [4Fe-4S] clusters to FdnI, which is the membrane anchor subunit consisting of four transmembrane helices and contains two *b*-type haems. FDH-N forms a complete redox loop with nitrate reductase A (NarGHI) that contributes to the proton gradient across the membrane. The crystal structure of FdnGHI demonstrates where the MGD cofactor and Fe-S clusters are coordinated and provide a molecular view of how the redox loop is completed during electron shuttle (Jormakka et al., 2002).

FDH-O/Z consists of the FdoGHI subunits that are highly similar to FdnGHI in terms of size, composition, and structural fold (Abaibou et al., 1995). It likely forms a redox loop with nitrate reductase Z in the same manner that FDH-N does with nitrate reductase A (reviewed in Sawers, 1994). FdoG contains the twin-arginine motif, and requires molybdenum and selenocysteine incorporation, and is considered the catalytic subunit (Sawers et al., 1991;

Pommier et al., 1992; Abaibou et al., 1995). FdoH and FdoI likely perform similar functions as FdnH and FdnI as they were found to be of similar size and have similar structural folds based on recognition by FdnHI-specific antibodies (Abaibou et al., 1995).

Early studies on *E. coli* mutants deficient in formate dehydrogenase activity isolated two classes of mutants, one involving *fdhD* and the other *fdhE*, coding for two accessory proteins of the FDH's (Mandrand-Berthelot et al., 1988). The two proteins were required for FdnGHI activity but not cytochrome incorporation, indicating a role in the cytoplasmic biogenesis of the FdnGH subunits. FdhD and FdhE do not appear to control transcription of *fdnGHI* (Stewart et al., 1991). FdhD/E does not have a direct role in incorporation of the selenocysteinyl residue as incorporation occurs during mRNA transcription (Schlindwein et al., 1990). FdhE was recently demonstrated to interact with the catalytic subunits FdnG and FdoG (Lüke et al., 2008; Chan et al., 2009). An interesting observation in the study by Lüke et al (2008) showed that the interaction of FdhE with FdnG excluded the interaction between FdnG and FdnH, suggesting that the interacting sites may overlap or induce a conformation in FdnG to occlude its binding site to FdnH. From this observation, the authors propose that the interaction between FdhE and FdnG prevents premature association of FdnGH prior to complete folding of FdnG.

Biochemical studies show cytoplasmic localization of the FdhD and FdhE and they are the largest of the known REMP chaperones (Schlindwein et al., 1990), and share 11% and 18% sequence identity and similarity, respectively (Chan et al., 2009). Less information is available about FdhD, but a recent study showed that FdhE adopts monomeric and homodimeric forms *in vitro* while dimerization *in vivo* appeared to be stabilized under anaerobic conditions (Lüke et al., 2008). FdhE was also shown to bind a ferric iron through conserved cysteine residues, but the function of the ligated iron is still not understood (Lüke et al., 2008).

Hydrogenase (HyaE HybE)

Three hydrogenases have been identified in *E. coli* and are denoted hydrogenase-1, -2, and -3. Hydrogenases-1 and -2 catalyze the oxidation of hydrogen (H_2) to protons (H^+) whereas hydrogenase-3 catalyzes the opposite reduction reaction. Hydrogenases-1 and -2 are membrane-anchored heterodimers that are transported by the Tat pathway to the periplasm (reviewed in Wu et al., 2000). Both enzymes consist of a large and small subunit that contain nickel and iron-sulfur cofactors as with other dimeric hydrogenases in other bacteria (Przybyla et al., 1992). Hydrogenase-1 consists of HyaAB whereas hydrogenase-2 consists of HybOC. The small subunits HyaA and HybO contain the consensus twin-arginine motif at their N-terminus and a C-terminal membrane anchor helix (Dubini et al., 2002). Studies have demonstrated co-targeting and co-translocation of the motif-lacking Hyb as a 'hitchhiker' with HybO, suggesting that this mechanism applies to both hydrogenases (Rodrigue et al., 1999). The large subunits HyaB and HybC are the catalytic centers for hydrogen oxidation. Crystal structure of hydrogenase from *Desulfovibrio gigas* shows nickel coordination by the large subunit and one [3Fe-4S] and two [4Fe-4S] centers in the small subunit (Volbeda et al., 1995).

The solution structure of the *E. coli* accessory protein HyaE was determined by NMR to have a thioredoxin fold, but lacked the canonical C-X-X-C motif typically found in the active site of thioredoxin proteins (Parish et al., 2008) (Figure 2F). HyaE was shown to interact with the twin-arginine leader of HyaA and potentially HybO (Dubini and Sargent, 2003). Conserved Glu and Asp residues on the surface of the recently determined HyaE structure were postulated for binding to the positively charged arginines in the leaders (Parish et al., 2008). There is no structure currently available for HybE but it was demonstrated to interact with the leader of HybO and HybC (Dubini and Sargent, 2003; Chan et al., 2009). In the study by Dubini and Sargent, HyaE and HybE also appeared to interact with themselves as well as each other, which have been suggested that they have cooperative functions to work together for maturation of the hydrogenases in a large complex. Despite increasing evidence to suggest that HyaE and HybE participate through ‘cross-talk’ for maturation of all three hydrogenases, they have low sequence homology at 12% and 19% identity and similarity, respectively (Chan et al., 2009). They also do not share any similar motifs, which raises the question of how specificity of cross-recognition towards the three hydrogenases is conferred (Turner et al., 2004).

REMP Classification and Structural Analysis

Bioinformatics

In 2004, two studies examined the amino acid sequences of accessory proteins that bind to twin-arginine signal peptides (Ilbert et al., 2004; Turner et al., 2004). At the time these proteins were loosely referred to as the TorD family. The Turner et al. study proposed that any protein involved in the maturation process of a complex redox enzyme but not part of the holo-enzyme itself be called a Redox Enzyme Maturation Protein or REMP, and classified a large number of proteins as such. This study took the known *E. coli* twin-arginine peptide binding protein sequences, which were “Blasted” (BLASTp) against the databases and the sequence similarities were analyzed. Based on the cofactor contained within the REMP substrate or functionality of the REMP substrate, the REMPs were classified into either a hydrogenase or molybdoprotein super-family. REMPs found to bind molybdoprotein substrates were classified into three families (based on the *E. coli* protein included): DmsD, NapD and FdhE. The NapD and FdhE families were not sub-classified further; however, the DmsD family was further classified into 3 clades (based on the *E. coli* protein included): DmsD, TorD and NarJ. The protein sequences within each clade were aligned and analyzed for defined signature sequence motifs and the *E. coli* protein sequence motifs were highlighted. *E. coli* DmsD family members were predicted to be mostly alpha-helical by secondary structure prediction algorithms and included DmsD, YcdY, TorD, NarJ and NarW (Turner et al., 2004). When looking at the *E. coli* REMP phylogenetic tree and considering the classifications above; the molybdoenzyme REMPs NapD and FdhE are located on the same side of the phylogenetic tree as the hydrogenase REMPs HyaE and HybE, opposite all *E. coli* DmsD family members. Interestingly, these same 4 proteins (NapD, FdhE, HyaE and

HybE) were predicted to contain beta structure in addition to alpha helix, contrasting the all alpha-helical nature of the proteins classified to DmsD family members (Turner et al., 2004).

In the study by Ilbert et al. (2004) only protein sequences with pre-determined biochemical information and those proteins found to bind to molybdoenzyme twin-arginine leader peptides were analyzed, thus a much smaller number of proteins were classified as members of the TorD family. These proteins [sequences] were then classified based on the characteristics (and classification) of the molybdoenzyme that the protein interacted with, and represented an extension of their group's previous definition of the TorD Family (Tranier et al., 2003). This group divided the TorD family into 4 clades, according to the molybdoenzyme classification types (I, II or III) of the DMSO reductase family, which is based on cofactor composition, and organization of the protein domain around the MGD cofactor (McEwan et al., 2002). The newly defined fourth clade (IV) contained TorD homologues that could not be related to any specific molybdoenzyme family member and were exemplified by the *E. coli* protein YcdY and shown to have the closest relationship to Type II TorD homologues (ie. DmsD). Types I and III TorD family members contain the molybdoenzyme itself and the TorD family member (TorD homologue) are located within the same operon (ie. *torCAD*, type III), while Types II and IV have separate operons for the molybdoenzyme and the gene of the TorD homologue.

The accessory proteins found to bind the twin-arginine leader peptides of molybdoenzymes can be correlated between the two papers. The TorD clade (Turner et al., 2004) can be correlated to Group III proteins of the DMSO reductase family (Ilbert et al., 2004) while types II and IV (Ilbert et al., 2004) would both be included within the DmsD clade (Turner et al., 2004). The NarJ clade, within the DmsD family, identified by Turner et al. (2004) is not mentioned by Ilbert et al. (2004). Both papers defined sequence motifs, slightly different amongst each clade/type. Overall, the Turner paper includes a much more diverse set of sequences than in the Ilbert paper focusing only on the TorD Family.

It is worth noting that there are REMP substrate molybdoenzymes of each clade/type that are not translocated (Sargent 2007). The enzyme substrates are assembled first, then targeted and translocated later. Therefore, at this time it is not always possible to extrapolate the type of molybdoenzyme with its localization within the cell, and likely not with the corresponding function of each REMP.

Structures

There are currently 7 REMP protein structures available (Figure 2 and 3). Almost all the different REMP protein families are represented. Structures from X-ray diffraction of crystals have been solved representing 3 different clades of the DmsD family – TorD, DmsD and NarJ. *Shewanella massilia* TorD was the first REMP structure published. It was found to contain a unique fold that is primarily helical, with two domains that undergo domain swapping (Tranier et al., 2003). *Archaeoglobus fulgidus* NarJ (Kirillova et al., 2007) and *E. coli* DmsD (Stevens et al., 2009) crystallized as dimers with no domain swapping and *S. typhimurium* DmsD (Qiu et al., 2008) was monomeric. Each monomeric domain has a very similar architecture and is displayed in Figure 2.

The *E. coli* NapD solution structure was determined using nuclear magnetic resonance and is significantly different from the DmsD/TorD family members (Maillard et al., 2007). NapD is a significantly smaller protein and belongs to a distinct protein family and adopts a mixed alpha-beta structure (Figure 2E). The NapD protein interacted specifically with a region of the NapA signal peptide (Maillard et al., 2007). The NapD residues most affected by NapA leader-peptide binding were also examined and included S9, V11, E33, A35, S37, Q43, E49, L74 and Y76 (Maillard et al., 2007). These residues are highlighted in Figure 3 and show the twin-arginine leader forming a helix lying along a beta-sheet surface. For comparison, the modeled twin-arginine leader peptide bound to DmsD is also shown in Figure 3. The peptide in this case is in an extended conformation lying across a groove in the protein.

The REMPs HyaE (pdb:2HFD) from *E. coli* and *Pseudomonas aeruginosa* FdhE (pdb:2FIY) display very different structures. Despite their assumed similar function, these proteins adopt independent folds where only DmsD, NarJ and TorD have similar architecture, which was suggested by the bioinformatic analysis establishing the family (Turner et al., 2004). Regardless of whether the twin-arginine-leaders from each substrate enzyme have a final teleological purpose to target to the Tat system or to facilitate cofactor loading, the structure of the twin-arginine signal peptides must be fine tuned to fit only one type of structure and REMP.

Concluding Comments

Of the REMPs DmsD and TorD are the most thoroughly studied. DmsD research has focused on its ability to bind the DmsA twin-arginine leader peptide (Chan et al., 2008; Oresnik et al., 2001; Sarfo et al., 2004; Winstone et al., 2006). Characterization of DmsD has shown it to exist in multiple folded forms when aerobically expressed and purified (Sarfo et al., 2004). Both *E. coli* and *S. massilia* TorD were also shown to exist in multiple folded conformations (Tranier et al., 2003; Tranier et al., 2002). TorD and DmsD have both been shown to bind to a molybdopterin biosynthetic enzyme which provides the hypothesis that the REMPs may form a scaffold for folding and cofactor loading of the reductase; ie. involved in co-binding of the twin-arginine-leader substrate upon translation in concert with interacting with Moco biosynthetic machinery.

Early studies suggested that the interaction between TorD and TorA, unlike DmsD, was not mediated by the twin-arginine signal peptide but with the mature protein sequence (Pommier et al., 1998). TorD has been reported to interact specifically with the unfolded form of TorA within the cytoplasm and assist with the insertion of the molybdopterin cofactor (Ilbert et al., 2003; Pommier et al., 1998). Similarly, NarJ was shown to recognize the nascent unfolded topology of its molybdoenzyme NarG (Blasco et al., 1998). Additionally, NarJ was required for the insertion of the molybdopterin cofactor into NarG (Blasco et al., 1998). NarJ was recently shown to interact with a NarG N-terminal leader peptide *in vitro* with both an *in vitro* far-western analysis and ITC (Chan et al., 2006). NarG and NarZ also have a molybdopterin cofactor but the N-terminal region (vestige signal sequence) is not cleaved (Blasco et al., 1990). The structure of the nitrate reductase complex

(NarGHI) was solved (Bertero et al., 2003) and confirmed that the N-terminal region (a vestige twin-arginine-leader) was not cleaved. These results suggest that the REMP has multiple interactions with their substrates and with other enzymatic events leading to their substrates maturation.

A model of the pathway of maturation of the example *E. coli* redox enzyme DMSO reductase is cartooned in Figure 4. It has been hypothesized that the REMP may interact with the twin-arginine-leader immediately after it appears from the ribosome tunnel, remaining bound throughout the folding and cofactor loading of its cognate substrate and continue to be associated until immediately prior to translocation. This interaction is likely important for multiple functions within the cell providing protease protection of the twin-arginine-leader, maintaining the substrate in a cofactor competent state, insertion of cofactor, proofreading of final folding and stability to the substrate – hitchhiker couple, providing targeting to the membrane via TatBC recognition complex and finally release to allow translocation to occur. The specificity and strength of this interaction would likely need to vary during the protein folding cycle of the substrate (and DmsB association) to allow these multiple functions to take place and provide for various interaction partners to aid in this maturation pathway.

With the concept of REMPs now accepted in the field and with several groups actively researching these proteins it is worth reviewing the type of questions being perused about their function and their relationship to the Tat system. Such questions include: What is the physiology of ‘REMP’ mutants? Is there a level of gene regulation and if so how does it occur? How common are REMPs within an organism? How common are REMPs in other bacterial species? What are the key residues in the REMPs that are involved in leader peptide binding? How specific is the interaction between the REMPs and their target proteins? When do REMPs bind the leader? What are the residues in the twin-arginine-leader that play a role? How do REMPs distinguish between twin-arginine-leaders and Sec-dependent leaders or does it? What is the structure of the twin-arginine-leader bound to the REMP? At what stage in the process does the REMP release the substrate peptide? What is the energetics of peptide binding and release? Do REMPs interact with the Tat translocase and if so what is the nature of this interaction? What other proteins are involved in the functionality pathway? This list demonstrates how little we know about these chaperones, yet their answers will ultimately lead to a more complete understanding of the physiology and biochemistry of organisms that respire with alternative oxidoreductase enzymes.

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Chapter V

Role of Molecular Biology in Molecular Imaging

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USA

Abstract

Recent progress in molecular biology has provided new insights into the molecular basis of diseases and molecular targets for diagnosis and therapy of human diseases. Molecular imaging is a research discipline aimed at development and testing of novel tools, reagents and methods to image specific molecular pathways *in vivo* that are key targets in disease process and appear much earlier than anatomical and physiological changes. The advancement in the field of imaging and therapy of diseases is mainly due to the vast information available from molecular biology research on new targets with specific ligands and methods to evaluate their application in *in vitro* and *in vivo* systems. Improvement in imaging modalities like single photon emission computed tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging (MRI), computed tomography (CT) and optical imaging (OI) has also contributed in the progress of molecular imaging. First section of this chapter is focused on molecular imaging, different approaches adopted for development of molecular imaging agents and recent imaging modalities and their applications.

Molecular biological techniques used in *in-vitro* diagnostics are being adapted to the special requirements of imaging diagnostics and high affinity imaging is achieved based upon receptor-ligand, antigen-antibody, transporter-substrate and enzyme-substrate interactions. Development of newer approaches based on reporter gene concept are solely dependent on molecular biology research tools. Small animal models of human

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diseases have become available after completion of human genome project. Noninvasive imaging of molecular, genetic and cellular processes in animal models complements established *ex vivo* molecular biological assays and imaging provides a new dimension to understanding of various diseases. Role of molecular biology research in molecular imaging is discussed in second section of this chapter. Development of imaging agents based on peptides and role of molecular biology methods in identification of their targets to development of labelled ligands and their evaluation in *in-vitro* and *in-vivo* systems is also discussed.

Chapter concludes with the applications of molecular imaging in diagnostics, gene therapy and drug development. Advancements in biology and medicine is possible due to synergism between various new disciplines, especially molecular biology research has contributed significantly towards progress of molecular imaging.

Keywords: Molecular imaging, Gene therapy imaging, Molecular targeting.

1. Introduction

Starting with the elucidation of structure of DNA by Watson and Crick in 1953, discipline of molecular biology has made remarkable progress leading to completion of several genome sequences especially the human genome [Venter JC et al. 2001]. Such advancement has resulted in realization of molecular biology discipline into more specialized technologies like genomics, proteomics and functional genomics. Molecular biology research has its influence on many other fields of biology and medicine and molecular biology protocols and techniques are being utilized in various other new and emerging fields such as molecular imaging.

Molecular biology deals primarily with understanding the interactions between various biomolecules like nucleic acids, proteins and their regulation within the cell. While molecular imaging is emerging biomedical research discipline that involves studying those interactions between biomolecules using high affinity probes for understanding the molecular basis of disease, diagnosis of disease and estimating the outcome of therapy. As microscopy helps in understanding of biology based on observation of tissues, cells and sub cellular organelles, molecular imaging contributes in visual representation, characterization and quantitation of biological processes at the cellular and sub cellular levels within living organisms. Present imaging technologies are based mainly on nonspecific physiological, anatomical and metabolic changes that differentiate diseased from normal tissue rather than identifying disease specific molecular events. Those changes are late manifestations of molecular changes that truly represent a diseased state. Direct imaging of molecular changes using specific molecular probes provides precise anatomical localization and quantification along with opportunity to investigate the time course (dynamics) of disease specific molecular events in the intact organism. Molecular imaging allows intervention at a predisease stage giving possibility for better management of the disease. Additionally, development of new drugs and effects of therapy can be monitored by molecular imaging [Massoud, TF & Gambhir, SS. 2003, Weissleder, R & Mahmood, U. 2001].

The term molecular imaging came into use in mid to late 1990s referring to the use of radioactive ^{131}I for assessment of benign and malignant thyroid disease. While certain imaging techniques that may be defined as ‘molecular’ were developed much earlier like imaging with radiolabelled peptides and monoclonal antibodies. Today, molecular imaging agents are being developed to image and measure the most fundamental biological processes ranging from transcription, translation, signal transduction pathways and synthesis and metabolism of substrates that perform cellular functions. Success has also been achieved in translation of experimental molecular biology assays for imaging and development of imaging systems for examination of molecular events in animal models. Recent advancements in molecular imaging are bridging the gap between research in animal models of diseases and its translation into clinical applications.

2. Molecular Imaging

Molecular imaging involves specific localization or accumulation of signal emitting probe within the cell or tissue where the target is expressed. Molecular imaging probes are composed of an affinity component that interacts with the target and a signaling component that is useful for imaging. Successful development of a molecular imaging agent requires selection and development of a high affinity probe with good pharmacokinetics in animal model of disease, ability of probe to overcome biologic delivery barriers (vascular, interstitial, cell membrane), amplification mechanisms (chemical or biological) and availability of sensitive, fast, high resolution imaging technique.

Molecular imaging is outcome of molecular targeting that involves specific accumulation of a probe by virtue of its interaction with a molecular species that is distinctly present, over expressed or absent in a diseased state. Molecular species could be a DNA, RNA or its protein product, a gene product of normal sequence and structure aberrantly expressed in a given tissue or a transcriptionally normal gene product whose structure or function has been modified by abnormal RNA splicing or posttranslational processing. Molecular targeting agents differ from conventional physiologic tracers as physiologic tracers are directed towards processes characteristic of a normal tissue or cell type and are dependent on the interaction of multiple gene products or on nonspecific bulk processes such as diffusion, membrane permeability or electrostatic interactions [Cunningham, SHB & Adelstein, SJ. 2003].

High affinity ligands developed for molecular targeting are evaluated in animal models of disease. These probes must demonstrate the ability to reach the intended target at sufficient concentration and for sufficient length of time to be detected *in-vivo*. A number of strategies are employed like peg-ylation to decrease immunogenicity and increase bioavailability, use of ‘chase’ compounds to eliminate unbound affinity ligands [Goodwin, DA et.al. 1988] and locoregional delivery to improve targeting.

Amplification strategies have also been developed for optimal visualization of molecular imaging agents like improving target concentration by pretargeting using avidin-biotin amplification systems [Barbet, J et.al. 1998, Goodwin, DA et.al. 1998], improving kinetics [Hu, S et.al. 1996], ability of probes to change their physical behavior after target interactions

as in fluorescent dequenching [Tijuvajev, JG et.al. 1996, Gambhir, SS et. al. 1999, Weissleder, R et. al. 1999] and by increasing R2 or R1 in magnetic resonance imaging [Weissleder, R et. al. 2000, Moats, RA et .al. 1997].

A number of sensitive, fast, high resolution imaging techniques like single photon emission computed tomography (SPECT), positron emission Tomography (PET), optical fluorescence imaging (OFI), optical bioluminescence imaging (BLI), magnetic resonance imaging, (MRI) computed tomography (CT) and ultrasound are available for molecular imaging. Different modalities give information about anatomy, physiology, metabolism and molecular changes to varying extent. Combining complementary modalities add anatomical and physiological information to molecular imaging studies, correlate two distinct biological measurements in time or allow simultaneous imaging of multiple molecular targets or probes. Results of imaging of animal models of human disease with improved spatial resolution can be directly translated into clinical applications in humans [Levin, CS. 2005, Massoud, TF & Gambhir, SS.2003].

2.1. Molecular Imaging Approaches

Noninvasive *in-vivo* imaging of molecular events can be achieved either by direct or indirect approach. The direct approach involves use of a labelled ligand that directly interacts with the target with high selectivity and specificity. Direct imaging can be done by using probes targeted towards proteins like receptor-ligand, antigen-antibody, enzyme-substrate, transporter-substrate, aptamers or probes targeted towards mRNA like labelled antisense oligodeoxynucleotides, peptide nucleic acids (PNA) or molecular beacons explored recently for imaging applications. Direct imaging can be further subdivided into three classes. First is based on probes which directly help in visualization of target expression, i.e. direct target imaging. Another class is of pathway probes which help in visualization of target function and the third class involves direct cell labeling to visualize pathological or therapeutic events.

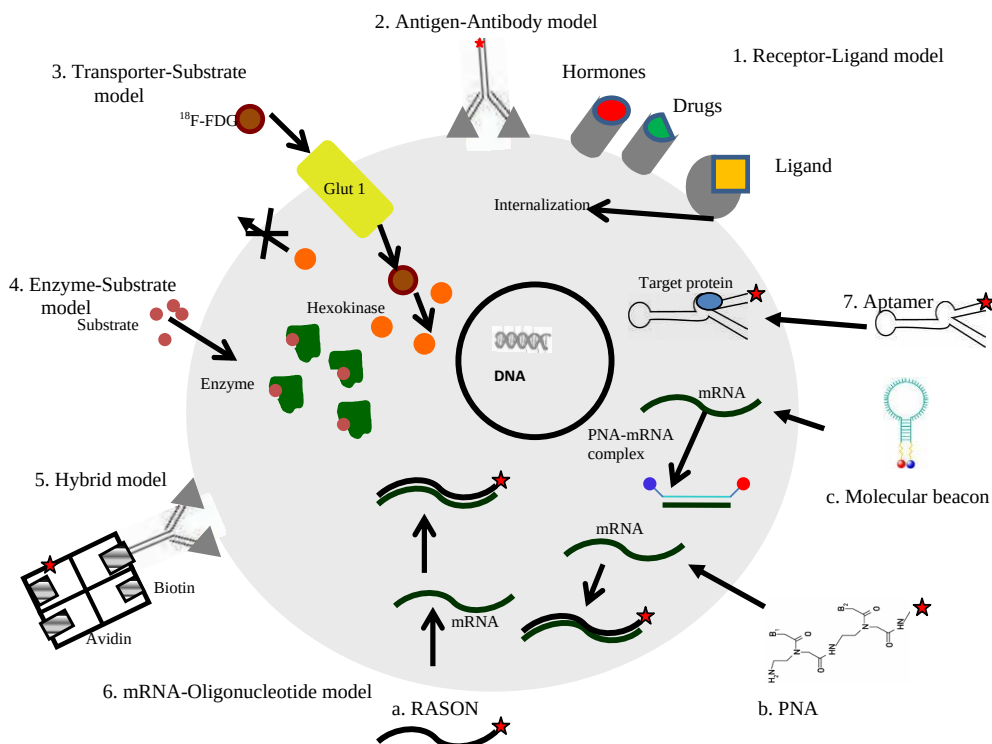
2.1.1. Direct imaging approach

Direct target imaging

Several mechanisms of accumulation of molecular imaging agents are reported in the recent past [Pauwels, EKJ et.al. 1998]. Property of diseased tissue to over express specific receptors, antigens, transporter proteins and enzymes (activation) is mostly exploited for development of imaging agents. Figure 1 depicts the most common mechanisms used for direct target imaging.

In the receptor-ligand model, one labeled ligand binds with high affinity to a specific site on a target known as receptor. Specificity and affinity of the receptor-ligand interaction as well as receptor density contribute to the success of imaging. Peptide receptor based imaging and therapy is the most successful and is widely practiced in clinics. Over expression of receptors of somatostatin (SST), vasointestinal peptide (VIP), bombesin (GRP), cholecystokinin (CCK) in various tumors is exploited for development of peptide based imaging agents. Targeting of tissues overexpressing receptors for drugs and hormones such

as estrogen receptors overexpressed in breast cancer cells is also achieved by this approach [Weiner, RE & Thakur, ML. 2005, Mintun, MA et.al.1988, Petra PH et.al. 2006].



1. Receptor-ligand model: Ligands, drugs or hormones specific for receptors, labelled either with radionuclide, fluorescent dyes or contrast agents, bind to the target receptors.
2. Antigen–Antibody model: Labelled antibodies target cells expressing specific antigens.
3. Transporter–substrate model: Labelled substrate gets incorporated into the target cell based on specificity and overexpression of transporter protein.
4. Enzyme–substrate model: Labelled substrate or inhibitor of enzyme is used to image enzyme activity in target cell.
5. Hybrid model: Intermediate molecule along with affinity component binds to the target and imaging is achieved with another labeled molecule specific for the intermediate.
6. mRNA-Oligonucleotide model
 - a) RASON, b) PNA, c) Molecular beacon
7. Aptamers: Labeled oligonucleotides specific for target in the cell are used for imaging.

Figure 1. Different mechanisms for direct target imaging.

The antigen-antibody model is similar to receptor-ligand model but follows more complex stoichiometry and binding kinetics. Antibodies are typically divalent and possibility of cross-linking can lead to an interaction that is stronger than would be predicted from the direct binding affinity. Specificity, affinity, density of antigenic epitopes and effective strength of interaction termed avidity are critical parameters for development of antibodies as imaging agents.¹³¹I-labeled tositumomab (Bexxer) used for detection and treatment of Non Hodgkin's lymphomas expressing CD20 antigens [Kaminski, MS et.al. 2005] and ¹¹¹In labeled Capromab pendetide (ProstaScint) for prostate cancer expressing prostate specific

membrane antigen [Petronis, JD et.al. 1998] are US food and drug administration (FDA) approved agents based on antigen-antibody interactions. Further details on recent antibody based imaging agents is given elsewhere [Albrecht, H & DeNardo, SJ. 2006].

In the transporter-substrate model, signal molecule called substrate is concentrated within a cell or tissue compartment via a mechanism in which the activity of the target molecule is the rate-limiting factor. In this model, the substrate must have a restricted interaction with a specific transporter and transporter must be uniquely present or absent or overexpressed in the disease tissue. Excessive uptake of ^{18}F -2-fluoro-2-deoxyglucose (^{18}F -FDG) in tumors is mainly due to over expression of GLUT-1 and GLUT-3 transporters compared to healthy tissue [Su, T-S et.al. 1990, Waki, A et.al.1998].

In the enzyme-substrate model, the target molecule is an enzyme that chemically modifies the signal molecule, fixing its biodistribution. A single molecule of enzyme can interact with many molecules of substrate, multiplying the signal many fold. The key issue is delivery of substrate as many desirable enzymes are restricted to specific intracellular compartments where the access to substrates is tightly controlled. Protease, mainly matrix metalloproteinase(MMP) that are over expressed and involved in tumor formation and angiogenesis are reported to be imaged by substrate peptide labeled with fluorescent probes [Bremer, C et.al.2001]. Radiolabelled enzyme substrates and inhibitors are also reported for many processes such as hypoxia and apoptosis imaging [Nunn, A et.al. 1995, Lahorte, CMM et. al. 2004]

In a complex or hybrid model, targeting is two step process where an intermediate molecule binds to the target first then in second step, the signal molecule reacts with the intermediate molecule which fixes its biodistribution. As an example the intermediate molecule may be a biotinylated antibody and the signal molecule may be a streptavidin linked chelator labeled with radioisotope or fluorescent label. This approach is expected to improve the specificity of targeting with agents that have a high affinity but clear slowly from the blood pool or have slow penetration into tumors. It can also improve the sensitivity of receptor targeting, if the intermediary binds the receptor first and then amplifies the signal by catalytically acting on the signal molecule as a substrate [Goldenberg, DM et.al.2003, Paganelli, G et.al.1988].

Gene/mRNA-oligonucleotide model is utilized for imaging of endogeneous gene expression. Northern analysis and *in situ* hybridization carried out in molecular biology research is translated for *in vivo* hybridization using radiolabelled antisense oligodeoxynucleotides (RASON) or oligonucleotides labeled with fluorescent labels that contain complementary sequence of mRNA to be imaged. In antisense-approach exquisite specificity of nucleic acid base-pair binding is exploited for targeting specific sequences [Gambhir, SS et.al.1999, Tavittian, B et.al.1998].

To overcome limitations of oligonucleotide probes such as stability, newer molecules that are nuclease resistant and are not substrates of RNase H are being explored. Peptide nucleic acid (PNA) is a nucleic acid analogue in which the sugar phosphate backbone of natural nucleic acid has been replaced by a synthetic peptide backbone usually as N-(2-amino-ethyl)-glycine units resulting in an achiral and uncharged mimic. PNA is capable of sequence specific recognition of DNA and RNA obeying the complementary base pairing rule. It is chemically stable and resistant to hydrolytic (enzymatic) cleavage and thus not

expected to be degraded inside a living cell [Ray, A & Norden, B. 2000]. PNAs linked with radionuclides or contrast agents are being exploited for early detection of cancers [Tian X et.al. 2007a, Tian X et.al. 2007b].

Molecular beacons are single stranded oligonucleotide hybridization probes that form a stem and loop structure (hairpin) structure with an internally quenched fluorophore whose fluorescence is restored when they bind to target nucleic acid sequences. Currently, molecular beacons are being explored for imaging of oncogene mRNA in tumors [Paroo, Z & Corey, DR. 2003].

Newer approaches like aptamers are based on single stranded oligonucleotides that have a complex 3 dimensional structure and can interact with suitable molecular targets with an affinity and specificity comparable to that of receptor ligand binding. Systematic evolution is an empiric technique for selecting oligonucleotides that will bind to a given target, using a repetitive process of selective binding and amplification by polymerase chain reaction. Like phage display, SELEX offers the potential of rapidly designing radiolabelled ligands for almost any target [Tuerk, C & Gold, L.1990, Missailidis, S & Perkins, A. 2007].

All models discussed above are being employed in molecular biology techniques for *in-vitro* diagnostics and research. After suitable optimization to meet the *in-vivo* requirements, these probes may be explored for *in vivo* molecular imaging.

Pathway probes

Direct visualization of target expression is complemented by imaging of target function. Assessment of the functional response to a drug target interaction, the flux through a signal transduction pathway can be considered more relevant than the visualization of the target expression or the receptor occupancy by the drug as such. A number of processes like proliferation, apoptosis, angiogenesis and hypoxia play a very significant role during diseases like cancer and small molecular probes to track these processes give very valuable information regarding disease status and progression [Rudin, M et.al. 2005, Kelloff, GJ et.al. 2005].

Proliferation imaging: From the beginning of molecular biology research, [^3H] thymidine incorporation in the cells has been the gold standard for assessing proliferation *in-vitro*. Same principle was utilized for *in-vivo* imaging after labeling thymidine with positron emitting nuclides. ^{11}C -thymidine, ^{124}I -iododeoxyuridine(^{124}I -IUDR) and ^{18}F -3'-deoxy-3'-fluorothymidine (^{18}F -FLT) are being used for estimation of proliferation and have potential in following response to therapy. Similarly increased protein synthesis and amino acid transport in proliferating tumor cells was studied *in vivo* using labeled methionine, tyrosine, glutamate and phenylalanine [Pauwels, EKJ et.al.1998].

Apoptosis imaging: Apoptosis plays a very important role in physiological processes. Excessive apoptosis leads to many diseased conditions like neurodegenerative diseases while insufficient apoptosis result in tumor development and autoimmune diseases. In the recent past, major thrust in molecular biology research is on understanding the pathways that lead to apoptosis and molecules involved in those pathways. Such research is also helpful in identification of potential biomarkers for apoptosis imaging. Annexin V is the most studied

and popular agent that binds to phosphatidylserine (PS) exposed to the outer layer of cell membrane during apoptosis and is the basis of standard assay method for *in-vitro* estimation of apoptosis. Utilizing the same principle, radiolabelled annexin V is being used for *in vivo* estimation of apoptosis [Yagle, KJ et. al. 2005]. Other agents explored for apoptosis imaging are anti-PS monoclonal antibodies, radiolabelled caspase inhibitors and substrates and mitochondrial membrane permeability targeting radioligands [Lahorte, CMM et. al. 2004].

Angiogenesis Imaging: Angiogenesis is another key oncologic process for tumor growth and for the initiation of metastasis. Apart from methods to assess blood volume and flow by imaging modalities, targeted probes are being utilized to visualize molecular effectors of angiogenesis like VEGF and $\alpha_v\beta_3$ integrins [Miller, JC et. al. 2005, Chen, X et. al. 2004, Meoli, DF et.al. 2004]. E-selectin and vascular cell adhesion molecule1 are also being explored as targets for imaging angiogenesis [Hsu, AR & Chen, X. 2008].

Hypoxia Imaging: Hypoxia is prevalent in nearly all tumors and identifying hypoxia is having prognostic value for radiation and chemotherapy especially in case of sarcoma, glioma, cancers of the uterine cervix, lung and head and neck cancers [Rajendran, JG & Krohn, KA. 2005, Eschmann, SM et.al. 2007]. ^{18}F -misonidazole is most promising hypoxia targeting agent used in humans. Its uptake in hypoxic cells is dependent on the reduction of the nitro group on an imidazole ring and is inversely proportional to intracellular O_2 concentration [Graham, MM et. al. 1997]. Second generation agents include fluoroerythronitroimidazole, fluoroetanidazole and diacetyl-bis (N4-methylthiosemi carbazone) copper (II) [Dearling, JL et. al. 1998].

Multidrug resistance imaging: Emergence of multidrug resistance (MDR) is a major obstacle to successful chemotherapy of cancer. Resistance is conferred by overexpression of transmembrane glycoprotein (Pgp), the product of MDR1 gene. Several transport substrates for Pgp were radiolabelled and characterized. $^{99\text{m}}\text{Tc}$ -sestamibi and $^{99\text{m}}\text{Tc}$ -tetrafosmin are found to be promising agents for prediction of MDR in a variety of cancers [Sharma, V. 2002, Del Vecchio, S; 1997, Chen, WS et.al.2000]. Recently ^{18}F -paclitaxel also showed promising results in preclinical studies [Kiesewetter, D & Eckelman, W. 2001]. Several agents, including ^{11}C -Colchicine, ^{11}C -Verapamil and ^{11}C -daunorubicin have been evaluated for the quantification of Pgp mediated transport with PET *in vivo* [Hendrikse, NH et.al. 1999].

Cell labeling

Many pathological events involve cell migration such as the infiltration of monocytes and lymphocytes to the site of inflammation or metastatic sites in cancer. Radiolabelling of cells for study of cell trafficking *in vivo* especially for inflammation and infection imaging is extensively reported [Thakur, ML. 1981, Thakur, ML & MacAfee, JG. 1976]. Using ultrasmall superparamagnetic particles of iron oxide (USPIO), which are internalized by phagocytotic macrophages, the migration of monocytes to sites of inflammation has been visualized by MRI in models of EAE [Dousset, V et.al.1999] brain ischemia [Rausch, M et. al.2002] soft tissue inflammation, kidney transplantation and antigen induced rheumatoid arthritis

[Beckmann, N et. al. 2003], providing relevant mechanistic information on inflammatory processes.

New approaches like cell therapy for treatment of degenerative disease using stem cells and targeting of tumor cells with lymphocytes to modulate or cure disease are being explored. The ability to repeatedly monitor the location of engrafted cells, their viability and their expansion in cell number is useful for estimating their effectiveness. Recently cell labelling approach is being utilized for tracking stem cells *in vivo*. USPIO labeling is also applied to monitor the migration of labeled stem cells to the site of ischemic brain damage [Hoehn, M et. al.2002] or to track progenitor cells in a model of traumatic spinal cord injury [Bulte, JW et.al. 1999]. An alternative approach to cell labeling is based on gene marking by stable transfection with reporter genes like visualization of migration of CD4⁺ T lymphocytes marked with luciferase to the brain of mice with experimental autoimmune encephalomyelitis (EAE) [Costa, GL et. al.2001] or in a murine model of collagen induced arthritis [Nakajima, A et. al.2001].

Applicability of direct imaging probes is limited because for each target, its own specific probe is required. Therefore indirect imaging techniques based on reporter genes have been developed for quantifying target expression.

2.1.2. Indirect imaging approach

Indirect imaging approach is applied in cases where specific ligands or probes are not available to define the magnitude of target expression. It is also very time consuming and inefficient to develop and validate new probes for each target. Alternatively it is both feasible and reasonable to develop and validate indirect imaging strategies using a marker/reporter gene in combination with the gene of interest. Reporter gene approaches are standard molecular biology tools to study gene expression both *in-vitro* in cultured cells and *in-vivo* in whole organisms to monitor gene expression [Gambhir, SS et.al. 1999]. Figure 2 depicts reporter gene approach for imaging gene expression.

The reporter gene along with gene of interest is first introduced into the target tissue by viral or nonviral vectors using molecular biology techniques. The promoter or enhancer elements can be cloned into these vectors to drive the transcription of a reporter gene. The promoter activity can be constitutive (always on), inducible (turned on or off) or tissue specific (expressed only in heart, liver or other organ). Translation of the mRNA leads to a reporter protein that can interact with the reporter probe. The signals can then be detected by various imaging modalities such as BLI, PET or MRI. Three types of reporter genes are used for imaging in SPECT and PET [Herschman, HR. 2004]. The reporter gene can encode for an enzyme that is capable of trapping a specific tracer through the action of that enzyme [Massoud, TF & Gambhir, SS. 2003, Haberkorn, U et.al. 1996]. Wild type or a mutant HSV1-tk reporter genes are most commonly used in PET imaging using radiolabelled probes like ¹⁸F-labeled penciclovir derivative, ¹⁸F-fluoro-3-hydroxymethylbutyl guanine(¹⁸F-FHBG) and ¹²⁴I-labeled uracil derivative, ¹²⁴I-iodo-2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil(¹²⁴I-FIAU).

Second type of reporter gene encodes for an intracellular and/or extracellular receptor capable of binding a tracer such as human dopamine D2 receptor(D₂R) or the human somatostatin receptor subtype 2 (SSTR2). Probes such as ¹⁸F-fluoroethyl spiperone (¹⁸F-

FESP) and ^{11}C -raclopride are available for clinical imaging of dopaminergic system with PET while octreotide labeled with $^{99\text{m}}\text{Tc}$, ^{111}In , ^{64}Cu and ^{68}Gd are useful for SSTR2 imaging [Zinn, KR & Chaudhari, TR. 2002]. Preclinical evaluation of ^{18}F labeled carbohydrate octreotide analogs is reported for PET imaging [Wester, HJ et.al.2003].

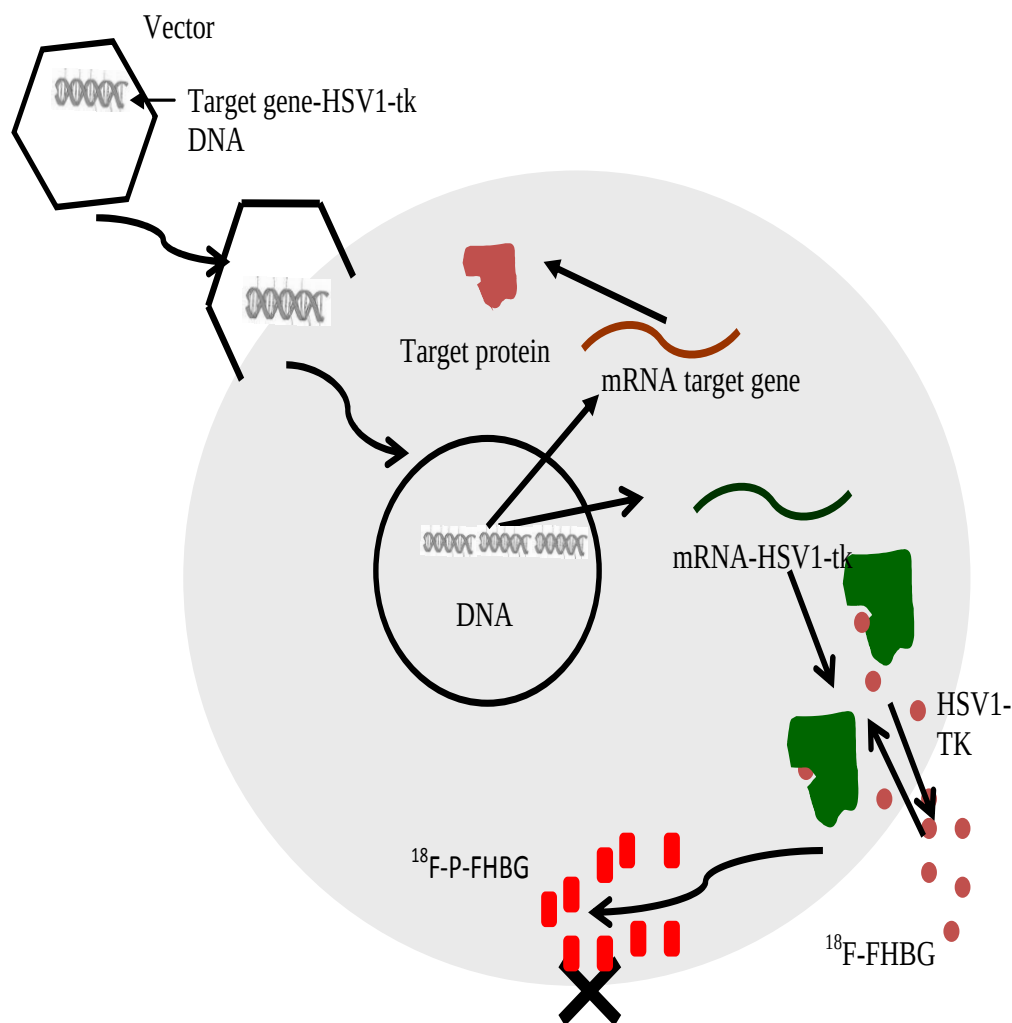


Figure 2. Reporter gene approach for imaging gene expression.

Third type of reporter gene can be a gene encoding a transporter protein that specifically incorporates a labelled tracer into the cell. Sodium iodide symporter (NIS) is reported for trapping ^{131}I especially for diagnosis and therapy of thyroid cancers [Haberkorn, U et.al.2001]. NIS gene transfer using tissue specific promoters provides a way of selectively targeting NIS gene to malignant cells, thereby maximizing tissue specific cytotoxicity and minimizing toxic side effects on nonmalignant cells [Kim, EE. 2003].

The reporter gene can provide information about the regulation of DNA by upstream promoter [Boecker, W et. al. 2004], the fate of intracellular protein trafficking [Paulmurugan, R et.al. 2002] and the efficiency of vector transduction into cells [Wu, JC et. al. 2001].

Likewise, the reporter probe itself does not have to be changed if one wishes to study a new biological process, which saves valuable time needed to synthesize, test and validate new imaging agent. However, the main disadvantage is that it is a surrogate marker of the physiologic process of interest rather than a direct measurement of receptor density, intracellular enzyme or mRNA copies as for direct imaging which are likely to be more clinically relevant. Other issues such as the safety of chromosomal integration in the cellular system, the potential for host immune response against reporter genes and the possibility of reporter gene silencing needs to be investigated. A very recent application of indirect approach is development of dual-luciferase reporter system for *in vivo* visualization of microRNAs (miRNAs) to understand the differentiation of grafted cells in vivo [Lee, JY et.al. 2008]

2.2. Molecular Imaging Techniques

In recent years, considerable efforts have been directed towards development of state of the art imaging technologies. The combined ability to perform tomographic assays in intact living subjects, followed by three dimensional reconstruction of images after acquisition and simultaneous quantification of various biological processes in an organ or in whole body is being possible by recent imaging modalities. Current techniques provide improved resolution and much detailed images of tissues and organs. Table 1. compares current imaging modalities and gives applicability of various modalities for obtaining physiological, anatomical and molecular information.

2.2.1. Radionuclide imaging

Radionuclide imaging by either PET or SPECT is the most popular among imaging modalities. PET records high energy γ rays while SPECT involves detection of low /medium energy γ emission. In PET, probe is labeled with a positron emitting isotope, positron annihilates with electron to produce two γ rays of 511KeV at $\sim 180^\circ$ apart. Coincidence detection of both γ rays in PET followed by reconstruction of image gives localization of radiolabelled probe. While SPECT involves use of probe labelled with γ ray emitting isotope and imaging is achieved by using gamma camera. Development of various radiopharmaceuticals for PET and SPECT studies is due to availability of a number of positron emitters like ^{18}F , ^{11}C , ^{13}N , ^{64}Cu , ^{124}I and γ emitters like $^{99\text{m}}\text{Tc}$, ^{111}In , ^{125}I with suitable decay characteristics and amenable chemistry for radiolabelling. Improvement in imaging instrumentation of PET and SPECT had added impact on applications of radionuclide imaging. Further details of instrumentation, image generation and reconstruction in PET and SPECT is discussed elsewhere [Levin, CS. 2005]

2.2.2. Optical imaging

Optical imaging techniques like *in-vivo* bioluminescence imaging and fluorescence imaging have already been developed for *in-vitro* and *ex-vivo* applications in molecular and cell biology. An extension of this concept is applied for non invasive imaging of biological processes in living subjects [Contag, CH & Bachmann, MH. 2002]. Bioluminescence

imaging requires cellular expression of an enzyme known as luciferase by incorporating gene for the enzyme in cells or animal model of disease. Enzyme reaction using substrate results in emission of visible light called bioluminescence that is used to monitor cellular or genetic activity of every cell that expresses the enzyme. A highly sensitive charge coupled device (CCD) converts light image to digital image [Levin, CS. 2005].

In fluorescence imaging, an excitation light of one wavelength in the visible range of 395-600nm, illuminates the living subject and a CCD camera [Golden, J & Ligler, F.A 2002] collects an emission light of shifted wavelength. Targets for fluorescence imaging may be endogenous molecules such as collagen or hemoglobin, fluorescent proteins like Green fluorescent protein and related molecules or optical contrast agents with fluorescent molecules [Lippincott-Schwartz, J et.al. 2001, Remington, SJ.2002]. Recently, investigators have developed strategies for imaging near-infrared fluorescence with emission wavelength between 650-900nm to image fluorescence in deeper tissues with minimal auto fluorescence from nontarget tissue. Fluorophores for NIRF imaging include Cy5.5, Alexa dye series, iodocyanine green and quantum dots [Luker, GD & Luker, KE 2008]. A newer approach to fluorescence imaging of deeper structures uses fluorescence mediated tomography [Ntziachristos, V & Wessleder, R. 2002].

2.2.3. Magnetic resonance imaging

Physiological, molecular and anatomical information can be extracted simultaneously by magnetic resonance imaging (MRI) and is exquisitely sensitive to soft tissue differences and abnormalities [Högemann, D & Basilion, JP.2002]. The principle of MRI is that unpaired nuclear spins called magnetic dipoles such as hydrogen atoms in water and organic compounds align themselves when placed into a magnetic field. In an MRI scanner, a strong magnet produces magnetic field surrounding the subject under investigation. An important function of the scanner is to determine the rate at which these dipoles relax to their baseline orientation; this measurement is translated into an MR signal [Jacobs, RE & Cherry, SR. 2001]. The two most important timing parameters are T1 and T2 weighting. MRI contrast agents have been developed to enhance signal differences and to further highlight the abnormality. Paramagnetic metal cations such as gadolinium or dysprosium or superparamagnetic contrast agents such as iron oxide nanoparticles can be used as compartmental, targeted or smart probes with this technique [Högemann, D & Basilion, JP 2002, Massod, TF & Gambhir, SS. 2003]. An interesting new extension of MRI technique for small animal imaging is magnetic resonance microscopy [Johnson, GA et.al. 2002] and magnetic resonance spectroscopy [Stegman, LD et.al. 1999].

2.2.4. Computed tomography

Computed tomography imaging involves generation of 3D images from a large series of 2D X ray images taken around a single axis of rotation [Dendy, P & Heaton, B.1999]. High-resolution micro CT scanners are available for anatomical imaging of small animals [Paulus, MJ et.al. 2001]. Direct imaging of molecular events is not possible by CT but it is mainly utilized to acquire anatomical details of the diseased tissue and complements the functional information obtained by other modalities. Unlike MRI, CT has relatively poor soft tissue contrast and iodinated contrast media is often used to delineate organs and tumors.

2.2.5. Ultrasonography

Ultrasonography is most widely used clinical imaging modality because of its low cost, availability and safety. Ultrasound images are obtained when high frequency (20kHz) sound waves are emitted from a transducer placed against the skin and the ultrasound is reflected back from the internal organs under examination. Contrast in the images obtained depends upon the imaging algorithm used, backscatter, attenuation of the sound and the sound speed. Recent emerging concepts are use of targeted ultrasonic contrast agent for molecular imaging of specific cell surface receptors [Dayton, PA & Ferrara, KW. 2002, Lanza, GM & Wickline, SA. 2001] and ultrasound biomicroscopy [Turnbull, DH & Foster, FS. 2002]. Figure 3 shows multiple imaging modalities available for molecular imaging.

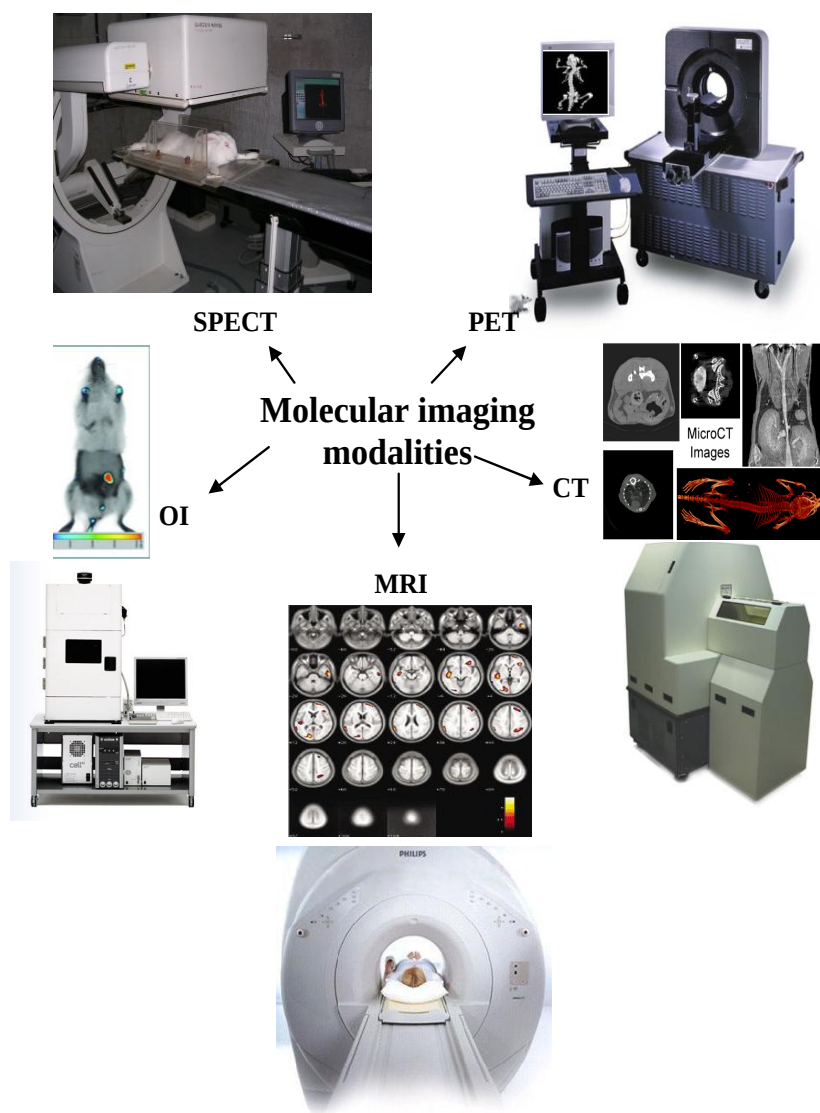


Figure 3. Different molecular imaging modalities.

The various existing imaging technologies differ in five main aspects: spatial and temporal resolution, depth of penetration, energy expended for image generation, availability of injectable/biocompatible molecular probes and the respective detection threshold of probes for a given technology. Spatial resolution is a measure of the accuracy or detail of graphic display in the images expressed in mm. It is the minimum distance between two independently measured objects that can be distinguished separately while temporal resolution is the frequency at which the final interpretable version of images can be recorded from the subject once the imaging process is initiated. Sensitivity is the ability to detect a molecular probe when it is present relative to the background measured in moles per liter. Advancement is also achieved in image generation from single image as in planer imaging to two and three dimensional tomographic imaging. Planer imaging is fast but gives low resolution. Tomographic imaging allows virtual slice of the subject to be obtained and is usually quantitative and capable of displaying internal anatomic structures and functional information but requires longer acquisition times and higher energy expenditure. Volumetric image acquisition shows a volume of interest in all three dimensions and results in highest spatial information content. Further reviews on molecular imaging techniques can be found elsewhere [Weissleder, R. 2002, Weissleder, R & Mahmood, U. 2001].

Each imaging modality has its own advantages and disadvantages. Combining complementary modalities add anatomical and physiological information to molecular imaging studies, correlate two distinct biological measurements in time or may allow simultaneous imaging of multiple molecular targets or probes. The greater flexibility offered by small animal imaging research has resulted in the development of several high resolution dual and tri-modality systems such as SPECT/ CT, SPECT/Optical [Peter, J et. al. 2005], PET/optical [Alexandrakis, G et.al. 2005], PET/SPECT and PET/MRI [Shao, Y et. al. 1997] [Grazioso, R et.al. 2005]. Such multimodality systems facilitate a range of *in-vivo* strategies to obtain rich, correlative information about the molecular basis of disease and enhance interpretation and quantification capabilities of data from the individual modalities involved [Culver, J et.al. 2008]

3. Role of Molecular Biology Research in Molecular Imaging

Molecular imaging is greatly benefited due to advancements in cell and molecular biology techniques. The human genome project and recent research in molecular biology already had a considerable influence on the current growth rate of laboratory diagnostics. Molecular biological techniques used in *in-vitro* diagnostics are being adapted to the special requirements of imaging diagnostics and molecular imaging protocols are being created. Identification of new targets for earlier disease detection, evaluation of specific molecular markers for therapy assessment, use of imaging for drug screening and imaging of gene expression are just a few of the applications.

Molecular imaging is a rather new discipline of experimental research that bridges basic and applied research applications. Its goal is noninvasive characterization and measurement of biological processes in living subjects at both cellular and molecular level. [Högemann, D & Basilion, JP. 2002, Phelps, ME.a 2000]. Present advancement in molecular imaging

appear analogous to recent development of methods to visualize gene expression by hybridization to arrays of DNA carried on chips. Functional genomics is a systemic effort to understand the function of genes and gene products by high throughput analysis in biological systems using automated procedures. The analysis of gene expression patterns using molecular biology approaches is likely to contribute to a more meaningful understanding of complex molecular pathways. Comparative genomics integrates different types of information derived from various animal models to generate a synergistic platform for understanding of complex disease mechanisms. Molecular imaging approaches may help in validating such research in *in vivo* systems [Massoud, TF & Gambhir, SS. 2003, Phelps, ME. b 2000].

3.1. Role in Development of Targeting Agents

Completion of human genome project has led to understanding of function of gene products and their role in development of disease. This knowledge is facilitating the discovery of new informative targeting agents, ligands or biomarkers that can be used for earliest detection of disease and for the creation of new classes of drugs directed at new therapeutic targets.

Molecular biology techniques like gel electrophoresis and blotting techniques are useful in isolation, purification and characterization of ligands. The discovery of techniques for manipulation of nucleic acids, restriction enzyme systems, DNA sequencing, reverse transcription, polymerase chain reaction, expression cloning and DNA microarrays has resulted in availability of methods for isolation, manipulation, identification and characterization of sub micromolar quantities of biomolecules. In principle, molecular imaging is based on the same fundamental biological techniques used for decades in *in-vitro* diagnostics, particularly peptide and antibody technologies. The detected cellular parameters such as cell surface receptors and enzymatic activities are also identical [Cunningham, SHB & Adelstein SJ. 2003].

The acquisition of *ex vivo* or *in vitro* information using cell extracts or in intact living cells in cell culture, has become easy because of the availability of variety of molecular biology reagents, protocols and instruments. However, laboratory diagnostics differs from molecular imaging in toxicological aspects, anatomical transport barriers in *in-vivo* systems such as the blood brain barrier and need for amplification strategies to detect minimal target concentrations, usually in the pico to nanomolar range. Biocompatibility and directional transport of imaging agent to the target are decisive factors for the clinical success of a molecular imaging agent [Hengerer, A & Mertelmeier, T.2001].

Table 1. Comparison of current imaging modalities

Imaging Modality	Region of EM spectrum used for image generation	Type of molecular probe	Spatial resolution	Temporal Resolution	Sensitivity mole/L	Tissue penetration depth	Amount of probe
Positron Emission Tomography (PET)	High energy γ rays from annihilation of positron with electron	Radiolabelled, direct or indirect	1-2mm	10 sec to min	10^{-11} - 10^{-12}	No limit	ng
Single photon emission computed tomography (SPECT)	Lower energy γ rays	Radiolabelled, direct or indirect	1-2mm	min	10^{-10} - 10^{-11}	No limit	ng
Optical bioluminescence imaging (BLI)	Visible light	Indirect	3-5mm	sec to min	Possibly 10^{-15} - 10^{-17} , Not well characterized	1-2cm	μ g to mg
Optical fluorescence imaging (OFI)	Visible light or near infrared	Direct or indirect	2-3mm	sec to min	Possibly 10^{-9} - 10^{-12} , Not well characterized.	<1cm	μ g to mg
Magnetic resonance imaging (MRI)	radiowaves	Direct or indirect	25-100 μ m	min to h	10^{-3} - 10^{-5}	No limit	μ g to mg
Computed tomography (CT)	X-rays	---	50-200 μ m	min	Not well characterized	No limit	Not applicable
Ultrasound	High frequency sound waves		50-500 μ m	sec to min	Not well characterized	mm to cm	μ g to mg

Table 1. (Continued)

Signal quantitation capabilities	Principal use	Ability for human imaging	Advantages	Disadvantages	Total Cost
High	Direct target imaging, pathway probes, indirect imaging	Yes	High sensitivity, isotopes can substitute naturally occurring atoms, quantitative translational research	PET cyclotron or generator needed, relatively low spatial resolution, radiation to subject.	High
Medium-high	Direct target imaging, pathway probes, cell labelling, indirect imaging	Yes	Many molecular probes available, can image multiple probes simultaneously, quantitative translational research	Relatively low spatial resolution, radiation to subject	Medium-high
Low-medium	Indirect imaging by reporter/gene expression, cell trafficking	Yes but limited	High sensitivity, quick, easy, low cost, relative high throughput.	Low spatial resolution, current 2D imaging only, relatively surface weighted, limited translational research.	Low
Low-medium	Direct target imaging, indirect imaging by reporter/gene expression, Cell trafficking	Yes but limited	High sensitivity. quantitative translational research possible.	Relatively low spatial resolution, surface weighted.	Low
medium	Direct target imaging, indirect imaging by reporter/gene expression, Morphological	yes	Highest spatial resolution, combines morphological and functional imaging.	Relative low sensitivity, long scan and postprocessing time, high quantity of probe needed.	High
Not applicable	Morphological	Yes	Bone and tumor imaging, anatomical imaging	Limited “molecular” applications, limited soft tissue resolution, radiation to subject.	Medium-high
Low	Morphological	Yes	Real time, low cost.	Limited spatial resolution, mostly morphological.	Low

Principles utilized in *in-vitro* diagnostics are being explored for *in-vivo* imaging. Like cancer biologists have known for decades that neoplastic degeneration is associated with increase in glycolysis and loss of tricarboxylic acid TCA cycle [Warburg, O. 1931]. Glucose consumption is further amplified by the activation of the hexose monophosphate shunt, increased hexokinase activity [Monakhov, NK et.al. 1978] and overexpression of GLUT 1 and GLUT 3 transporters. This principle was utilized for measurement of cerebral glucose consumption in rodents by autoradiography studies using ^{14}C -deoxyglucose [Sokoloff, L et. al. 1977]. Later the same molecule was labeled with ^{18}F for *in vivo* imaging [Ido, T et. al.1978, Som P et.al. 1980] and ^{18}F -FDG has become the most promising molecular imaging probe for PET studies of cancer and for the study of normal functions and diseases of brain and heart. ^{18}F -FDG, an analog of glucose enters cell through facilitated diffusion and is subsequently phosphorylated to ^{18}F -FDG-6-phosphate by hexokinase. ^{18}F -FDG-6-phosphate is not metabolized further because of strict structural and geometrical requirements of glucose 6-phosphate isomerase. Thus, ^{18}F -FDG-6-phosphate being negatively charged remains metabolically trapped within the cells and high uptake of ^{18}F -FDG due to increased glucose metabolism allows detection and staging of the disease [Gallagher, BM et.al.1978, Hoffman, JM & Gambhir, SS. 2007].

Similarly, molecular imaging agents based on receptor-ligand, antigen- antibody reaction, transporter-substrate, enzyme-substrate and hybrid systems were developed when high specificity and sensitivity was achieved in molecular biology or *in-vitro* diagnostic assays. RASONS, antisense oligonucleotides and molecular beacons initially discovered for *in-situ* hybridization assays, SNP detection, real time nucleic acid detection and PCR quantification are being explored for *in vivo* applications.

3.2. Role in Development of Animal Models of Disease

Use of animals with many characteristics shared with humans has become fundamental to all aspects of biomedical research from the study of basic biological mechanism to the understanding of disease pathology and the development of new medicines for both human and veterinary use. Breakthrough in immunology with identification of nude and severe combined immunodeficiency syndrome mice (SCID) gave possibility of development of animal models of various diseases in such immunocompromised animals [Killion, JJ. 1999]. Development of a number of tumor targeting agents is being possible due to availability of such animal models for various types of cancers in nude mice.

Creation of first transgenic mice in 1980 provided new insight to the development of animal models for various types of diseases [Palmiter, RD. 1985]. With the completion of human genome project, all the genes identified could be suitably modified either by introducing specific mutations in the genes that will lead to knockout animals or by introduction of foreign genes to have specific characteristics in transgenic animals. Transgenic animals have become very valuable tools for understanding the role of genes in specific diseases and for development of more effective drugs for treatment. Noninvasive imaging to study biochemistry and physiology of the brain, heart, musculoskeletal and metabolic systems and cancer biology is being possible by use of transgenic or gene “knock-out” mouse [Budinger, TF et.al. 1999]. Huntington’s disease,

parkinson's disease, multiple sclerosis, congestive heart failure and creative kinase gene knock outs are being utilized for understanding and development of therapeutic agents for such diseases.

Transgenic," Knockout" and "Knock-in" murine models are extensively used in cancer research. Models exist in which oncogenes can be activated ubiquitously, conditionally inactivated and reporter genes can be monitored during tumor initiation, progression and metastasis. In many instances, it would be of great value to be able to noninvasively monitor the growth of local tumors, the site, timing and extent of metastasis and/or the response to alternate therapies [Herschman, HR. 2004]. Nowadays, gene therapy vectors are being increasingly used to generate animal models for human disease through adult transgenesis that does not require the genetic manipulation of germline. More recently use of siRNA technology has allowed predictable knocking down of dominant disease causing genes by specific mRNA silencing [Jacobs, AH et. al. 2005].

Noninvasive imaging of molecular, genetic and cellular processes in such animal models complements established *ex vivo* molecular biological assays and imaging provides a new dimension to our understanding of various diseases.

3.3. Development of Peptide Based Molecular Imaging Agents

Monoclonal antibodies when discovered were considered as potential magic bullets for application in cancer diagnosis and therapy but because of higher molecular weight and slower pharmacokinetics they didn't became popular for *in-vivo* applications. An alternative to radiolabelled antibodies appeared in the form of small radiolabelled peptide, a somatostatin analog, which led to major breakthrough about 20 years ago. On the basis of the finding that many human tumors overexpress certain types of receptors like SST, VIP, CCK, bombesin (GRP) and neurotensin receptors, it has been possible to develop a method for localizing these tumors and their metastases by *in-vivo* receptor scintigraphy. Regulatory peptides have some inherent advantages as targeting agents because of their small size, excellent permeability, minimal side effects and no antigenicity. Easy synthesis and coupling with chelators for radiolabelling, high affinity receptor binding and rapid clearance from the body are features that contribute towards successful development of peptide based diagnostic and therapeutic agents. A number of peptide analogues are synthesized and labeled to obtain probes with optimal targeting properties.¹¹¹Indiethylenetriamine pentaacetic acid-D-Phe (1)-Octreotide (Octreoscan) is most popular imaging agent for detection of neuroendocrine tumors. Detailed description of peptide based targeting agents is described elsewhere [Reubi, JC. 2003. Weiner, RE & Thakur, ML. 2005].

However, development of such probes or agents requires extensive *in-vitro* evaluation of incidence and density of peptide receptor expression in human tumors and their metastases before *in-vivo* investigations can be performed in humans [Reubi, JC. 2003]. Such data on receptor expression helps in identifying types of tumors that can be targeted using peptide based imaging agents. Detection of receptor expression in primary human tumors, estimation of density, incidence and subtypes of receptor is being done by using basic molecular biology protocols and methods like receptor autoradiography, *in-vitro* binding assays, *in-situ* hybridization, real time PCR and immuno histochemistry.

In-vitro receptor autoradiography localizes and quantitates the peptide receptor. Pharmacological experiments using subtype selective analogs allow the gross identification of peptide receptor subtypes by their rank order of potencies in displacement experiments [Reubi, JC. 2001]. Receptor binding can also be quantitated using *in-vitro* binding assays. In contrast to receptor autoradiography performed on tumor tissue, binding assays are performed on homogenates and usually express the receptor density per mg of tissue or protein. Number of receptors per cell can be calculated when cell cultures are used for such experiments. Receptor immunohistochemistry identifies the receptor protein with a much better cellular resolution than autoradiography. *In-situ* hybridization, northern blots, RNase protection assays, RT-PCR and real time PCR all identify mRNA of receptor. PCR and RNase protection assays are methods with a very high sensitivity but without morphological correlates. However, *in-situ* hybridization is a morphological method with a lower sensitivity than RT-PCR.

Once information on types of receptors expressed on different types of tumors is available, next step is identification and development of suitable ligands for *in-vivo* applications. Molecular biology research has resulted in availability of information on structure and sequence of biomolecules especially peptides, proteins and nucleic acids. Extensive work to elucidate the structural and functional domains of receptors involved in ligand binding is reported by NMR measurements, X-ray crystallography and by indirect methods like site directed mutagenesis [Pohl, E. 1995]. Peptide ligands specific for receptors overexpressed on tumors are selected and suitably modified to meet *in vivo* application requirements. Receptor binding assays like saturation assays and competition binding experiments are done to calculate affinity of radiolabelled probes for receptors. Characterization of labelled probes also involves use of techniques like gel electrophoresis to establish *in vivo* stability of the probe and study interaction with serum components [Zhang, K et.al. 2007]. Animal tumor models are developed by injecting tumor cell lines overexpressing receptors for peptides in nude mice. Transgenic animals with tumors are also utilized to evaluate peptide based imaging agents.

Table 2. Examples of a few molecular imaging probes

Molecular target/process	Imaging probes
ONCOLOGY	
Small-molecule probes	
Proliferation	^{18}F -2-fluoro-2-deoxyglucose (^{18}F -FDG), 2- ^{11}C -Thymidine, 1-(2'-fluoro- β -D-arabinofuranosyl)thymine(^{18}F -FLT), ^{124}I -iododeoxyuridine (^{124}I -IUDR).
Apoptosis	$^{99\text{m}}\text{Tc}$ -Annexin V, ^{18}F -Annexin V.
Hypoxia	^{18}F -misonidazole, ^{64}Cu (II)-diacetyl-methylthiosemicarbazone (^{64}Cu -ATSM) (Cu-
Pharmacokinetics	^{11}C -Temozolomide, ^{13}N -Cisplatin.
Multidrug resistance	$^{99\text{m}}\text{Tc}$ -Sestamibi, $^{99\text{m}}\text{Tc}$ -tetrafosmin, ^{11}C -Verapamil, ^{11}C -Daunorubicin, ^{11}C -Colchicine
Breast Cancer (estrogen receptor)	$^{16\alpha}$ -[^{18}F]fluoro-17 β -estradiol (FES)
Prostrate cancer (androgen receptor)	$^{16\beta}$ -[^{18}F]fluoro-5alpha-dihydrotestosterone (FDHT)
Peptide probes	
Somatostatin receptor	^{111}In -diethylenetriamine pentaacetic acid-D-Phe(1)-Octreotide, ^{111}In -Pentetreotide (Octreoscan), $^{99\text{m}}\text{Tc}$ -depreotide (Neotect), ^{111}In -DOTA-Lanreotide

Vasoactive intestinal peptide receptor	^{123}I - Vasoactive intestinal peptide, $^{99\text{m}}\text{Tc}$ -TP3654
Bombesin,gastrin releasing peptide receptor	$^{99\text{m}}\text{Tc}$ -Bombesin
Cholecystokinin receptor	^{111}In -diethylenetriamine pentaacetic acid-minigastrin
Integrin receptor	^{18}F -Arg-Gly-Asp (RGD)
Cathepsin proteases	NIR fluorochrome labeled cathepsin B & D
Antibody probes	
Angiogenesis	^{111}In -anti-endoglin antibodies, Paramagnetic nanoparticles using antibodies to integrin $\alpha_v\beta_3$, vascular cell adhesion molecule1 and E-selectin.
CEA	$^{99\text{m}}\text{Tc}$ -Arcitumomab (CEA scan), ^{111}In -Satumomab pendetide (OncoScint CR/OV)
Prostrate specific membrane antigen	^{111}In -Capromab pendetide(ProstaScint)
CD20	^{131}I -labeled tositumomab (Bexxer)
Cardiology	
Myocardial infarction (tissue viability)	^{18}F -FDG
Myocardial angiogenesis ($\alpha_v\beta_3$ targeting)	^{111}In -RP748
Atherosclerosis	$^{99\text{m}}\text{Tc}$ -Annexin V, ^{18}F -Annexin V
Neurology	
Alzheimer's disease(dementia)	^{18}F -FDG, ^{11}C -flumazenil, ^{11}C -N-methyl-4-piperidinyI-acetate or propionate (^{11}C -MP4A & ^{11}C -PMP), N-methyl- ^{11}C -2-(4'-methyl-aminophenyl)-6-hydroxybenzothiazole(^{11}C -PIB)
Parkinson's Disease(Dopamine receptors)	^{18}F -6-fluorodopa (^{18}F -DOPA), ^{11}C -dihydro-tetrabenazine (^{11}C -DHTBZ), ^{11}C or ^{18}F -labeled 2 β -carbomethoxy-3 β -(4-fluorophenyl) tropane (^{11}C or ^{18}F -DHTBZ), ^{11}C -raclopride, ^{18}F -spiperone, ^{123}I -iodobenzamide.
Gliomas	^{18}F -FDG, ^{18}F -FLT, ^{11}C -methionine (^{11}C -MET)
Infection/Inflammation	$^{99\text{m}}\text{Tc}$ -Bectumomab (Lymphoscan), $^{99\text{m}}\text{Tc}$ -Fanolesomab, $^{99\text{m}}\text{Tc}$ -Sulesomab
arthritis	^{111}In -E-selectin antibodies, $^{99\text{m}}\text{Tc}$ -CD4 antibodies

Among various receptor targeting agents, somatostatin receptor scintigraphy and therapy is most successful. Somatostatin consists of a family of a 14 amino acid (Somatostatin-14) and a 28 amino acid (Somatostatin-28) peptide. Somatostatin can be considered to be a neurotransmitter, a neurohormone or a hormone acting via autocrine or paracrine mechanisms [Patel, Y. 1999]. Molecular biology of somatostatin receptors is extensively studied and reported. To date, five human somatostatin receptor subtypes (sst₁, sst₂, sst₃, sst₄ and sst₅) have been cloned and characterized [Reisine, T & Bell, GI. 1995, Reubi, JC. 2003]. These subtypes belong to a superfamily of G protein-coupled receptors that can functionally couple to various intracellular effector systems.

Somatostatin receptor expression was studied by receptor autoradiography studies in the 1980s and early 1990s. A very high incidence and often a high density of somatostatin receptors have been found in neuroendocrine tumors. Nonneuroendocrine tumors including brain tumors, breast carcinomas, lymphomas, prostatic, ovarian, gastric and hepatocellular carcinomas were also shown to overexpress somatostatin receptors. Various radioligands were used in binding studies, either natural somatostatin labeled with ^{125}I or synthetic small sized analogs such as ^{125}I -Tyr³-octreotide, ^{125}I -MK-678 or ^{125}I -RC-160 which label only selected somatostatin receptor subtypes [Hoyer, D. 2000]. Analysis of mRNA, immunohistochemistry with selective antibodies and autoradiography with subtype selective ligands have recently been introduced and have been able to confirm the previous data and extend results by determining the subtypes

involved. Recently, reports on measurement of sst₂ receptor by real time PCR indicates that mRNA could be quantified precisely [Pinzani, P. 2001].

Somatostatin is having short biological half life as it is amenable to be degraded by proteases and hydrolytic enzymes. A lot of research work is done to make stable somatostatin analogues with optimal binding properties and improved *in vivo* pharmacokinetics. Analogues were then suitably labeled either directly or after linking with suitable chelating agents. Radiolabelled somatostatin analogues were then tested by carrying out binding studies in cell lines and tumor tissue homogenates. Promising analogues were tested in animal models like nude mice bearing tumors over expressing somatostatin receptors or in transgenic animal models. Such research has led to development of ¹¹¹In-DTPA-Octreotide (octreoscan) that has become the most widely used molecular imaging agent for somatostatin receptor scintigraphy [Krenning, EP. 1995] and ⁹⁰Y-DOTA-Tyr³-Octreotide (⁹⁰Y-DOTATOC) for radiotherapy.

Hence, molecular biology research and methods greatly contribute in development of molecular imaging agents. Development of newer approaches based on reporter gene concept are solely dependent on molecular biology research tools.

4. Applications of Molecular Imaging

Molecular imaging creates a possibility of achieving several important goals in biomedical research and medicine like assessment of disease progression and imaging drug effects at molecular and cellular level for optimization of drug and gene therapy. *In-vivo* imaging methods also help in studying specific cellular and molecular processes like gene expression and following trafficking and targeting of cells. Molecular imaging creates a possibility of achieving all the goals of imaging in a rapid, reproducible and quantitative manner so as to monitor time dependent experimental, developmental, environmental and therapeutic influences on gene products in the animal models or patients.

4.1. Molecular Imaging in Diagnostics

A number of molecular imaging probes are reported at different phases of development that image specific molecular pathways *in vivo*, enabling visualization of phenotypic expression of key targets in the disease processes. A list of molecular imaging probes for various molecular targets is given in Table 2 [Kelloff, GJ et.al.2005]. Detailed list of current molecular imaging agents for specific applications is given elsewhere [Massoud, TF & Gambhir, SS 2003, Neves, AA & Brindle, KM 2006]

In future, molecular imaging will serve as a supplement to other imaging and diagnostic procedures for earlier and more specific diagnoses and will assist the physician to provide optimal treatment that is tailored to the clinical history of each patient. Encouraging advances have already been realized in animal research.

4.2. Molecular Imaging in Gene Therapy

Gene therapy has emerged as a new and promising therapy for many diseases. Currently gene therapy based anticancer strategies involve replacement of tumor suppressor genes, use of antisense agents, drug sensitization by transduction with suicide genes, genetic immunotherapy, transfer of genes involved in biology of tumor growth etc. However, gene therapy is associated with inefficient incorporation of transgene into desired cells and inadequate level of expression. To ensure patient safety, it is necessary to determine biodistribution of transgene and its expression. In the clinical settings, these studies would imply unacceptable repetition of invasive procedures with no information on whole body gene distribution and expression. Thus, there is urgent need to develop sensitive and reproducible noninvasive imaging method that could be repeatedly and safely performed in patients. Advances in molecular biology are making it possible to develop assays for imaging specific molecular processes including those directly related to gene expression. Imaging gene expression entails determining the locations of cells expressing particular gene of interest as well as monitoring the magnitude and persistence of gene expression. The gene of interest may be an exogenous gene or an endogenous gene introduced into the organism or tissue of interest [Gambhir, SS. 2000]. Molecular imaging has emerged as a promising technology for *in vivo* mapping of gene expression and provides promising tools for progress of molecular medicine and gene therapy. Excellent reviews on gene therapy imaging for oncology, neurology and cardiology are available [Penuelas, I et.al.2005, Jacobs, AH et.al.2005, Wu, JC & Yie-Herttuala, S.2005].

4.3. Molecular Imaging in Drug Development

Drug targets derived from the human genome are largely unexplored with 30,000 human genes that code for almost 100,000 proteins. It is clear that drug interactions in living subjects cannot be completely understood using *in-vitro* research methods. To increase the success rates of clinical trials, it is necessary for potential drugs to be tested first within the intact living system before clinical trials on humans can be considered. In research, cell cultures consisting of cells isolated from tissue and grown outside the organism in culture flasks are often used for initial testing. The imitations of such artificial systems are becoming more and more apparent, since isolated cultivated cells adjust to the altered environmental conditions and are not true representative of the organism of interest. As a result, animal experiments are indispensable for many pharmaceutical reasons and are important, despite the ethical concerns that exist for animal experimentation. Small animal models already constitute an established research tool for the biological validation of new therapies. Furthermore in basic research, small animal models such as transgenic and knock out mice are indispensable. To be able to obtain kinetic measurements of a drug action with several measurement values over the course of a specific time frame, a corresponding number of animals must be treated as specified in the experimental protocol. Afterwards, the experimental animals must be killed successively in many cases in order to perform an exhaustive molecular analysis. In this event, *in vivo* molecular imaging techniques allow repetitive observations of biological processes in the same animal. Small

animal imaging technologies include micro-MR, micro-CT, micro-SPECT, micro-PET, micro-US and various optical technologies. Level of resolution with some small animal imaging modalities is now approaching the size of individual cells. Molecular imaging with small animals can substantially improve the efficiency of biological validation of drug molecules and shorten the time required for drug approval while simultaneously contributing to limit the cost linked to preclinical development [Rudin, M & Weissleder, R 2003].

5. Conclusion

Molecular imaging reaped benefits of the advancements in molecular biology and many other related fields. From identification of targets to development of labelled ligands and their evaluation in *in-vitro* and *in-vivo* systems, molecular biology tools are being utilized for development of molecular imaging agents. However, molecular imaging can now be exploited for advancement of research in molecular biology. Visualization and quantitation of relevant molecular and physiologic variables such as altered cellular metabolism and proliferation, gene expression, protein-protein interactions and enzymatic expression that contribute to human disease is possible by molecular imaging. Molecular imaging can also be used to address basic scientific questions like transcriptional regulation and signal transduction and will be helpful in developing treatment strategies based on gene therapy. In future, molecular imaging may become a key technology in translational research.

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Chapter VI

Yeast Protein Aggregates, Containing Chaperones and Glucose Metabolism Enzymes

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Abstract

Three groups of proteins associated with misfolded protein depending aggregates were identified in *Saccharomyces cerevisiae* cells by using a new approach: comparative analysis of crude lysate pellets of isogenic yeast strains differing by their prion composition or adenine biosynthesis pathway characteristics. 2D electrophoresis followed by MALDI analysis of a recipient [*psi⁻*] strain and of [*PSI⁺*] cytoductant permitted identification of 53 proteins whose aggregation state depended on prion content or red pigment accumulation in yeast cells. Further studies allowed identifying an overlapping group of 38 proteins whose aggregation state responded to a shift of prion(s) content and also a rather similar group of more than 40 proteins whose aggregation state depended on accumulation of red pigment. In all these cases nearly one half of the identified proteins belonged to a functional group of chaperones and enzymes involved in glucose metabolism. Notable were proteins involved in oxidative stress response and in translation. The prion dependent group also contained a proteinase. These results are comparable with recent literature data on various misfolded proteins containing aggregates in yeast cells. Being not dependent on cloned heterologous genes, our approach permits a conclusion about universal presence of glucose metabolism enzymes in such aggregates. Most of the identified proteins, although behaving like prions in some experiments (for example, being “transmittable” by cytoduction), seem to

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be just amyloid-associated and mobilized to pellets in response to presence of prion fibrils. Our model experiments demonstrate that red pigment binds insulin fibrils and blocks their interaction with Thioflavine T. This allows concluding that red pigment impedes mobilization of some prion-associated proteins to prion-containing aggregates and so makes them to appear as pigment depending ones. Also there are some proteins (e.g. Sod1p and Cus1p) that themselves can be “clients” of a hypothetical prion generation pathway dealing with not NQ-rich proteins in yeast.

Introduction

Detecting abnormal (mutant or damaged) proteins cellular surveillance system attempts refolding such polypeptides, then destroying the remaining ones, and eventually, when all these attempts fail, mobilizing them to aggregates (that in lower eukaryotes are not passed to daughter cells). Notable among such abnormal proteins are amyloids forming stable β -sheet rich fibrillar structures. At present, about 20 diseases related to amyloid production are described (Uversky, 2008; Uversky, Oldfield, Dunker, 2008). The best known among them are Spongiform Encephalopathies, Huntington’s disease, Alzheimer’s disease, Parkinson’s disease, type 2 diabetes, Amyotrophic Lateral Sclerosis and others (Wickner et al., 2008). Some of these diseases appear to be infectious and are shown to be caused by amyloid fibrils that in these cases (Griffith, 1967; Prusiner, 1999) are called prions (protein infectious). Recent progress in understanding prions is mainly connected with studying prionizable proteins in cells of yeast *Saccharomyces cerevisiae*, an eukaryotic microorganism that is a subject to powerful methods of molecular genetics. Yeast prions were described more than 40 years ago as cytoplasmic (“non-Mendelian”) heritable determinants, but were recognized as such by Wickner (1994, for a review see the papers by pioneers of yeast prion studies Chernoff et al., 2002; Chernoff, 2004; Wickner et al., 2007; Wickner et al., 2008). In two cases, the function of normal form of yeast prionizable proteins is well known. Sup35p (also denoted eRF3) is one of the translation termination factors. Its inactivity due to prionization (like mutation in *SUP35*) leads to an increased read-through of translation termination codons. This permits the assay of the Sup35p prionization by resulting nonsense suppression. The heritable cytoplasmic factor of the nonsense suppression is called [*PSI*⁺] factor. The cells carrying or not carrying this factor were called [*PSI*⁺] and [*psi*⁻], correspondingly.

Ure2p is a negative regulatory factor in nitrogen catabolism, which inhibits utilization of poor nitrogen sources when a rich nitrogen source is available. Conversion of Ure2p to the prion form denoted [*URE3*] eliminates nitrogen catabolite repression and enables yeast cells to utilize both rich and poor nitrogen sources. Ure2p is one of the best characterized prion proteins in yeast (Lian et al., 2006, 2007).

Later there was discovered a hereditary cytoplasmic factor [*PIN*⁺] whose presence leads to enhancement of the rate of [*PSI*⁺] appearance *de novo*. Its name is derived from “[*PSI*⁺]-inducibility”. The [*PIN*⁺] phenotype is due to amyloidization of Rnq1p, a protein rich in N and Q residues. Swi1p, subunit of a chromatin remodeling factor, has been shown to give rise to a prion [*SWT*⁺] with a phenotype due to deficiency of the normal form of the protein (Du et al., 2008). The prion-like behavior has also been described for [*NU*⁺] derived from New1p (see Derkatch, Liebman, 2007). Both Swi1p and New1p overproduction mimics the [*PIN*⁺]-

phenotype. Recently it has been established that any asparagine and glutamine rich prion facilitates induction of other prions in the same cell, but some cases of negative interaction have also been described (Derkatch, Liebman, 2007). In 2009, two new prions were demonstrated in yeast—[MCA], a product of prionization of metacaspase Mca1p, a protein supposed to participate in yeast apoptosis (Nemecek et al., 2009), and [OCT⁺]*—*a result of prionization of the yeast global transcriptional co-repressor protein Cyc8p (Patel et al., 2009).

A detailed study of [PSI⁺] and [URE3] factors permitted deriving the following genetic criteria that are proposed to distinguish the prion-based inheritance from the traditional nucleic acid-based one: (a) if a prion is cured, it can arise again in the cured strain, provided the corresponding gene is not deleted; (b) overproduction of a potentially prionizable protein increases the frequency of the prion arising *de novo* and (c) if the prion phenotype is due to the absence of the normal form, then the phenotype of mutation of the gene for the protein should be similar to that of the prion. (For review see Wickner et al., 2008, Chernoff et al., 2002; Chernoff, 2004).

Amyloids are self-propagating in a sense that they are able to recruit monomers (soluble forms) of the protein and to convert them to amyloid. Prions have a special position among amyloids, as they are infective and/or able to support the self-propagating amyloid state in successive generations. This is possible owing to assistance of several special cellular proteins that are not amyloids, but affect folding and generation of “seeds” (sometimes called propagons) that pass to daughter cells to serve as matrices in amyloid self-propagation. The most important among these proteins are chaperones (see Chernoff, 2004; Cox et al., 2007; Kushnirov et al., 2007; Rikhvanov et al., 2007; Tuite, Cox, 2007; Wickner et al., 2007; Wickner et al., 2008). The first chaperone whose role in prion life cycle (mainly in propagation) was firmly established is Hsp104p. This chaperone disassembles protein aggregates formed from denatured proteins under stress (e.g., heat shock) conditions and thereby increases survival. Hsp104p functions to break up large [PSI⁺] (and probably other prion) aggregates for resolubilization, producing seeds that are transmissible to daughter cells during cell division. A loss of function in Hsp104 eliminates [PSI⁺], [URE3], and [PIN⁺]. Curiously the same effect for [PSI⁺] is caused by Hsp104p overproduction. Guanidine hydrochloride (GuHCl) applied at low doses also leads to such a curing. The effect of GuHCl turned out to be due to inhibition of Hsp104p (Chernoff et al., 1995, Kryndushkin et al., 2003, Takahashi et al., 2007, for review, see Cox et al., 2007; Kushnirov et al., 2007; Rikhvanov et al., 2007).

Apart from Hsp104p, other chaperon families, like Hsp70 and Hsp40, seem to affect prion fibrils assembly and propagation (Chernoff et al., 1999; Jung et al., 2000; Jones, Masison, 2003; Sharma et al., 2009). Also, some changes in prion protein amino acid sequence can alleviate the dependence of prion propagation on Hsp104p (Alexandrov et al., 2008). Recently it has been shown that chaperone Ydj1p cures [URE3] by binding to the native protein Ure2p and thus reducing incorporation into amyloid (Lian et al., 2007). Chaperones also seem to be important factors in forming aggregates connected with prion amyloids. The recent study from Liebman’s laboratory (Bagriantsev et al., 2008) have shown that some chaperones like Hsp104p, Sis1p, Sse1p, and Ssa1p/Ssa2p interact preferentially with the prion versus nonprion form of Sup35p. The authors have demonstrated that Sup35p amyloid fibrils (polymers) connect with each other and with other proteins (among which the

chaperones are the most abundant) to form $[PSI^+]$ aggregates. They emphasize the difference between the Sup35p polymers and aggregates (containing, together with Sup35p polymers, also other proteins). The prion life cycle allows different pathways connected with many different chaperones.

Prion life cycle starts with prion fibril generation from seeds, and ends by degradation or formation of large aggregates unable to be transmitted to daughter cells (Wang et al., 2007, 2008). Not only prion fibrils, but also proteins involved in prion refolding, degradation, stabilization and aggresome formation are mobilized to the final prion aggregates leading to prion detoxification. List of the functions needed for prion life cycle has been certainly not closed yet. Finding new prion-associated proteins can lead to opening new ways of manipulating prions, involving suppression of prion propagation, minimizing their interaction with other molecules, decreasing toxicity, etc. These proteins are ready-made tools for affecting prions. It is not surprising that during the year since we started our own study, several groups have turned to study of amyloid-associated proteins in yeast cells (Bagriantsev et al., 2008; Erjavec et al., 2007; Wang et al., 2007, 2008).

Good object for looking for such proteins are prion-containing aggregates. The cellular systems recognizing and curing misfolded protein molecules seem to take prion fibrils together with any interacting molecules to press them all to thick neutralized packets unable to be transmitted to daughter cells. The process starts when misfolded molecules attract proteins destined to neutralize them, and these complexes attract other protein molecules. To analyze the end product of the process, we collected aggregates of crude lysate pellets that sediment during gentle centrifugation (we must not damage even loosely attached molecules). To discard the aggregates that do not involve prions, we purified the prion-containing aggregates genetically.

Materials and Methods

Strains and Plasmids

Strain AH/B (*MATa ade1-14 ura3 his3 lys9 kar1 [psi⁻ pin⁻]*) was kindly provided by Dr. A. Borchsenius. Strain K5-5 (*MATa his4 ade2 kar1-1 [psi⁻]*) and K5-13 (*MATa his5 kar1-1 [PSI⁺]*)—by Dr. B.F. Yarovoi, 168t (*MATa his5 [PSI⁺]*) and YTK79 (*MATa ura3 leu2 his3 trp1 lys2*) by Dr. T. S. Karpova. The strain 74-D694 (*MATa ade1-14 ura3 his3 trp1 leu2 [PSI⁺ weak PIN⁺]*) and its isogenic derivative OT56 (*MATa ade1-14 ura3 his3 trp1 leu2 [PSI⁺ strong PIN⁺]*), were kindly donated by Dr. L. Mironova and Dr. G. Zhouravleva. Other variants of these strains differing by presence of the *PSI* ($[PSI^+_{strong}]$ or $[PSI^+_{weak}]$) factors were obtained during this study. Strain GT26 (*MATa, aro7-1cys1-72 his4-166 leu2-1 lys2-187 met8-1 trp5-48 ura3-1 [PSI⁺ PIN⁺]*) and its isogenic *psi⁻ pin⁻* derivative GT27 (Chernova et al., 2003) were kindly donated by Prof. Y. Chernoff. The plasmids used include pRS316-GAL-*SUP35(URA3)*, pYS-GAL-*HSP104(URA3)* containing *SUP35* or *HSP104* gene under control of inducible GAL1,10 promoter and a matching control plasmid pRS316-GAL(*URA3*) that were described in (Chernoff et al., 1999) and were provided to us by Dr. G. Zhouravleva.

Using the above-mentioned strains and plasmids, a set of various transformants and cytoductants was constructed (Nevzglyadova et al., 2008). Cytoductants were designated to show clearly their origin. For example, in C6[*PSI*⁺]/(GT26)B, C6 is the short name of the cytoductant, [*PSI*⁺] is its plasmotype, B stands for the short name of the nucleus donor parent AH/B, and GT26 is the name of the other parent serving as the additional cytoplasm donor. The [*psi*⁻] derivatives obtained after GuHCl treatment of a strain are designated as in the following example: 74-C/GuHCl [*psi*⁻]. (In this case 74-C is a short name of transformant 74-C/SUP35.)

The Cultural Media and Basic Methods

The cultural media and basic methods of manipulating yeast cells are described by Sherman et al. (1986). Prion elimination by GuHCl was performed according Chernoff et al. (2002), and cytoduction—according to Zakharov and Yarovoy (1977), with some changes: zygotes were identified as characteristic dumb-bell shaped cells and then isolated by micromanipulation. [*PSI*⁺] cytoductants were picked as clones retaining nuclear markers of the [*psi*⁻] parent, but obtained the [*PSI*⁺] factor as judged by colony color on YEPD and confirmed by ability to grow without adenine.

Preparation and Analysis of Cell Lysate Pellets

Yeast cultures were grown on solid media for 5-6 days. The cells were harvested, normalized for optical density, washed in water, and lysed by vortexing with glass beads in a buffer A: 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA. Proteases were blocked by adding PSMF at a high concentration (10 mM). Since the goal of this study was to isolate the whole set of prion-containing aggregates contained in our pellet preparations, we did not use dithiothreitol (DTT) in the lysis buffer to avoid disturbing the connection of prionized proteins with various prion-associated ones. Cell debris and glass beads were removed by centrifugation at 100g for 1 min, and supernatants were 1.5-fold diluted at 0°C. The protein concentration in the diluted lysates to be compared was determined according to a modified method of Lowry (Severin and Solovyov 1989). The adjusted lysates were centrifuged at 1000 g for 30 min and the supernatant was removed. Each step of pellet washing involved resuspending and high vortexing followed by recentrifugation, but had some differences in different experiments. To compare the pellet proteins in agarose, we washed them once with PBS (150 mM NaCl, 25 mM Na₂HPO₄-NaH₂PO₄, pH 7.4). Before loading to polyacrylamide gel the pellets were washed with 8 M urea, then twice with PBS and three times with PBS containing 2% of sodium dodecylsulfate (SDS) at 37°C—to remove the loosely associated proteins from prion aggregates (Kryndushkin et al. 2003). The pellet protein extraction was carried out in buffer B (0.15 M Tris-HCl, pH 6.8, 2% SDS, 3% 50 mM DTT) for 15 min at 37°C.

Electrophoresis

To induce protein monomerization, the carefully washed pellet material extracted in buffer B was boiled for 10 min and after centrifugation at 1000g for 5 min the supernatant was used as an electrophoretic sample. The electrophoretic samples were separated by standard SDS-polyacrylamide (6.5-10% SDS-PAGE) procedure (Laemmli, 1970). The 1.8% agarose gels were used according to Jones and Masison (2003). The procedures of sample extraction and boiling coincided for polyacrylamide and agarose gels. Nevertheless, in the last case when pellet preparations were washed only once, boiling served mainly for release of prion oligomers and small polymers from larger aggregates incapable to enter the gel untreated, than for monomerization. For details see (Nevzglyadova et al. 2009c).

Measuring Fluorescence Intensity

Fluorescence intensity (IF) in crude lysate pellets stained with thioflavin T was measured as reported in our earlier paper (Nevzglyadova et al. 2009c). Before extraction the lysate pellets were washed once by PBS. This procedure causes loss of red color and so obviates red pigment effect on subsequent measurement. IF was determined at excitation 435 nm and emission 486 nm. Total protein in a pellet sample was determined by modified Lowry's method (Severin and Solovyov, 1989) and the fluorescence value per total protein in a sample (IF/prot) was calculated. Samples with and without red pigment were compared using $IF_{\text{white}}/IF_{\text{red}}$ ratio. Significance of differences in IF values was estimated using the sign test by Dixon and Mood (Sachs, 1972).

Model Experiments with Insulin Fibrils

Insulin fibrils were formed at pH 2 in buffer B_f (20 % CH₃COOH, 100mM NaCl) at protein concentration 2 mg/ml. The ratio of fibrils dissolved in acid buffer B_f to red pigment dissolved in water was equal to 1: 2. Optical density of the pigment stock solution determined at $\lambda = 490$ varied from 3 to 4. For experiments 1-2 we used 10 mcl and for 3-6—40 mcl of fibril solution. After incubating during half of an hour the samples were centrifugated at 1000g for 5 minutes, supernatant was discarded and pellet was dissolved in 500 mcl of 0,025 M TrisHCl, at pH = 7.5. After endogenous fluorescence measurement the probes were supplied with 3 mcl of 10^{-3} M Thioflavine T and the fluorescence was registered again. IF of the fibrils bound Thioflavine T fluorescence was calculated as a difference between second and first measurement. Such a procedure was considered to be necessary because if the share of fibrils autofluorescence did not exceed 1 % of total IF, the autofluorescence of fibrils + red pigment was larger by an order of magnitude and reached 15 %.

Red Pigment Purification

Crude lysate of red strain was cleared of debris and centrifuged at 1000g for 30 min. Supernatant was purified by chloroform: methanol (3:7) extraction. Supernatant: extractant ratio was 3:5. After centrifugation the upper phase was collected, loaded to SpeedVac and then applied to PD-10 column (Pharmacia, Sweden). Red fraction was eluted by H₂O, dried in SpeedVac and dissolved in minimal volume of water. The optical density of red pigment was measured at 490 nm.

Two-Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional electrophoretic separation of proteins included 2 stages: isoelectric focusing (IEF) and SDS-PAGE. IEF was performed in 18-cm IPG DryStrip gels, pH 3-10, with use of Ettan IPGphor II IEF system (GE Healthcare, former Amersham, UK) according to manufacturer's recommendations. Samples were dissolved in 350 µl of standard IEF buffer (8 M urea, 2% CHAPS, 2% IPG buffer, pH 3-10, 1.2% DeStreak reagent; all reagents were purchased from GE Healthcare), loaded onto ceramic strip holders, covered with IPG strips and IPG cover fluid. Strips underwent the active rehydration for at least 15 hrs at 50 volts, followed by IEF for 65 kV-hrs. In the second dimension, proteins were fractionated according to their molecular weights in the denaturing 10% PAGE (Laemmli 1970).

Mass Spectrometry and Protein Identification

1 mm³ gel samples carrying the spots were destained and dehydrated using incubation in 100 µl of 40% solution of acetonitrile in 0.1 M NH₄HCO₃ for 30 min at 37°C. 100 µl of acetonitrile was added. After removal of the acetonitrile, gel samples were dried and 4 µl of solution of 15 µg/ml modified trypsin (Promega) solution in 0.05 M NH₄HCO₃ was added. Following the incubation for 18 hrs at 37°C, 8 µl of 0.5% TFA in 10% acetonitrile was added and the resulting mixture was vigorously stirred. Supernatant was used for MALDI. 1 µl of the supernatant was mixed with 0.3 µl of 10 mg/ml solution of 2,5-dihydroxybenzoic acid in 20% acetonitrile with 0.5% TFA and spotted unto target.

The resulting tryptic peptides of each spot were analyzed with a BRUKER Ultraflex II MALDI-TOF/TOF mass-spectrometer equipped with Nd ultraviolet laser. After calibrating according to the trypsin autolysis peaks, the mistake of mass estimate did not exceed 0.007%.

Identification of proteins according to the peak-lists obtained was performed using Mascot program (<http://www.matrixscience.com>). Searching was performed against NCBI database for proteins of all organisms. In some cases, peak-lists for contaminating human keratin were removed and for the remaining ones, search was performed among *Saccharomyces cerevisiae* proteins. Cysteine modification (propionamide) and methionine oxidation were allowed, also maximum 0 or 1 missing cleavage site. Criteria for protein identification were a minimum of 5 matched peptides and probability greater than 0.95 (most obtained data exceed these criteria by far).

Results

To recognize among all the pellet proteins the prion-dependent ones, we compared pellet protein sets of isogenic strains differing by their prion composition. For this, we optimized the procedures of cell growth, lysate pellet fraction obtaining and washing, and pellet protein extraction to get maximal differences between the compared strains. The method of sample preparation that we have developed turned out to be adequate as it revealed Sup35p in the pellet fraction only in $[PSI^+]$ and not in $[psi^-]$ strains (Nevzglyadova et al. 2009a, 2009b, see also Table 1).

For construction of yeast strains differing by their prion composition, we used prionotropic treatments (treatments leading to modification of prion content of cells). To be able to select $[PSI^+]$ strains showing the nonsense-suppression, we applied overexpression of the *SUP35* gene located on a plasmid. To obtain $[psi^-]$ strains, we used GuHCl treatment or overexpression of the plasmid-born *HSP104* gene. Also we used cytoduction. As a result of cytoduction, two isogenic strains can be obtained: recipient and cytoductant; they share the same nuclear genotype and differ only by their “plasmatype”, a set of genetic elements localized in the cytoplasm. In our case this meant two strains differing only by their prion composition. Comparison of pellet proteins of such pairs of strains permitted identifying consequently the proteins responding to changes in the prion content. To identify the proteins that can be transmitted or lost (diminished in quantity) during prionotropic treatments, we used 2D electrophoresis followed by MALDI.

In this study we performed the following 2D electrophoreses: “A” for a strain 74-C/*SUP35* $[PSI^+]$ and its derivative 74-C/GuHCl $[psi^-]$ induced by GuHCl and “B” for the $[PSI^+]$ cytoductant C6 $[PSI^+]$ /(GT26)B and its $[psi^-]$ parental recipient strain B (shorthand for AH/B). Pair-wise comparisons of this kind could indicate how many (and which) pellet proteins respond to GuHCl treatment of cells or can be transmitted by cytoduction.

Figure 1 illustrates the 2D electrophoresis in experiment “B” that identifies the greatest number of proteins. The corresponding images for the experiment “A” are shown in Figure 2. More than 100 protein spots were selected for identification by MALDI analysis in all experiments. As a result, more than 50 individual proteins were identified—many of them repeatedly (Table 1).

In these experiments we used strains containing or not containing a well-known yeast prion—the $[PSI^+]$ factor. Like most other people working with $[PSI^+]$, we used strains containing a nonsense mutation in *ADE1*, coding for SAICAR synthase. This mutation leads to accumulation of 4-aminoimidazole ribotide (AIR) whose subsequent polymerization results in red pigment formation and storage in vacuole (Smirnov et al., 1967, Weisman et al., 1987, Chaudhuri et al., 1996).

Table 1. List of identified putative prion-associated proteins

Protein name	Spot No./Experiment	NCBI Acc. No	Class	MW ^{***} kDa	PI ^{**}	No. of Pep- tides	Seq. Coverage %
Sup35p			Prion [PSI ⁺] protein	detected by Western blot			
Ctt1p	34c3 /B	NP_011602	Response to stress	64.543	6.09	8	18
Sod2p	54-2 /B	NP_011872	Oxidative stress response	25.758	8.49	7	46
Ahp1p	56-2 /B	NP_013210	Oxidative stress response	19.103	5.01	10	53
Yhb1p	42-2 /B	NP_011750	Oxidative stress response	44.618	5.86	11	40
Tsa1p	55a2 /B	NP_013684	Oxidative stress response Unfolded protein binding Chaperone	21.576	5.03	10	62
Ssa1p (HSP70 Family)	5 /A 33a /B	NP_009396	Chaperone	69.725	5.00	28 23	48 46
Ssa2p (HSP70 family)	5 /A 33a /B	NP_013076	Chaperone	69.427	4.95	28 19	53 41
Ssb1p (HSP70 family)	6a/ A	NP_010052	Chaperone	66.561	5.32	29	56
Ssb2p (HSP70 family)	33b /B 41-3 /B	NP_014190	Chaperone	66.554	5.37	21 9	50 24
Sse1p (HSP70 family)	32a /B	NP_009728	Chaperone	77.361	5.12	13	25
Hsp60p	34a /B	NP_013360	Mitochondrial Chaperone	60.714	5.23	24	45
Eno2p	39-2 /B	NP_012044	Co-chaperone Glucose metabolism	46.885	5.67	14	40
Eno1p	59-a2 /B 60-2 /B	NP_011770	Glucose metabolism	46.601	6.04	8 10	20 27
Adh1p	10 /A 42-4 /B 42a2 /B	NP_014555	Glucose metabolism	36.826	6.21	11 6 11	37 25 36
Adh4p	44-2 /B	NP_011258	Glucose metabolism	50.586	8.64	11	27
Ald4p	8-2 /A 36-3 /B	NP_015019	Mitochondrial Glucose metabolism	56.688	6.31	20 8	36 22

Table 1. (Continued)

Protein name	Spot No./Experiment	NCBI Acc. No	Class	MW ^a kDa	PI ^{**}	No. of Pep- tides	Seq. Coverage %
Cdc19p	35a2 /B	NP_009362	Glucose metabolism	54.564	8.0	16	38
Fba1p	11a2 /A 44-3 /B	NP_012863	Glucose metabolism	39.596	5.51	8 8	20 30
Glk1p	36-5 /B 36a2 /B	NP_009890	Glucose metabolism	55.342	5.8	8 16	21 38
Lat1p	36-4 /B	NP_014328	Glucose metabolism Pyruvate	51.786	7.6	8	18
Pdc1p	8-1 /A 36-2 /B	NP_013145	Glucose metabolism Pyruvate	61.457	5.8	27 21	47 44
Pgk1p	38b2 /B	NP_009938.2	Glucose metabolism Kinase	44.552	6.73	20	57
Tdh3p	45a2 /B	NP_011708	Glucose metabolism ROS metabolism	35.724	6.46	15	56
Tpi1p	52-2 /B	NP_010335	Glucose metabolism	26.795	5.86	5	28
Aco1p	4 /A 30b2 /B 31a /B	NP_013407	Tricarboxylic acid cycle	85.315	8.17	31 18 7	41 27 13
Fum1p	38-2 /B 38a2 /B	NP_015061	Tricarboxylic acid cycle	53.146	8.48	12 13	31 34
Shm2p	9 /A 9-1 /A	NP_013159	One-carbon metabolism	52.186	6.98	25 17	48 37
Ach1p	34c2 /B	NP_009538	Mitochondrial acetate metabolism	58.731	6.3	12	31
Pab1p	6 /A	NP_011092	Poly(A) binding Translation initiation	64.304	5.71	38	23
Rpl4a	43-2 /B	NP_010295	Translation Ribosomal protein	38.907	10.64	5	24
Tef1p or Tef2p	9-2a /A	NP_015405 NP_009676	Translation elongation	50.001	9.14	16	37

Table 1. (Continued)

Protein name	Spot No./Experiment	NCBI Acc. No	Class	MW [*] kDa	PI ^{**}	No. of Pep- tides	Seq. Coverage %
Tif35p	47-2 /B	NP_010717	Translation initiation	30.483	6.42	11	48
Tma19p	16a /A	NP_012867	Translation Oxidative stress response	18.729	4.41	11	47
Tuf1p	39-3 /B	NP_014830	Mitochondrial translation elongation	47.942	6.19	11	27
Act1p	40-3 /B 41-2 /B	NP_116614	Actin CytoskeletonOxidative stress response	41.678	5.44	5 13	16 43
Tub2p	8a /A	NP_116616	Beta-tubulin Cytoskeleton	50.890	4.64	23	43
Psa1p	11a1 /A	NP_010228	GDP-mannose biosynthesis Cell wall maintenance Stress response	39.541	5.95	8	29
Sec53p	15 /A 51-2 /B	NP_116609	GDP-mannose biosynthesis Protein targeting to ER Protein folding	29.057	5.14	16 11	50 42
Ilv5p	43a2 /B	NP_013459	leucine, isoleucine, and valine biosynthesis	44.341	9.10	18	47
Leu1p	3 /A	NP_011506	leucine, isoleucine, and valine biosynthesis	85.741	5.61	31	33
Sam2p	40-2 /B	NP_010790	Methionine metabolism	42.229	5.18	7	23
Cpr1p	19 /A 58-2 /B 58a2 /B	NP_010439	Posttranslational protein modification Chaperone	17.380	6.9	7 8 7	37 37 37
Pst2p	17 /A 55-2 /B	NP_010315	Posttranslational protein modification Oxidative stress response	20.953	5.46	9 10	65 75
Fpr1p	62-2 /B	NP_014264	Chromatine architecture Peptidyl-prolyl cis-trans isomerase Protein folding	12.019	5.71	5	50

Table 1. (Continued)

Protein name	Spot No./Experiment	NCBI Acc. No	Class	MW [*] kDa	PI ^{**}	No. of Peptides	Seq. Coverage %
Gsp1p	14 /A	NP_014125	RNA processing Nuclear organization	24.795	6.11	7	30
Apa2p	11 /A	NP_010819	Nucleotide metabolism	36.397	4.96	14	48
Por1p	49-2 /B	NP_014343	porin Mitochondrial organization	30.410	7.70	12	49
Qcr7p	20 /A 59a3 /B	NP_010818	Respiration	14.556	5.62	8 6	69 43
Cyb2p	35a3 /B	NP_013658	Respiration	65.498	8.59	18	31
Cox4p	59-2 /B	NP_011328	Respiration	17.132	6.41	5	49
Gor1p	42-3 /B	NP_014125	Respiration	38.807	5.93	10	40
Fmp12p	38-3 /B	NP_011842	Mitochondrial Unknown function	53.102	7.21	6	16
Ynl010w	50-2 /B	NP_014388	Unknown function	27.463	5.28	7	37

* Theoretical molecular weight, ** theoretical isoelectric point.

Experiment A. Selected were the spots present in strain 74-C/*SUP35* [*PSI*⁺] and absent in 74-C/*GuHCl* [*psi*⁻].

Experiment B. Selected were the spots present in strain C6 [*PSI*⁺]/(GT26)B and absent in the recipient strain B (shorthand for AH/B). Spot numbers in experiment B correspond to spot numbers in Figure 4 (top panel).

Strains carrying this mutation are most convenient for prionization studies involving Sup35p (also known as eukaryotic translation termination factor eRF3). Prionization of Sup35p inactivates the majority of Sup35p molecules and this results in a partial loss of translation termination activity leading to the low level translation through the nonsense codon. As a result there appear sufficiently full-sized and functional SAICAR-synthase molecules to lift the block of adenine biosynthesis and hence stop the accumulation of the red pigment. By this way the color of yeast colonies (together with related ability to grow in minimal medium without adenine) allows making quantitative conclusions about Sup35p prionization.

Quite unexpectedly we recognized that red pigment itself affects amyloidization in yeast cells. Amyloid bound thioflavine T fluorescence in the lysates of red yeast strains carrying mutations in genes *ADE1* or *ADE2* turned out to be drastically enhanced when the cells were grown in media containing high concentration of adenine (100 mg/l) that blocks accumulation of the pigment. Additional blocks at first stages of purine biosynthesis de novo also impede red pigment formation and enhance Thioflavine T fluorescence. At the same time induction of mutations in genes *ADE1* or *ADE2* in originally white prototrophic strains leads to considerable drop of fluorescence (Table 2). A fraction of protein polymers was studied by agarose gel electrophoresis (Figure 3). After losing red color, even if retaining the original [*psi*⁻] phenotype, the cells nevertheless contain considerable amount of protein polymers with high molecular weight, migrating in agarose gel above the band of the molecular weight marker apoferritin (MW = 450 kDa) (Nevzglyadova et al., 2009c). Taken together the data on fluorescence intensity and on agarose electrophoresis confirm our inference that red pigment diminishes amyloidization of yeast proteins.

Model experiments with insulin fibrils, formed at pH 2 in buffer B_f demonstrate that purified red pigment binds fibrils and blocks their interaction with Thioflavine T (Table 3). In all 6 independent comparisons fibrils that were incubated with red pigment exhibited considerable drop of IF. As one can see in the B/P column, undiluted pigment (P) diminishes IF ca. 8 times, diluted preparation (P/2)—5 times. Reiterated measurements of the sample after 2-3 hours gave the same IF value. These results permitted us to suppose that red pigment interacts with insulin fibrils and it is this interaction that diminishes Thioflavine T binding to the same fibrils and leads to the drop of IF.

The data on the influence of red pigment forced us to reconsider our previous data and we repeated the study reported in Table 1 growing the used strains at high concentration of adenine that inhibits synthesis of the red pigment. These data were reported by Nevzglyadova et al. (2009a, 2009b) and are included in Table 4 of this chapter (column 3).

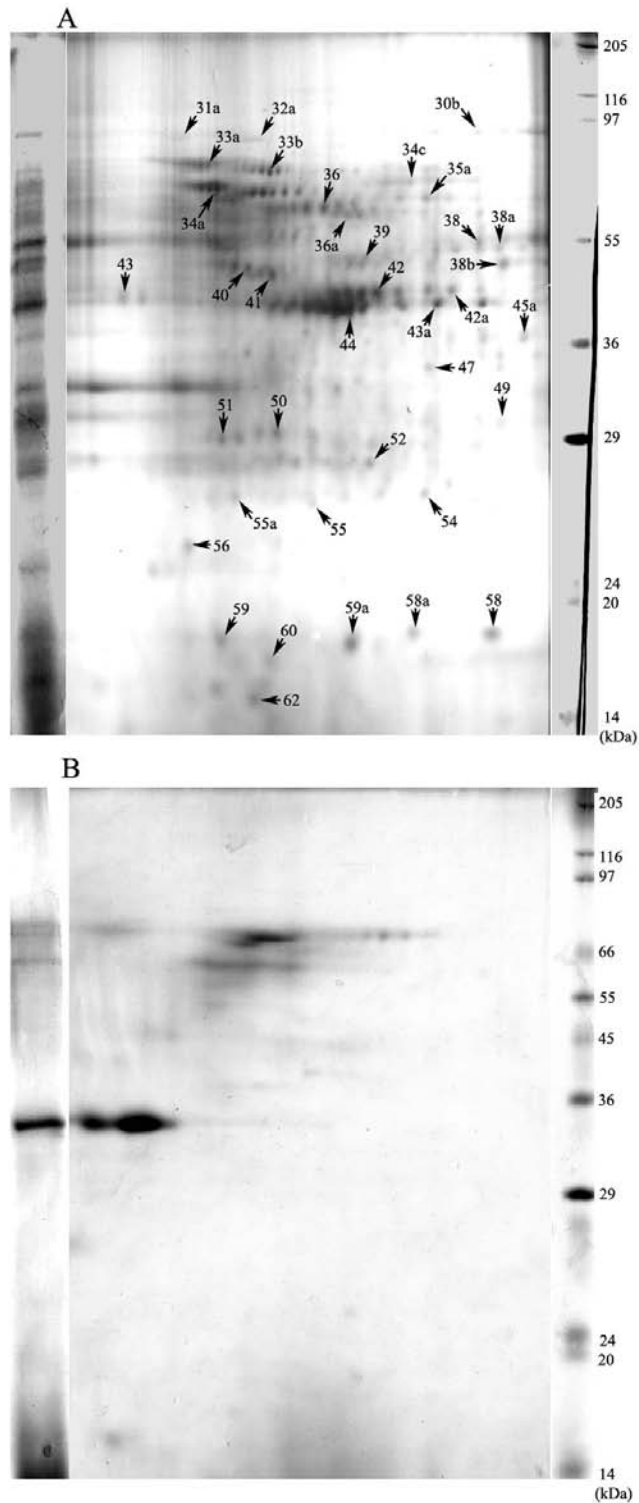


Figure 1. A and B. Coomassie blue stained 2-D electrophoresis patterns of pellet proteins from strains C6 [*PSI*⁺]/(GT26)B and AH/B [*psi*⁻] correspondingly. Left vertical line represents 1D electrophoresis of the sample, right lane—molecular weight markers.

Table 2. Intensity of fluorescence of Thioflavine T stained pellet proteins in isogenic pairs of strains differing by red pigment accumulation.

Colony color	Strain	Adenine genotype	[PSI]-factor	IF	IF white /IF red, %	IF/prot	IF (prot) white / IF (prot) red, %
Red White	K5-5 K5-5/w1	<i>ade2</i> <i>ade2ade5,7</i>	(—) (—)	284.6 495.9	174	3558 5700	160
Red White	AH/B AH/B/w1	<i>ade1</i> <i>ade1ade6</i>	(—) (—)	246.2 446.0	181	3075 6282	204
Red White	74-D694/Gu 74-D694/Gu/w1	<i>ade1</i> <i>ade1ade6</i>	(—) (—)	227.6 321.3	141	1897 2677	141
Pink White	74-D694w 74-D694w/w1	<i>ade1</i> <i>ade1ade6</i>	(+weak) (+weak)	347.8 787.9	226	524.5 1762	336
Red White	K5-13/r1 K5-13	<i>ade2</i>	(+) (+)	322.2 2265	703	4079 39070	958
Red White	K5-13/r3 K5-13	<i>ade2</i>	(+) (+)	212.4 2266	1067	2590 39070	1508
Red White	GT26/r1 GT26	<i>ade1</i>	(+) (+)	419.7 1517	361	8931 58350	653
Red White	168t/r1 168t	<i>ade1</i>	(—) (—)	364 1617	444	3370 15400	457
Red White	YTK79/r1 YTK79	<i>ade1</i>	(?) (?)	181.7 1996	1098	3187 22170	696

Mutant strains are marked as r with a corresponding number for red mutants and w—for white ones.

Corresponding genotype changes were determined using standard testers for adenine biosynthesis genes. Confidence limits for a two-sided sign test at 1 % level are 1 and 8. The difference between white and red clones is significant at $p < 0.01$.

By genetic criteria the proteins identified in our experiments reported here formally behave like prions, as they are eliminated by GuHCl treatment and/or are not detected in recipient strains, but revealed both in donors of the cytoplasm and in cytoductants. Still we have no evidence in favor of them being prions. The data present in the *Saccharomyces* Genome Database clearly indicate that most of them are prion-associated proteins. Indeed, more than 50% of the proteins identified in our study unequivocally belong to the groups previously shown to directly or indirectly interact with prionizable proteins. It seems to be also important that unlike prionized proteins they were able to enter polyacrylamide gel without preliminary boiling. (True prions were also found in our studies (Nevzglyadova et al., 2009a, 2009b). Prionizable protein Rnq1p was detected in the pellet fraction by 2D electrophoresis and MALDI, and other prion Sup35p—by Western blot.)

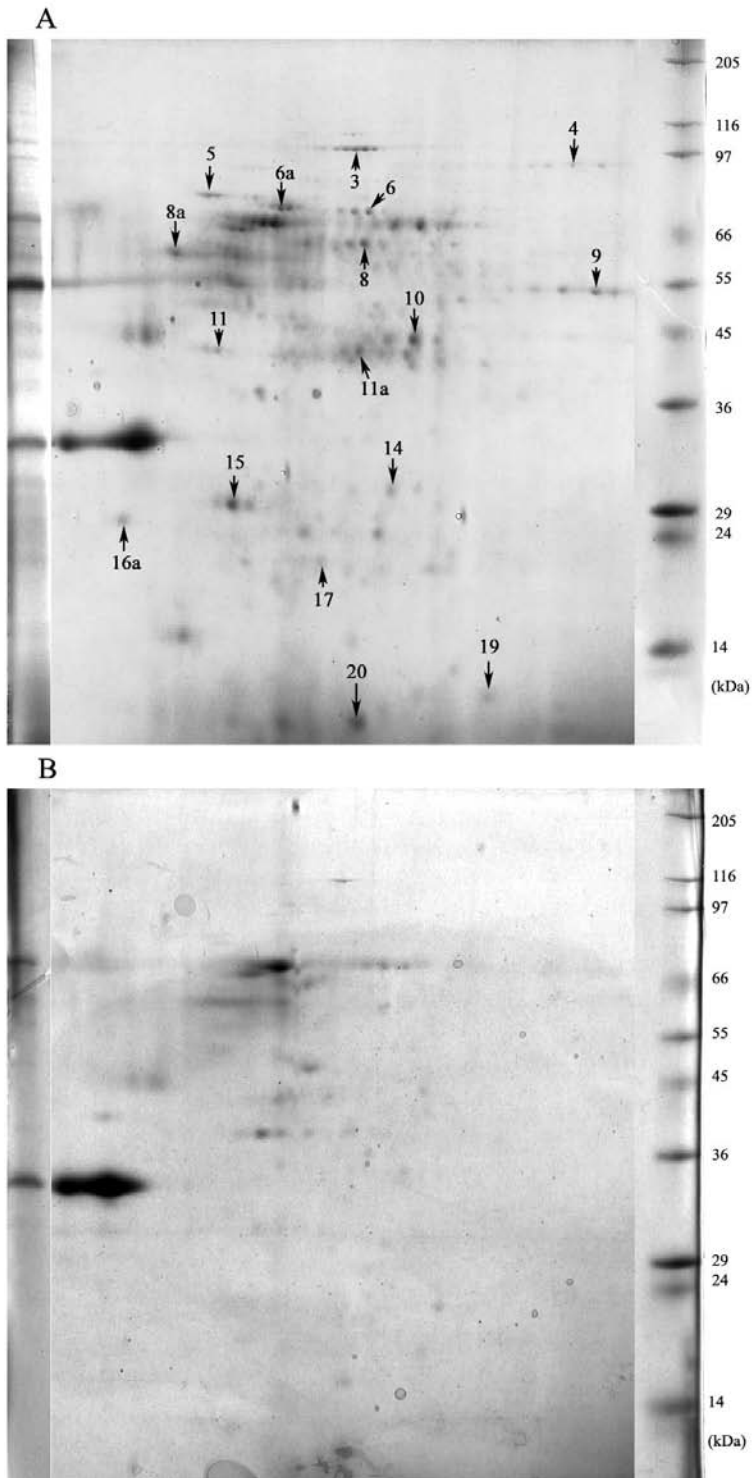


Figure 2. A and B. Coomassie blue stained 2-D electrophoresis patterns of pellet proteins from strains 74-C/*SUP35* [*PSI*⁺] and 74-C/*GuHCl* [*psi*⁻] correspondingly. Left vertical line represents 1D electrophoresis of the probe, right lane—molecular weight markers.

We would like to suggest a hypothesis concerning the putative prion-associated proteins detected in our studies. Despite the considerable uncertainty about the particular mechanism of prion-depending recruitment of each protein to pellet, we would like to note that the Kryndushkin's protocol (Kryndushkin et al., 2003) permitted us to show that many pellet proteins were connected with small polymers/aggregates that are trapped on the top of the polyacrylamide gel (Nevzglyadova et al., 2009a, 2009b). So to explain the simulation of cytoplasmic inheritance by the proteins identified in our work, we suggest mobilization of these proteins to prion containing aggregates. This hypothesis does not exclude the possibility of multiple pathways of different proteins to the aggregates responding to prion presence (or generally speaking—to protein misfolding). We suppose that the intimate mechanism of the apparent transmission of electrophoretic protein patterns in cytoduction is as follows. A donor cell transmits to the recipient some infective prion “seeds” that lead to prionization of molecules of the potentially prionizable protein (Satpute-Krishnan & Serio, 2005). This in turn leads to mobilization to the prion aggregates of a set of additional non-prion proteins having suitable affinities. In the absence of prions the recipient strain synthesizes all the potentially mobilizable proteins, but they remain soluble and are not recruited to the pellet fraction. Consequently, prion curing (elimination) will lead to the absence of mobilization of these sets of putative prion-associated proteins.

Table 3. Red pigment effect on fluorescence intensity of insuline fibrils stained by Thioflavine T.

№ of experiment	B	P	P/2	P/4	B/P (%)
1	6218	798.5			780
			1342		460
2	3314	315			1050
			462		720
				842	390
3	23769	5027			470
			6582		360
4	23769	3475			680
			4626		510
5	23497	6953			340
			7771		300
6	20612	1647			1250
			2707		760

Confidence limits for a two-sided sign test at 1 % level are 0 and 6. The difference between red pigment and buffer is significant at $p < 0.01$.

We also looked for pellet proteins that are different in white and red strains. 2D electrophoresis and subsequent MALDI analysis permitted identifying 46 proteins in strains AH/B/w, K5-13, and GT26 that were classified as pigment-dependent proteins as they were missing in the corresponding red strains (Nevzglyadova et al., 2009c). No established prions

were identified in this group. (Nevertheless, one of the identified proteins, Sod1p, has been shown to be capable for amyloidization in mammalian cells (Elam et al., 2003; Banci et al., 2008). We cannot exclude the possibility that the same is possible for yeast cells). The remaining pigment-depending proteins seem to be close to the true prion-associated proteins identified by Nevzglyadova et al., 2009a, 2009b. The data on pigment dependent proteins are also shown in Table 4 (column 2).

Taking into account the drop of amyloidity in red strains when compared with the white ones and effect of red pigments on model fibrils, we came to conclusion that the pigment-dependent proteins are really amyloid-dependent. The affinity to amyloid (or to other amyloid-associated proteins) mobilizes them to pellet fraction of crude lysates. Red pigment accumulation impairs association between the proteins identified in our study and amyloids blocking the corresponding sites on amyloid. As a result, the presence of these proteins in pellet fraction depends on accumulation of red pigment and is lower in red cells than in the white ones.

Possibly, the red pigment binding to amyloid fibrils leads indirectly to a decrease of amyloid levels, via interfering with prion polymer contacts with chaperones effecting generation of seeds (propagons) needed for prion spreading (Chernoff et al., 1995; Jung et al., 2000; Jones, Masison, 2003; Kryndushkin et al., 2003; Song et al., 2005; Rikhvanov et al., 2007). Indeed, we have demonstrated that the presence of 8 chaperones and co-chaperones in the pellet fraction was inhibited by red pigment (Table 4, for more details, see Nevzglyadova et al., 2009c). This seems to confirm our hypothesis. At the same time we must add that red pigment does not block propagation of at least some prions as the red strains still contain [*PSI*⁺]-factor. (Possibly this is due to compartmentalization of most of the red pigment to vacuoles, leaving only a trace of the pigment to affect prion amyloids.)

In all three groups: prion dependent, red pigment dependent and prion + pigment dependent proteins—nearly one half of the identified ones belonged to a functional group of chaperones and enzymes of glucose metabolism. Also notable were proteins involved in oxidative stress response, in protein degradation and translation.

Recently several laboratories have characterized many proteins associated with purified aggregates containing a [*PSI*⁺] prion (Bagriantsev et al., 2008) or a prion-related polyglutamine domain of huntingtin exon 1, cloned in yeast (Wang et al., 2007, Wang et al., 2008).

In these studies, like in our work, all the characterized sets of proteins contain numerous chaperones and co-chaperones. Demonstrating many chaperones, our data are compatible with the results by Bagriantsev et al. (2008). But if Bagriantsev et al. restricted their study to description of the abundant chaperone molecules, Wang et al. (2007, 2008) documented that the studied polyglutamine aggregates (like our pellet proteins) also contain a surprising number of proteins participating in glucose metabolism, including Adh1p, Fba1p and Eno2p. These rather impressive similarities between the results of Wang et al. and ours allow concluding the isolated proteins to be derived most likely from similar molecular complexes, either aggresomes (Wang et al., 2008) or some other aggregates involved in amyloid/abnormal polypeptide metabolism.

Table 4. Comparison of misfolding-dependent aggregate proteins identified in different studies.

Chaperones						
Eno2p	Eno2p	Eno2p	Eno2p	Eno2p	Eno2p	---
--	--	Hsp104p	--	--	--	Hsp104p
Ssa1p	Ssa1p	Ssa1p	Ssa1p	--	Ssa1p	Ssa1p
Ssa2p	Ssa2p	--	Ssa2p	--	--	Ssa2p
Ssb1p	--	Ssb1p	Ssb1p	--	Ssb1p	Ssb1p
Ssb2p	--	--	--	--	--	--
--	--	Ssc1p	--	Ssc1p	Ssc1p	--
Sse1p	--	Sse1p	--	Sse1p	Sse1p	Sse1p
--	Cdc48p ^{&}	--	--	Cdc48p	--	--
--	--	--	Sis1p	Sis1p	--	Sis1p
--	--	--	Sgt2p	Sgt2p	--	
--	--	--	--	Rgt3p	--	
--	--	--	--	Dsk2p	--	
Cpr1p	--	--	--	--	--	
--	Cpr3p	--	--	--	--	
--	Hsc82p ^{+,&}	--	--	--	--	
--	--	--	--	Hsp82p	--	
--	Hsp78p	Hsp78p	--	--	--	
Hsp60p	--	--	--	Hsp60p	Hsp60p	
--	--	Hsp42p	Hsp42p	Hsp42p		
--	Hsp26p	Hsp26p				
Sec53p [*]	--	Sec53p [*]				
Fpr1p [*]						
Tsa1p						
10: 53 = 19%	7.5:48 =16%	9.5: 46= 21%	6.5:16 =40%	10.5:25 =42%	5.5: 15 =37%	100%
Prion+ Pigment Dependent ¹	Red Pigment Dependent ²	Prion Dependent ³	103Q ⁴	103QP ⁵ (aggresome)	Carb. ⁶	Bagr. Chaperones ⁷
Glycolysis, TCA						
Tdh3p	Tdh3p	Tdh3p	Tdh3p	Tdh3p	Tdh3p	
Eno2p	Eno2p	Eno2p	Eno2p	Eno2p	Eno2p	
Pgk1p	Pgk1p	Pgk1p	Pgkp72	Pgk72p	--	
Tpi1p	Tpi1p	Tpi1p	--	--	Tpi1p	
Fba1p	Fba1p	Fba1p	Fba1p	Fba1p	Fba1p	
Adh1	Adh1	Adh1p	Adh1p	Adh1p	Adh1p	
Adh4	--	--	--	--	--	
Pdc1p	--	Pdc1p	--	--	Pdc1p	
Ald4p	Ald4p	Ald4p				
Cdc19p	Cdc19p	Cdc19p				

[illegible]

8.5:53 =16%	7.5:48 =16%	7.5:46 =16%	?	?	?
Prion+ Pigment Dependent	Red Pigment Dependent	Prion Dependent	103Q	103QP	Carb.
Translation					
--	Efb1p	--	--	--	--
--	--	--	--	--	EF1alpha
Tef1p	--	--	--	--	Tef1p
Tef2p?	Tef2p	--	--	--	--
Pab1p	--	--	Pab1p	--	--
--	--	--	Rpl2Ap or Rpl2Bp	--	--
Rpl4Ap	--	--	Rpl4Ap	--	--
--	--	--	Rpl8Bp	--	--
--	--	--	Rpl9Ap	--	--
--	--	--	Rpl10p	--	--
--	--	--	Rps6Bp	--	--
--	--	--	Grs1p	--	--
--	--	--	--	--	Nog2p
Tuf1p	Tuf1p	--	--	--	--
--	Asc1p	--	--	--	--
Tif35p	--	--	--	--	--
Prion+ Pigment Dependent	Red Pigment Dependent	--	Prion Dependent	--	103QP
Others					
--	--	Rnq1p Prion	--	--	--
--	--	--	YPR154wp Prion?	--	--
--	--	--	Ynl208wp Prion?	--	--
--	--	Pep4p ^{&}	--	--	--
Ilv5p	--	Ilv5p	--	Ilv5p	--
Leu1p	--	--	--	--	--
--	--	Leu4p	--	--	--
--	--	Lpd1p	--	--	--
--	--	Cus1p	--	--	--
--	--	Msc1p	--	--	--
--	--	Qcr2p	--	--	--
Qcr7p	--	Qcr7p	--	--	--
Por1p	Por1p	Por1p	--	--	--
Ach1p	Ach1p	--	--	--	--
--	Ura1p	--	--	--	--
--	--	--	Bmh1p	Bmh1p	--
--	--	--	--	Mdr1p	--
--	--	--	--	Nsp1p	--
--	Atp1p	--	--	--	--

	Atp2p	--	--	Atp2p	Atp2p
--	--	--	--	Ptk1p	--
--	--	--	--	Ygr192cp	--
--	--	--	Ybr011cp	--	--
--	--	--	Yol162wp	--	--
--	--	--	--	--	Met6p
--	--	--	--	--	Tkl1p
Shm1,2p	Shm1,2p	--			
--	Ura1p	--			
--	Ipp1p				
Sam2p	Sam2p	--			
--	Fsh1p	--			
--	Gdh1p	--			
--	Rgi2p	--			
Tub2p					
Gsp1p					
Apa2p					
Cyb2p					
Cox4p					
Gor1p					
Fmp12p					
Ynl010wp					
Prion+ Pigment Dependent	Red Pigment Dependent	Prion Dependent	103Q	103QP	Carb.

+ closely related to Hsp82

++ involved in stress resistance

* involved in protein folding

& involved in protein degradation

Prion+ Pigment Dependent¹—proteins whose presence in aggregates depends on prion status of yeast cells and/or red pigment accumulation. Data of this study (see Table 1).

Red Pigment Dependent²—proteins whose presence in aggregates depends on red pigment accumulation. Data by Nevzglyadova et al. (2009b).

Prion Dependent³—proteins whose presence in aggregates depends on prion status of yeast cells. Data by Nevzglyadova et al. (2009, 2009a).

103Q⁴—proteins of aggregates dependent on polyglutamine (huntingtin 1st exon) amyloids. Data by Wang et al. (2007, 2008).

103QP⁵ (aggresome)—aggresome proteins. Data by Wang et al. (2008).

Carb.⁶—carbonylated proteins associated with Hsp104-containing protein aggregates. Data by Erjavec et al. (2007).

Bagr. Chaperones⁷—proteins of purified [PSI⁺] containing aggregates. Data by Bagriantsev et al. (2008). Authors relate only data concerning of major constituents of the aggregates—chaperones.

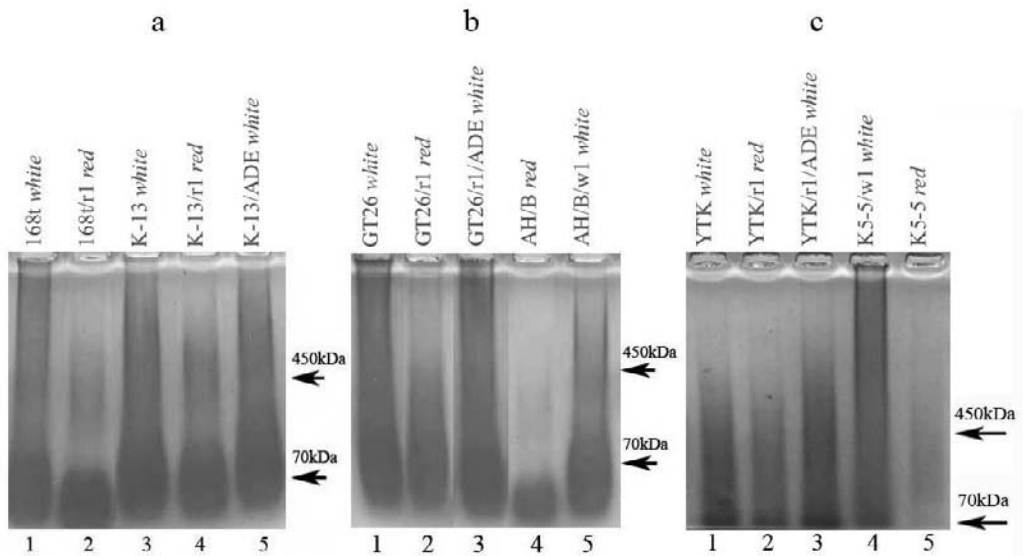


Figure 3. 1.8% agarose gel electrophoresis patterns of pellet proteins from crude lysates of isogenic white and red strains.

Wang et al. (2008) explain the presence of glycolytic enzymes in polyglutamine aggregates either by their sensitivity to oxidation and propensity for aggregation or by association of glycolytic enzymes with huntingtin that was established in mammalian cells. Our results seem to favor rather strongly the first explanation, as we isolated the glycolytic enzymes in experiments that did not include cloning huntingtin coding exons.

In this connection, important seem to be data by Erjavec et al. (2007) who have demonstrated that a subset of glycolysis-related proteins was oxidatively damaged (carbonylated) during replicative aging. This was also the case for several chaperones, but not true for a control group of other abundant cell proteins. The carbonylated proteins were found to be associated with Hsp104-containing protein aggregates (and were shown to be retained in aggresome like manner in the progenitor cell during cytokinesis). The data by Erjavec et al. (2007) certainly provide evidence against involvement of glycolytic proteins in the pellet fraction solely due to their abundance in cells. It is quite remarkable that out of 6 carbonylated glucose metabolism proteins of Erjavec's list, all were also identified in our study (Eno2p, Pdc1p, Fba1p, Adh1p, Tdh3, Tpi1p). In this connection it is also possibly worth repeating that several proteins involved in oxidative response were found in our prionotropic treatment dependent pellet protein preparations: Sod2p, Yhb1p, Act1p, Tma19p, Tdh2p, Tdh3p. We would like to add that Ssa1p, Ssb1p, Ssc1p, Sse1p and Act1p identified in our experiments appear in the short list of 5 carbonylated heat-shock and 4 other proteins by Erjavec. The data by Erjavec are clearly related to aggresome concept. Also Cdc48p, the key component of aggresome machinery (Wang et al., 2008), was identified in our studies as red-pigment dependent protein.

As a result of the recent studies, it is tempting to divide proteins found in all these studies (1) to proteins, like prions and possibly oxidatively damaged glucose metabolism enzymes,

that are subject of some manipulations destined to disintegrate or neutralize misfolded proteins and (2) to other proteins that serve as instruments of these manipulations, like chaperones, other proteins affecting protein folding, like Sec53p, and proteins involved in protein degradation. (Such a classification is most likely an oversimplification. We know that Eno2p is both an enzyme of glucose metabolism and a co-chaperon. Rikhvanov et al. (2007) suggested a fascinating possibility that prions have turned the chaperon machinery destined to protect cells from aggregated proteins into a tool for propagation of prion aggregates. In view of all this, we prefer to suggest that the border between active and passive participants of prion-containing aggregates is rather arbitrary and that many of the seemingly passive proteins of the aggregates can play a functionally important role in certain situations. Nevertheless the classification seems to be helpful for systematization of the variety of proteins in aggregates.)

We suggest that many proteins playing role in attempts of refolding amyloids and damaged polypeptides, then of destroying the remaining ones, eventually, when all these attempts fail, are themselves mobilized to aggresomes together with their clients. Indeed, our data show the presence in psi-dependent aggregates of chaperones belonging to different families and of Sec53p and Fpr1p implicated in protein folding. Various groups identified several proteins involved in protein degradation, like Cdc48p and Hsc82p-Hsp82p (that seem to be among the most remarkable proteins identified in our studies). Also a *sensu stricto* proteinase Pep4p was identified in our experiments. It remains unclear, if Pep4p is a representative of proteinases and its discovery suggests that more are still to be discovered, or is Pep4p an isolated special case. What is possibly important in the case of Pep4p is that it is related to a non-amyloid [*beta*] prion of yeast (Roberts, Wickner, 2003; Wickner et al., 2006). Vacuolar protease A encoded by *PEP4* is involved in proteolytic activation of protease B. In deletion mutants *pep4*, protease B can be activated autocatalytically. During growth on nonfermentable carbon sources when gene coding protease B (Prb1p) is derepressed, such self-activation can proceed indefinitely. Our result of finding Pep4p among amyloid-associated proteins suggests that amyloid and non-amyloid prions can share some prion associated proteins and hence be more than just formally connected. Also worth of mentioning seems to be the fact that vacuole is the receptacle of red pigment that according to our data presented here is functionally related to prion life cycle.

Interesting is the presence of oxidative stress response proteins Sod1p, Sod2p, Tma19p, Yhb1p, Act1p, Tsa1p, Pst2p and others among the identified prion-associated pellet proteins. Also one can note that Tdh2p and Tdh3p are involved in Reactive Oxygen Species (ROS) metabolism and Zeo1p, Psa1p, Gvp36p and some other proteins seem to be implicated in stress response (Table1). This agrees with results of recent studies showing a significant overlapping between prion effects and oxidative stress (see Cashikar et al., 2005; Erjavec et al., 2007; Herczenik & Gebbink, 2008; Sitia & Molteni, 2004; Watt et al., 2005). Finding Sod2p also reminds that a not infectious age-related degenerative disease—amyotrophic multiple sclerosis—involves accumulation of aggregated superoxide dismutase of *SOD1* with amyloid-like structure (Banci et al., 2008; Elam et al., 2003, see also Glabe, 2008). This raises an intriguing possibility that Sod1p and may be also Sod2p are involved in some way in amyloid production in yeast cells. Regardless of the issue of their group loyalty, the superoxide dismutases are among most interesting proteins found in our study and described

in this chapter. Even if not amyloid-producing themselves, these proteins demonstrate that at least some of the prion-dependent proteins found in our study are oxygen stress related. Other authors cited in Table 4 have managed to identify only Tdh3p and Act1p (maybe also Tkl1p) belonging to the promising group of proteins. Possibly this demonstrates some unique possibilities provided by our approach.

There is a unique protein found in our work. Cus1p, having monomer with M.W. around 50kDa, apparently moved in SDS-PAGE at the 205 kDa level (Nevzglyadova et al., 2009a, 2009b). This reminds of behavior of prefibrillar oligomers described by (Glabe, 2008). The possibility of amyloidization of Cus1p and Sod1/2p in yeast cells seems to deserve a further study. It is an especially intriguing possibility as Cus1p, and Sod1/2p, are not rich in asparagine and glutamine (NQ), when all so far identified yeast amyloid prions contain NQ-rich sequences. Nevertheless, there seems to exist a NQ independent prionization pathway in yeast. Taneja et al. (2007) have demonstrated that a not NQ-rich prion domain of *Podospora* HET-s fused to GFP propagates as a prion in yeast cells. If Cus1p and Sod1/2p be indeed established as prions, they will be the first not asparagine- and glutamine-rich native prions of yeast.

Also we have found in our experiments Pab1p, a poly(A)-binding protein that is characteristic of stress granules (Buchan et al. 2008). This can argue in favor that one more form of protein aggregates—stress granules—are possibly finding their way to aggresomes. There are alternative possibilities, as, according to published data, Pab1p is able to interact with Sup35p (the *Saccharomyces* Genome Database data, discussed by Nevzglyadova et al., 2009a, 2009b).

Finding Pab1p and Pep4p suggests some not yet investigated connections between different aggregates and groups of proteins.

We would like to add here that several years ago we (Nevzglyadova et al., 2004) demonstrated that nuclear transmission during budding of yeast heterocaryons changes drastically after treatment of parental strains with GuHCl. The transmission was related to apoptotic death of one of the nuclei. This led us to tentative conclusion (Nevzglyadova et al., 2004) about a link between apoptosis and prionization in yeast cells. Recent data show (Wang et al., 2008) that aggresomes, the last receptacle of yeast prions in aged mother cells is connected with spindle pole body, controlling the nuclear distribution in heterokaryon. Also, one of apoptosis related proteins—Mca1p—was shown to be able of prionization (Nemecek et al., 2009). So it seems to be worth of mentioning that one of the proteins identified in this study (Cit1p) is (according to the *Saccharomyces* Genome database data discussed in Nevzglyadova et al., 2009a, 2009b) able to interact with Mca1p.

All this indicates that the list of yeast prions and their roles in cells is open to serious revisions. This statement was recently confirmed by the discovery of prionization of yeast global transcriptional co-repressor protein Cyc8p (Patel et al., 2009). We would like to add that interaction pattern of this prion puts it together with another prion Swi1p involved in transcriptional regulation, and the Cus1p protein identified in our study. Being able to interact also with Sup35p, Cus1p seems to be unically prionophilic, and as mentioned above, possibly prionizable itself.

The aggregates identified by us and by other authors are remarkably similar. All of them contain numerous chaperones, main cellular instruments aimed at misfolded proteins

refolding (though turned by prions into a tool for their own propagation (Rikhvanov et al., 2007)). Also they contain a special group of glucose metabolism enzymes, that are often damaged and carbonylated (marked for proteolytic degradation and/or being retained in mother cells). So we can say, that the aggregates, analyzed by various authors all contain both the main executors and the major clients of cellular protein quality control system. Our studies add to these groups some important ones—oxidative stress proteins and enzymes involved in protein degradation. The similarity of data of different studies clearly hints at multiplicity of overlapping flexible pathways leading to protein aggregation in response to oxidative stress and to protein misfolding (including the amyloid presence).

Conclusion

We would like to conclude that our methodical approach based on study of pellet protein constitution of strains that undergo different prionotropic treatments (“genetic purification”) seems to reveal the same core prion-associated proteins as other approaches more strictly based on biochemical techniques, such as the prion aggregate purification and protein carbonylation studies. At the same time, our approach is less restrictive and permits identification of a variety of unique additional proteins, like oxidative stress response proteins, Sec53p affecting protein folding, protease Pep4p—all likely to be responding to signals created by protein damage and misfolding. Since use of red pigment reveals that essentially the same proteins that were shown to be prion-associated also responded in the pigment dependent manner, our approach can be used for studying untraditional factors affecting amyloidization and hence seems to complement more conventional protocols (Kushnirov et al., 2006; Wang et al., 2007, Wang et al., 2008; Bagriantsev et al., 2008). Red pigment accumulating in *ade1* and *ade2* mutants seems to be worth of further study as an agent interfering with amyloid functions, causing an apparent drop of amyloidity.

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Chapter VII

Structure and Multiple Functions of Cyclophilin 40: A Divergent Loop Cyclophilin

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Abstract

The divergent loop cyclophilin, cyclophilin 40 (CyP40), initially discovered in association with the estrogen receptor, is now recognized as an immunophilin cochaperone common to all steroid receptors. This unique peptidylprolyl isomerase, the first tetratricopeptide repeat (TPR) cyclophilin to be identified, contains a C-terminal TPR domain through which it shares structural identity with FKBP52 and other partner cochaperones in steroid receptor-Hsp90 complexes. By dynamically competing for Hsp90 interaction, the cochaperones allow the receptors to establish distinct Hsp90-chaperone complexes, with the potential to exert tissue-specific control over receptor activity. CyP40 regulates Hsp90 ATPase activity during receptor-Hsp90 assembly. Functional deletion of the yeast CyP40 homologue, Cpr7, adversely affected glucocorticoid receptor and estrogen receptor α activity that could be fully restored, either with wild type Cpr7 or Cpr7 with a cyclophilin domain lacking isomerase activity. We draw parallels with the mechanism already established for FKBP52 and propose that the CyP40 divergent loop, within the N-terminal cyclophilin domain, interfaces with a contact surface on the steroid receptor ligand-binding domain to achieve an optimal orientation for receptor activity. The cyclophilin domain also mediates association with

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dynein, suggesting a role for CyP40 in nuclear translocation of steroid receptor-Hsp90 complexes from the cytoplasm, as proposed for other immunophilin cochaperones. CyP40 chaperone function has been mapped to a central linker region separating the cyclophilin and TPR domains. Although not essential for viability, CyP40 homologues are important for normal cell growth and mitosis in yeast and in progression of vegetative growth in plants. These may be linked to CyP40 client proteins other than steroid receptors, including diverse kinases and transcription factors. The recent development of CyP40 knockout mouse models provides an attractive opportunity to address fundamental questions regarding the physiological role of CyP40 in mammals.

Keywords: Cyclophilin 40; Hsp90; steroid receptors; immunophilin cochaperones; steroid hormone action.

Introduction

Cyclophilin 40 (CyP40) belongs to the widespread cyclophilin family of peptidylprolyl isomerases that have the capacity to catalyze the *cis-trans* isomerization of peptide-proline bonds, an activity that may be central to the numerous roles ascribed to cyclophilins in biological processes that include protein folding, chaperone activity, signaling, mitochondrial function, the stress response, gene expression and regulation of kinase activity (Galat 2003; Wang and Heitman 2005). By targeting the active site, cyclosporin A universally blocks cyclophilin isomerase activity, and its specific interaction with the prototypical cyclophilin, CyP18, yields a heteromeric complex that binds and inhibits the phosphatase, calcineurin, forming the basis of immunosuppression by the drug (Galat 2003; Wang and Heitman 2005). Since its discovery in association with the estrogen receptor in 1990 (Ratajczak et al., 1990), CyP40 has become recognized as an immunophilin cochaperone common to all steroid receptors, with the potential to exert tissue-specific influences over receptor function. This review provides a synopsis of what we currently know about this unique immunophilin, the first tetratricopeptide repeat (TPR)-containing cyclophilin to be identified.

Development of an estrogen-liganded affinity gel with low non-specific protein interaction properties, allowed our laboratory to use low salt buffers in a single step purification of the intact estrogen receptor α (ER α)-Hsp90 heterocomplex, together with associated cochaperone proteins, from bovine uterine cytosol. At this stage, in the late 1980s to early 1990s, the concept of dynamic assembly of steroid receptors with Hsp90 chaperone machinery was beginning to evolve from the Toft, Pratt and Smith laboratories (Smith 1993; Smith et al., 1990) (reviewed in Pratt and Toft 1997). Proteins that we recovered with purified bovine ER α , in addition to Hsp90, included CyP40 and p23, as well as FKBP52 and Hsp70 (Ratajczak et al., 1993; Ratajczak et al., 1990). In parallel, work from the Handschumacher laboratory revealed the isolation of a 40-kDa cyclophilin-related protein from calf brain using cyclosporin A affinity chromatography (Kieffer et al., 1992). These latter studies together led to the cloning of the cDNA of bovine (Ratajczak et al., 1993) and human (Kieffer et al., 1993) CyP40, respectively.

hCyP18	-----MVNPTVFDDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGF- 47
bCyP40	MSHPSPQAKPSNPSNPRVFFDVGGERVGRIVLELFADIVPKTAENFRALCTGEKGIGP 60
hCyP40	MSHPSPQAKPSNPSNPRVFFDVGGERVGRIVLELFADIVPKTAENFRALCTGEKGIGH 60
SpCyP5	-----MSTYAYFKISIDGKIQPTIYFELFDNVVPKTVKNFASLCNGFEKDG- 46
ScCpr6	-----MTRPKTFDDISIGGKPGGRIVFELYNDIVPKTAENFLKLCENAGMAK 48
ScCpr7	-----MIQDPLVYLDISIDKKPIGRIVCKLFREKAPKTTFNYFKLCAGDVKSPL 49
AtSQN	-----MGRSKCFMDSIGGELEGRIVIELYDDVVPKTAENFRLLCTGEKGLGP 48
	* * * * * * * * * *
hCyP18	-----YKGSCHFRIIPGFMCGGDFTRHN-----GTGGKSIYGEK----- 82
bCyP40	TT-GKPLH FKGCPFHRIIKKFMIQGGDFSNQN-----GTGGESIYGEK----- 102
hCyP40	TT-GKPLH FKGCPFHRIIKKFMIQGGDFSNQN-----GTGGESIYGEK----- 102
SpCyP5	-----RCLTYKGSRFHRVIKNFMLQGGDFTRGN-----GTGGESIYGEK----- 85
ScCpr6	TKPDVPLS YKGSIFHRVIKDFMCQFGDFTNFN-----GTGGESIYDEK----- 91
ScCpr7	KD-QQYLS YKNGGFHRVKNFMIOAGDIVFGTKDSSSSSVSGGCSIYADKEEVKTDE 108
AtSQN	NT-GVPLHY KGNRFHRVIKGFMIQGGDISAND-----GTGGESIYGLK----- 90
	* * * * * * * * * *
hCyP18	-----FEDENFILKHTGPGILSMANAG-PNTNGSQFFICTAKTEWLDGKHVVFGKVEK 135
bCyP40	-----FEDENFHYKHKDREGLLSMANAG-SNTNGSQFFITVPTPHLDGKHVVFGQVIK 155
hCyP40	-----FEDENFHYKHKDREGLLSMANAG-RNTNGSQFFITVPTPHLDGKHVVFGQVIK 155
SpCyP5	-----FEDENFELKHKDPFLLSMANAG-PNTNGSQFFITVPTPHLDGKHVVFGKVIQ 138
ScCpr6	-----FEDENFTVKHKDPFLLSMANAG-PNTNGSQAFITCVPTPHLDGKHVVFGQVIQ 144
ScCpr7	SFCYGNFEDENLG-EFVEPFTLGMANLGSPTNNNSQFFITTYAAPHNGKHSIFQGVVHG 167
AtSQN	-----PDENFELKHKRKMGLSMANSNG-PNTNGSQFFITTTRTSLDGHKVVFGVRVK 143
	* * * * * * * * * *
hCyP18	MNIVEAMER--FGSRNGKTSKKITIADCGGLE----- 165
bCyP40	MGVAKILEN--VEVKGEKPAKLCVIAECGELKEGDDWGIFFKDGSGDSHPD-----FPED 208
hCyP40	IGVARILEN--VEVKGEKPAKLCVIAECGELKEGDDGGIFFKDGSGDSHPD-----FPED 208
SpCyP5	KSTVRTIEN--LETKNDDPVVPVIEECGTCTKDQIEAPKPDVTDGSLEEFF-----D 189
ScCpr6	KRIVRLIENQQCDQENNKPLRDVKIDDGVLDDYQVPEAEATPTDEYGDNYEDVLKQD 204
ScCpr7	KSVVRTIENCRVDSG-VPESDVRISDCGVWEKTMG-VPLYNASNDQIGGDVYEEYPPDD 225
AtSQN	MGVVRISIEHVSIEEQS-CPSQDVVIDCGEIEPEG---ADDGICDFFKDGDVYPDWP-ID 197
	* * * * * * * * * *
hCyP18	-----ADVDLKDVKDKILLISEDKNIGNTFFKSQNWEMAIKKYTKVLRVYEG--SRAAEADAG- 265
bCyP40	ADIDLKDVKDKILLITEDLKNIGNTFFKSQNWEMAIKKYAEVLRVYDS--SKAVIETADR- 265
hCyP40	DYEGDKSETAIFKIASDLKGIANKQFAQQNLDTAVAKWQKALRYLMEYVVPNDSSKESPD 249
SpCyP5	EKVDLKNFDTVLKAIETVKNIGTEQFKKQNSVALEKYVKCDKFLKEY-FPEDLEKEQI- 262
ScCpr6	THFGDDDFGKALEAANI IKESGTLFLFKKDYDNAFFKYRKSILNYINEYMPEDVDKERN- 284
ScCpr7	LNESPAELSWMMETVDFVKAHGNHFKKQDYKMAIRKRYRKALRYLDICWEKEGIDEETS- 256
AtSQN	-----
hCyP18	-----AKLQPVALS CVLNI GACKLKMSDWQGA VDSCLEALEIDPS-----NTKALYRRAQGWQG 319
bCyP40	-----AKLQPVALS CVLNI GACKLKMSDWQGA VDSCLEALEIDPS-----NTKALYRRAQGWQG 319
hCyP40	-----AKLQPVALS CVLNI GACKLKMSDWQGA VDSCLEALEIDPS-----NTKALYRRAQGWQG 319
SpCyP5	FWKEYNALRYSIYANLALVALKQNKQAEAIRNANIVIEASNSTELEKQKAYRLGCAQGL 309
ScCpr6	--EKINQLKVSIPLNTAICALKLKDYKQVLVASSEVLYAADEAKAKALRYRLGLAYH 320
ScCpr7	--IQFINLKMKIYLNLSLVLFNLERYDDAIMYATYLLEMDNVNPRDQAKAYYRRGNSYLK 342
AtSQN	--TALRKTQSQIFTNAAACKLFGDAKGALLDTEFAMRDEN-----NVKALFRQQAQYMA 310
hCyP18	-----LKEYDQALADLKKAEIAPEDKAIQAELLKVQKIKAKQDKKEKAAAYAKMFA 370
bCyP40	-----LKEYDQALADLKKAEIAPEDKAIQAELLKVQKIKAKQDKKEKAVYAKMFA 370
hCyP40	-----LKEYDQALADLKKAEIAPEDKAIQAELLKVQKIKAKQDKKEKAVYAKMFA 370
SpCyP5	LKNFESEKALAKAG---NDPAISKLAIEIRQKKKDYKKRQKQKAYAKMFQ 356
ScCpr6	VNDTDMALNDLEMATTFQPNDAAILKAIHNTKLKRKQNEKAKKSLSKMFS 371
ScCpr7	KKRLDEALQDYIFCKEKNPDDEVIEQRIEYVNRILIEENKEKTRKNISKFFS 393
AtSQN	LNNVDAASLEKALQFEPNDAGIKKEYAAVMKKIAFRDNEEKQYRKMFV 361

Figure 1. Alignment of selected CyP40 homologues with CyP18. The sequences of bovine and human cyclophilin 40 (bCyP40 and hCyP40), wis2 from *S. pombe* (SpCyP5), Cpr6 and Cpr7 from *S. cerevisiae* (ScCpr6 and ScCpr7), SQN from *Arabidopsis thaliana* (AtSQN) and human cyclophilin 18 (hCyP18) were aligned using the Clustal W program (Thompson et al., 1994). Features common to CyP40-related proteins are highlighted in bold and include the divergent loop, a glutamate residue important for loop conformation, conserved cysteine residues involved in putative redox signaling or metal co-ordination and a histidine substituted for a tryptophan residue critical for prolyl isomerase activity. The tryptophan and other residues important for cyclophilin catalytic activity are highlighted with a bold asterisk. A high level of conservation is displayed among all of these proteins in their N-terminal cyclophilin domains.

CyP40: A Divergent Loop Cyclophilin

Although highly homologous to CyP18, the amino terminal cyclophilin domain places CyP40 among the ‘divergent loop’ sub-class of cyclophilins since it possesses an 8-residue insert (60PTTGKPLH67) that fits with a consensus ****GK*LH** sequence derived from multiple sequence alignments of mammalian, plant and yeast homologues (Figure 1). Highly conserved features of this cyclophilin subfamily include a glutamate residue that helps determine loop conformation, as well as two proximally located cysteines that may either participate in redox-signalling through disulphide bond formation triggered by oxidative stress or together with the histidine contained within the loop, may have a role in metal co-ordination (Dornan et al., 1999; Dornan et al., 2003; Taylor et al., 2001). Significant differences in loop conformations suggest that the protruding loop structure mediates a distinctive function among cyclophilin homologues that may include recognition of specific protein interaction targets (Dornan et al., 1999; Dornan et al., 2003). Within the N-terminal cyclophilin domain, residues important for peptidylprolyl isomerase activity and cyclosporin A binding are identical in CyP40, except for His141 which, although conserved among CyP40 homologues, remains a tryptophan residue in CyP18 and other cyclophilins (Figure 1), a modification that may account for the decreased catalytic activity and cyclosporin A-binding affinity of CyP40 (Hoffmann et al., 1995; Ratajczak et al., 1995).

CyP40 Structure

The domain architecture of CyP40 is characterized by the cyclophilin domain tethered to a C-terminal, 3-unit TPR domain by a 30-residue linker (Figure 2A). A shared identity between the TPR domains of CyP40 and FK506-binding protein 52 (FKBP52) represents the first significant homology between the cyclosporin A- and FK506-binding class of immunophilins (Kieffer et al., 1993; Ratajczak et al., 1993) and this conserved region allows both immunophilins to compete together and with other TPR-containing cochaperones for binding to Hsp90 (Ratajczak et al., 2003; Smith 2004). A charged α -helical region flanking the C-terminal end of the TPR domain and incorporating the charge-Y motif (Figure 2A) has a regulating influence over Hsp90 recognition by CyP40 and FKBP51 and FKBP52 (Allan et al., 2006; Cheung-Flynn et al., 2003). The 30-residue linker region (residues 186-215), also contributes to Hsp90 interaction by CyP40 (Ratajczak et al., 1995) and forms a hydrophobic substrate-binding cavity essential for CyP40 chaperone function (Mok et al., 2006) (Figure 2A). In addition to its overlapping roles associated with isomerase activity and cyclosporin A-binding, the CyP40 cyclophilin domain, independently of its catalytic function, links Hsp90-bound substrates, such as steroid receptors, to the cytoplasmic dynein motor protein involved in retrograde movement to the nucleus (Galigniana et al., 2002) (Figure 2A).

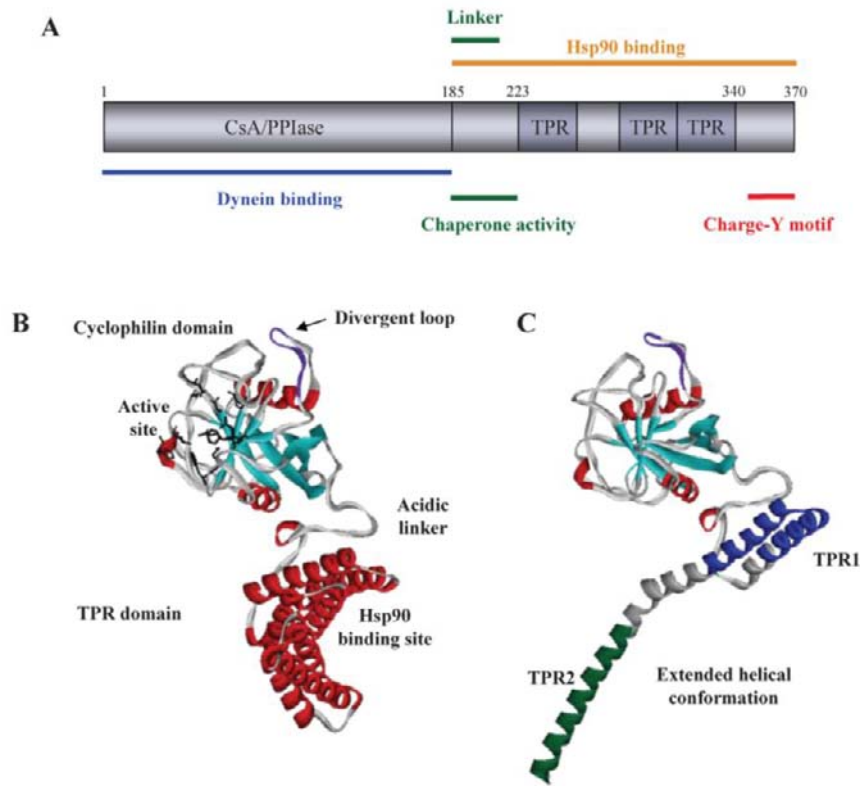


Figure 2. Functional domains and structure of CyP40. (A) The N-terminal half of CyP40 incorporates overlapping domains for cyclosporin A (CsA) binding, peptidylprolyl isomerase (PPIase) catalytic activity and interaction with cytoplasmic dynein and is separated from the TPR domain by a 30-residue linker containing elements essential for CyP40 chaperone function. Hsp90 binding is mediated by the core TPR domain, the linker region and the C-terminal charge-Y motif. (B) Structure of bovine CyP40 showing the divergent loop (purple) and selected active site residues (in black) within the cyclophilin domain, which is separated from the TPR domain by the 30-residue acidic linker (Dornan et al., 2003). The partially resolved final helix contains the conserved charge-Y motif. (C) Structure of CyP40 showing the alternative extended helical fold of the TPR domain. Only the first two units of the TPR domain (TPR1 – dark blue, residues 223-256 and TPR2 – green, residues 273-298) are visible (Taylor et al., 2001).

The X-ray structure of CyP40 (Taylor et al., 2001) clearly shows the additional loop within the cyclophilin domain protruding above the active site (Figure 2B). The structure confirmed a helix-turn-helix motif for each 34-residue repeat of the TPR domain, giving rise to six helices, the first three of which are more elongated than observed for tandem TPR sequences in PP5, a partner Hsp90 cochaperone (Das et al., 1998). An incompletely resolved seventh helix defines part of the C-terminal charge-Y motif. A second crystal form revealed a unique folding pattern of the CyP40 TPR domain in which the two helices of the first TPR motif were followed by a long extended helix (Taylor et al., 2001) (Figure 2C), similar to that seen for the peroxisomal TPR protein PEX5 (Kumar et al., 2001). Although a recent study of CyP40 in solution suggests the partially unfolded TPR domain is likely to be a crystallization artifact (Onuoha et al., 2008), observation of the extended ‘jackknife’ structures as an alternative to canonical antiparallel helix conformations suggests a level of flexibility among

TPR helical domains that may allow a variety of protein interactions with different biological consequences (Dornan et al., 2003; Smith 2004).

Interaction with Hsp90

The MEEVD peptide at the extreme C-terminus of Hsp90 is the common acceptor site for TPR-containing cochaperones, including Hop (Ratajczak et al., 2003; Smith 2004) and may dock in two alternate orientations in the CyP40 TPR domain, one of which corresponds to that defined for Hop-MEEVD interaction (Scheufler et al., 2000; Taylor et al., 2001; Ward et al., 2002). Both binding options maintain key electrostatic interactions that help form separate two-carboxylate clamps, thus revealing a broad specificity for the peptide-binding amphipathic groove (Taylor et al., 2001; Ward et al., 2002). Within this binding groove, five residues critical to CyP40 recognition of Hsp90 through the two-carboxylate clamp anchor were shown to be fully conserved in the TPR domains of PP5 and Hop and four of these residues were conserved in FKBP51 and FKBP52 (Ward et al., 2002). Additional CyP40 residues within the TPR domain contributing to Hsp90 binding efficiency, also displayed a high degree of conservation among the Hsp90 cochaperones.

While the core TPR domain contains residues essential for docking of the Hsp90 MEEVD peptide, high affinity Hsp90 interaction requires involvement of sequences within the 30-residue linker, as well as the C-terminal helix flanking the TPR domain (Ratajczak et al., 1995). Within this final helix, the charge-Y motif and an adjacent upstream element form part of an extended Hsp90-binding interface (Allan et al., 2006). Corresponding C-terminal regions within CyP40 and its partner immunophilins, FKBP51 and FKBP52, may help determine the Hsp90-binding specificities of these cochaperones (Allan et al., 2006; Cheung-Flynn et al., 2003). The CyP40 linker is structured through well-defined hydrogen bonds and is characterized by 11 acidic and 10 hydrophobic residues (Taylor et al., 2001). Mutation of selected acidic amino acids, including those forming salt bridges with specific basic residues within the TPR domain, failed to identify determinants that contribute to Hsp90 interaction (Ward et al., 2002). It is possible that an intact linker region is required to stabilize the TPR domain for enhanced Hsp90 interaction. Alternatively, the hydrophobic substrate cavity, proposed to mediate CyP40 chaperone activity (Mok et al., 2006), may provide a potential site for Hsp90 interaction within the linker, locking in a specific Hsp90 conformation conducive to client protein function.

Although the mammalian Hsp90 α and β isoforms are highly homologous, and equivalently expressed (Jakob and Buchner 1994), isolated chicken steroid receptors and bovine ER α complexes contain Hsp90 α as the preferred chaperone (Binart et al., 1989; Ratajczak et al., 1988). Binding studies of CyP40 with Hsp90 α and β C-terminal segments incorporating the Hsp90 dimerization domain failed to identify an isoform preference (Carrello et al., 1999), suggesting that this might be governed by the receptors themselves. Analysis of interactions between yeast Hsp90 and the yeast cochaperones, Sti1 and Cpr6, corresponding to the homologues of Hop and CyP40, respectively, revealed that two cochaperone molecules bind per Hsp90 dimer, with Sti1 itself existing in the dimeric form (Prodromou et al., 1999). Identical binding stoichiometry has been shown between human

CyP40 and Hsp90 proteins (Pirkel and Buchner 2001). Sti1 inhibits inherent Hsp90 ATPase activity, which is unaffected by Cpr6. However, displacement of Sti1 from Hsp90 by Cpr6 restored ATPase activity, revealing an important regulatory role for TPR cochaperones in Hsp90 chaperoning function. An updated model proposes the presence of two intermediate complexes during the ATPase cycle involving a shift from an open to a closed conformation upon nucleotide binding, resulting in reduced Sti1 binding and enhanced interaction with Sba1, the yeast p23 homologue (Johnson et al., 2007). A conformational change within the N-terminal catalytic loop favors Cpr6 interaction. ATP hydrolysis releases Sba1 and this is followed by additional steps resulting in loss of Cpr6 and Sti1 binding as Hsp90 reverts to the open conformation. Regulation of Cpr6 interaction by Hsp90 N-terminal conformational changes is consistent with previous observations that deletions within the chicken Hsp90 amino terminus and catalytic domain translate into altered interaction patterns with TPR cochaperones (Chen et al., 1998). It is now accepted that the TPR cochaperones make distinct and extensive contacts with Hsp90 that lead to differential modulation of Hsp90 chaperone function (Chen et al., 1998; Cheung-Flynn et al., 2003; Prodromou and Pearl 2003; Ramsey et al., 2000). In this respect, it is of interest that CyP40 and FKBP52, whilst displaying only a 30% homology within their TPR domains, showed similar interaction patterns with Hsp90 deletion mutants, suggesting a possible overlap in how they impact Hsp90 chaperone function (Chen et al., 1998).

A noncontiguous EEVD-like TPR recognition site near the ATP-binding pocket of Hsp90 has recently been identified for GCUNC45, a TPR cochaperone associated with the progesterone receptor (PR) at an early stage of PR-Hsp90 heterocomplex assembly (Chadli et al., 2008). FKBP52 and CyP40 are also able to interact at this site in a manner that is strongly regulated by nucleotide binding and changes in Hsp90 conformation. Client protein chaperoning by Hsp90 might then be coordinated through sequential TPR cochaperone interactions with the N- and C-terminal domains of the Hsp90 chaperone.

Role in Steroid Receptor Function

Although it is generally understood that the immunophilin cochaperones have a selective preference for specific steroid receptors, the presence of these TPR proteins in receptor-Hsp90 complexes at any one time may be influenced by their relative abundance, the dynamics of their exchange and incorporation into receptor complexes and their unique interactions with other components, including the receptors themselves (Ratajczak et al., 2003; Riggs et al., 2004; Smith 2004; Smith and Toft 2008). Results from available animal models argue against redundancy of the immunophilins and support tissue-selective and distinct modes of action for these cochaperones in steroid signaling (Cheung-Flynn et al., 2005; Tranguch et al., 2005; Yang et al., 2006). From long-held dogma, largely based on *in vitro* evidence, it is believed that while Hsp90 molecular chaperone machinery helps to fold the ligand-binding domain (LBD) of steroid receptors in a high affinity hormone binding conformation, the chaperone complex also restricts or represses receptor activity by preventing the conversion of receptor to a DNA-binding state (Picard 2006; Smith and Toft 2008). Our early results with highly purified ER α -Hsp90-CyP40 preparations, in which

receptor activation to the DNA-binding form required either urea- or heat-induced dissociation from Hsp90, were consistent with this model (Ratajczak et al., 1990).

The ability of steroid receptors to interact functionally with yeast Hsp90 facilitated studies demonstrating the requirement of Hsp90 for hormonal signaling through androgen (AR), glucocorticoid (GR) and estrogen receptors (ER α) (Fang et al., 1996; Picard et al., 1990). Subsequently, it was shown that deletion of Cpr7, but not that of its companion CyP40 yeast homologue, Cpr6, compromised the transcriptional activities of both GR and ER α (Duina et al., 1996a; Warth et al., 1997). While GR transcriptional function was partially restored by overexpression of the Cpr7 C-terminal domain that included the linker region thought to contribute significantly to Cpr7's potent chaperone function (Mok et al., 2006), full restoration could be achieved either by wild type Cpr7 or a Cpr7 protein with a PPIase domain disrupted for catalytic activity (Duina et al., 1998b). Although it has yet to be precisely defined, the PPIase domain then might have a conjoint role with the TPR-containing C-terminus in providing Cpr7 with the unique ability to influence Hsp90, and in turn, steroid receptor function. Application of an alternative yeast model which allowed the first demonstration of selective potentiation of GR, but not ER α , by FKBP52, failed to reveal any impact of Cpr7 or human CyP40 overexpression on receptor activity (Riggs et al., 2003). Using the same yeast assay with an *S. cerevisiae* strain deleted in Cpr6 and Cpr7, we found no discernable influences mediated by CyP40 or FKBP52 on the transcriptional activity of either wild type ER α or the ER α G400V mutant (Aumais et al., 1997) known to have a more stable interaction with Hsp90 (Cluning CL, Riggs DL, Smith DF and Ratajczak T, unpublished).

The Pratt laboratory has provided extensive evidence for FKBP52 involvement in hormone-dependent targeting of GR-Hsp90-immunophilin complexes from the cytoplasm to a nuclear location *in vivo*, a role that is mediated by interaction of the FKBP52 PPIase domain with both receptor LBD and the dynein motor protein complex (Galigniana et al., 2001; Harrell et al., 2004). Observations that CyP40 and PP5 are similarly associated with cytoplasmic dynein through their PPIase domains, have led to a model in which the immunophilin cochaperones are proposed to link steroid receptor-Hsp90 complexes to the dynein-dependent retrograde transport system for translocation to the nucleus (Galigniana et al., 2002).

Specific Functions

Although cyclophilins are ubiquitously expressed and highly conserved, suggesting they participate in important and related cellular functions, studies in *S. cerevisiae* have shown that none, including Cpr6 and Cpr7, are essential for viability (Dolinski et al., 1997). Cpr7 however, is required for normal cell growth (Duina et al., 1996b) and appears to be unique in this function since overexpression of either Cpr6 or human CyP40 failed to complement the slow growth phenotype in yeast deleted in this cyclophilin (Dolinski et al., 1998). Wis2, the *S. pombe* CyP40 homologue, efficiently suppresses a cell cycle defect in a mutant strain, suggesting wis2 has a regulatory role in mitosis (Weisman et al., 1996). The effects of both Cpr7 and wis2 are largely conferred by their C-terminal TPR-containing domains, consistent

with their role in modulating Hsp90 function (Dolinski et al., 1998; Duina et al., 1998b; Weisman et al., 1996).

It is of interest that while Cpr6 and Cpr7 bind Hsp90 with similar affinity, Cpr6 displays a higher catalytic activity than Cpr7. However, Cpr7 is the more potent chaperone (Mayr et al., 2000). On the other hand, human CyP40 outperforms both of its yeast homologues as a peptidylprolyl isomerase and has a chaperoning capability similar to Cpr7 (Pirkl and Buchner 2001). CyP40 chaperone function defined within the CyP40 linker is proposed to be mediated by a hydrophobic cavity formed by a linker strand, incorporating hydrophobic residues, closing up against TPR helices (Mok et al., 2006). Between the linker sequences for human and bovine CyP40 and the yeast homologues, Cpr6 and Cpr7, there is a partial conservation of hydrophobic residues forming the cavity and acidic residues proposed to stabilize the cavity structure through salt bridges with specific basic residues within the TPR domain (Mok et al., 2006; Taylor et al., 2001). Since the linker region is a major determinant of Hsp90 binding, the Hsp90 chaperone would appear to be the logical substrate for CyP40 chaperoning activity, although the receptor component within steroid receptor complexes cannot be excluded as a possible chaperone target.

Regulation

Analysis of selected human and rat tissues has determined CyP40 to be ubiquitously expressed (Kieffer et al., 1993; Kieffer et al., 1992). CyP40 is a heat shock protein, as evidenced by increased mRNA and protein turnover rate following heat shock and chemical stress (Mark et al., 2001). The yeast homologues, Cpr6 and wis2, were similarly shown to be heat-responsive, but Cpr7 is constitutively expressed (Dolinski et al., 1998; Warth et al., 1997; Weisman et al., 1996). Putative heat-shock elements in the 5' upstream regions of Cpr6 and wis2 (Warth et al., 1997; Weisman et al., 1996) and within the first intron of human CyP40 (Yokoi et al., 1996), may account for the heat inducibility of these cyclophilin genes. Typical of housekeeping genes, CyP40 lacks a TATA consensus sequence (Yokoi et al., 1996), but its basal expression is largely driven by the Ets-related transcription factor, GA-binding protein (GABP), through tandem elements immediately upstream of the transcription initiation site (Kumar et al., 2001b). The observed up regulation of CyP40 expression by estrogen in MCF-7 breast cancer cells (Kumar et al., 2001a) could not be ascribed functionally to potential estrogen responsive elements identified within the CyP40 promoter. Rather, the estrogenic influence is indirect and appears to result from mitogenic signals integrated through GABP, a key regulator of CyP40 expression (P Kumar, PhD thesis, 2001).

Cyp40 and Cancer

Although the TPR-containing immunophilins are ubiquitously expressed, changes in their protein levels leading to alterations in the balance of the cochaperones available for incorporation into steroid receptor complexes could impact on receptor function. In this regard, hormone-dependent cancers of the breast and prostate, in which ER α and AR are the principal targets of anti-hormonal therapies, are of paramount interest. We have shown that

CyP40 and FKBP52 expression is upregulated in breast cancer compared to normal tissue (Ward et al., 1999). A contrasting pattern of protein expression was seen for these two immunophilins in a panel of breast cancer cell lines, with FKBP52 being more highly variable and more highly expressed (by as much as 40-fold) than CyP40 (Ward et al., 1999). The presence of ER α was strongly correlated with higher levels of FKBP52 in the cell lines. A recent study by the Sanchez group has confirmed CyP40 as a component of AR protein complexes in LNCaP prostate cancer cells and revealed a substantial increase in immunophilin cochaperone protein levels, including CyP40, in prostate cancer cell lines compared to normal human prostate epithelial cells (Periyasamy et al., 2007). Peroxisome proliferator-activated receptor α (PPAR α), a nuclear receptor that mediates the carcinogenic effects of peroxisome proliferators in mice (Heuvel 1999), is also known to be associated with Hsp90, which has an inhibitory role in PPAR α activity (Sumanasekera et al., 2003). Peroxisome proliferator-induced liver tumours showed a dramatic increase in CyP40 protein expression, suggesting a link between peroxisome proliferator-induced growth and a possible mitogenic role for CyP40 (Miller et al., 2000), the latter being consistent with evidence of CyP40 involvement in c-Myb growth regulatory pathways (Leversson and Ness 1998).

The CyP40 gene has been mapped to 4q31.3 (Ratajczak et al., 1997), within a region (4q25-q34) previously demonstrating loss of heterozygosity (LOH) in cases of advanced breast cancer (O'Connell et al., 1994). A dinucleotide repeat polymorphic marker detected in the 5' flanking region of the CyP40 gene allowed allelic loss at the CyP40 locus to be determined in a cohort of breast cancer patients (Ward et al., 2001). Deletions in the region of the CyP40 gene are likely to be a late event in breast cancer progression. The possibility exists that genetic loss of CyP40 might compromise estrogen receptor function by altering immunophilin composition in receptor complexes, impacting antiestrogen resistance and resulting in phenotypic changes in breast cancer.

Other Interactors

Similar to other single TPR domain-containing cochaperones, CHIP (Connell et al., 2001) and α SGT (Angeletti et al., 2002), CyP40 binds both Hsp90 and Hsp70 through the common EEVD TPR recognition motif located at the C-terminus of the two major chaperones, but displays a preference for Hsp90 (Carrello et al., 2004). While CyP40 differs from CHIP and α SGT by not having a direct influence on Hsp70 ATPase activity, a modulating role for CyP40 in Hsp70 function, whereby it competes with other TPR-containing regulatory proteins, cannot be discounted. Cpr7 binds directly to Hsp104, a molecular chaperone conferring stress-induced tolerance in yeast (Abbas-Terki et al., 2001). Hsp90 competitively inhibits the interaction *in vitro* and may predominate over Hsp104 in Cpr7 complexes under normal metabolic conditions. A yeast two-hybrid screen identified both Cpr6 and Cpr7 as interaction targets of the yeast histone deacetylase, Rpd3, suggesting an involvement of these cyclophilins in the function of this global transcriptional regulator (Duina et al., 1996b). Only Cpr7 and not Cpr6 was found to be present in Hsp90 complexes with the yeast TPR-containing Sti/Hop homologue, Cns1 (Dolinski et al., 1998). Although Cns1 association with Hsp90 does not require Cpr7 (Marsh et al., 1998), observations that

cyclosporin A precludes Cpr7-Cns1 interaction and not the formation of Hsp90-Cpr7 complexes, suggests that Cpr7 binds Cns1 directly through its cyclophilin domain (Dolinski et al., 1998). The viability of yeast with impaired Cns1 function is dependent on both the isomerase and TPR domains of Cpr7, but not its catalytic activity (Tesic et al., 2003).

Other Substrates

While we are beginning to understand the functional significance of some of the preferred associations seen for steroid receptors with specific components of the Hsp90-Hsp70 assembly pathway, a variety of regulatory proteins, including diverse kinases, receptors and transcription factors appear to follow the same paradigm as client substrates of Hsp90 chaperone machinery (Nair et al., 1996). CyP40 cyclophilins have been shown to interact functionally with Hsp90-dependent kinases, with Cpr7 deletion in yeast resulting in loss of v-Src-mediated tyrosine kinase activity (Duina et al., 1996a), as well as causing defective mitogen-activated protein (MAP) kinase signaling (Lee et al., 2004). CyP40 (and FKBP52) has also been detected with selected kinases in separate Hsp90 heterocomplexes assembled with Cdc37 or the Cdc37-related cochaperone, Harc (Hartson et al., 2000; Nair et al., 1996; Scholz et al., 2001). Cpr7 has a major role in suppressing the heat shock response in yeast and acts together with Hsp90 to negatively regulate the activity of the heat shock transcription factor, HSF1 (Duina et al., 1998a). *In vitro* assembly of recombinant HSF1 revealed as components Hsp90 and TPR chaperone components analogous to those for steroid receptors, including CyP40 (Nair et al., 1996). Mutant p53 was found to be similarly associated with Hsp90 chaperone machinery in cell lysates (Whitesell et al., 1998). As previously demonstrated for GR (Galigniana et al., 2001), there is evidence that CyP40 and other TPR-containing immunophilins, such as FKBP52, may function through their PPIase domain to link Hsp90-p53 to the dynein motor complex involved in p53 cytoplasmic-nuclear translocation (Galigniana et al., 2004). It is intriguing that both CyP40 and FKBP52, in addition to caveolin, have been identified as components of a cytosolic lipoprotein-chaperone complex that translocates newly synthesized cholesterol from the endoplasmic reticulum to cell surface caveolae (Uittenbogaard et al., 1998). Although the precise role of the two immunophilins in cholesterol trafficking has not been defined, CsA treatment of cells disrupted association of CyP40 with caveolin and prevented rapid transport of cholesterol to caveolae membranes.

It is well established that the unliganded aryl hydrocarbon receptor (AhR) requires Hsp90 for dioxin binding and function *in vivo* and separate reports have indicated a dependence on Cpr7 for AhR signaling in yeast (Miller 2002; Yao et al., 2004). After ligand binding, AhR heterodimerizes with aryl hydrocarbon receptor nuclear translocator (Arnt) to become transcriptionally active (Furness et al., 2007). *In vitro* studies with recombinant AhR and Arnt proteins have suggested a role for CyP40 in the binding of AhR-Arnt to the dioxin response element (DRE) (Shetty et al., 2004). This effect of CyP40 appears to not involve Hsp90 and has been mapped to the linker separating the PPIase and TPR domains (Luu et al., 2008), a region previously shown to mediate CyP40 chaperone activity (Mok et al., 2006).

Chan and coworkers (Luu et al., 2008) have confirmed a negative impact of CyP40 siRNA knockdown on AhR signaling in MCF-7 breast cancer cells.

c-Myb is a homeodomain transcription factor that is critical for mammalian hematopoiesis and has a central role in determining differentiation, proliferation or apoptosis in immature and mature cells of myeloid lineage (Greig et al., 2008). c-Myb DNA-binding activity is controlled conformationally through intramolecular interactions between its N-terminal DNA-binding domain and a negative regulatory domain located at the C-terminal end of the protein. In *in vitro* studies, CyP40, through its TPR domain, was shown to interact with the DNA-binding domain of c-Myb and exposure of c-Myb in cell nuclear extracts to increasing amounts of recombinant full length CyP40 abolished c-Myb DNA-binding activity (Levenson and Ness 1998). A reversal of this effect by CsA implicated CyP40 catalytic activity in c-Myb inhibition. Since v-Myb, an oncogenic c-Myb derivative with alterations in the highly conserved DNA-binding domain known to enhance transforming activity, was unable to bind CyP40, it was concluded the v-Myb mutations negate the controlling influence seen with CyP40 over c-Myb function, allowing v-Myb to escape negative regulation. The significance of these observations needs to be tested *in vivo* (Hunter 1998). Specifically, it remains to be established whether CyP40 targets c-Myb in the cell to regulate its transcriptional activity.

An initial report from the Poethig laboratory on the function of the *Arabidopsis thaliana* CyP40 homolog, SQUINT (SQN), proposed a specialized role for CyP40 in plant development linked to progression of vegetative growth (Berardini et al., 2001). Additional genetic studies have now revealed that CyP40 promotes the activity of microRNAs in plants, and may chaperone Argonaute 1 (AGO1) or a protein that is critical for AGO1 function (Poethig RS, personal communication) (Smith et al., 2009). In this context, it is interesting that mammalian AGO2, another Argonaute protein family member, associates with Hsp90 (Maniataki and Mourelatos 2005; Tahbaz et al., 2001; Tahbaz et al., 2004), suggesting that Argonaute proteins might be functionally dependent on Hsp90, and opens the door for potential influences of Hsp90 cochaperones, including CyP40, following assembly of AGO-Hsp90-cochaperone complexes.

Future Prospects

While models developed in yeast provided an early insight into how CyP40 impacts on Hsp90-dependent signaling by steroid receptors (Duina et al., 1996a; Duina et al., 1998b), little headway has been made over the last 10-years with respect to how CyP40 influences the function of these receptors in the mammalian setting. This perplexing scenario has been evident over a period marked by important progress towards a better understanding of how receptor function is modulated by the CyP40 partner cochaperones, FKBP51, FKBP52 and PP5. These advances have been aided and will continue to be enhanced by the availability of mouse strains deficient in these TPR cochaperones (Cheung-Flynn et al., 2005; Tranguch et al., 2005; Yang et al., 2006; Yong et al., 2007a; Yong et al., 2007b). Most dramatic findings thus far have come from FKBP52 null mice, studies of which have revealed tissue-specific roles for the immunophilin in AR and progesterone receptor A (PRA) signaling (Cheung-

Flynn et al., 2005; Tranguch et al., 2005; Yang et al., 2006). The broad conclusion of these studies is that a TPR cochaperone may act as an essential regulator of a selected hormone receptor in one tissue, yet may be less crucial for receptor activity in another target tissue under the influence of a different mix of chaperones and cellular factors. Fundamental questions regarding the physiological role of CyP40 are likely to be best addressed by investigating the phenotype of distinct CyP40 mutant mouse lines now available to the Shou and Sanchez laboratories and through the collaborative efforts of the Picard and Ratajczak laboratories. These approaches will be complemented by studies of CyP40 knockdown using RNAi in human cell lines derived from different tissues to determine the impact of selective depletion of CyP40 on steroid receptor function in different cellular backgrounds.

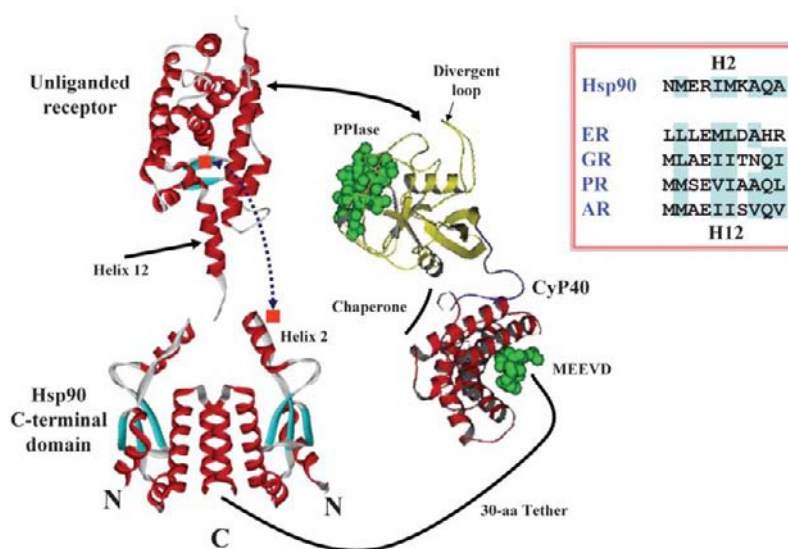


Figure 3. Model of CyP40 cochaperone interactions in apo-receptor complexes. The illustration shows unliganded receptor (represented by apo-retinoid X receptor- α) with helix 12 displaced from the ligand-binding pocket. The Hsp90 C-terminal domain, adapted from the structure of *E. coli* Hsp90, htpG (Harris et al., 2004), is shown as a dimer with the hydrophobic helix 2 proposed to form a client protein-binding surface. Helix 2 shares considerable similarity with helix 12 in steroid receptors (shown boxed) and may facilitate hormone binding by displacing this helix in receptor-Hsp90 complexes (Jackson et al., 2004). The Hsp90 MEEVD peptide (shown in space filling representation) is docked within the CyP40 TPR domain allowing the cyclophilin domain to act independently on the receptor LBD, either through its PPIase function (the active site is highlighted with a bound CsA molecule in space filling representation) or through direct contact with the divergent loop. CyP40 chaperone function may target either receptor or Hsp90.

From a mechanistic sense, further structural studies are required to present a clearer picture of how CyP40 interfaces with Hsp90 and with steroid receptors in *apo*-receptor complexes. By way of example, the successful production and assembly of a ternary Hsp90-Cdc37-Cdk4 complex and elucidation of its 3-dimensional structure have been key to understanding the Hsp90/Cdc37-dependent activation of protein kinases (Vaughan et al., 2006). Although the similarity in domain structure between CyP40 and FKBP52 is limited to

both having PPIase and TPR domains at their N- and C-terminal ends, respectively, it is tempting to apply to CyP40 a similar mechanism through which FKBP52 is understood to potentiate steroid receptor activity. Since FKBP52 catalytic activity is not required for potentiation, it has been proposed that the Hsp90-associated cochaperone facilitates contact with the receptor LBD through a loop that overhangs the FK1 PPIase pocket and hence stabilizes a receptor orientation favorable to high affinity hormone binding (Riggs et al., 2007). For cyclophilins there is a growing consensus that, rather than functioning as prolyl isomerases, the primary and normal function of the enzyme active site is to mediate specific protein-protein interactions (Gamble et al., 1996; Ke and Huai 2004; Luban 1996; Schreiber and Crabtree 1992). As for FKBP52, it is possible that in the absence of a role for its isomerase function, CyP40 uses the 'divergent loop' to form part of a binding interface that serves to anchor the receptor LBD in an optimal hormone binding conformation. In the dual domain structure of CyP40, the TPR domain, which accommodates the Hsp90 acceptor site, is clearly separated from an outward-facing isomerase active site, permitting both domains to function independently. Figure 3 provides an illustration of how CyP40 might help achieve a high affinity hormone binding state for ER α with receptor-Hsp90 complexes.

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Chapter VIII

Free-Living Protists as a Model for Studying Heat Shock Proteins in the Cell

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Abstract

Heat shock proteins (HSP) are closely involved in response of organisms to adverse natural and anthropogenic factors. Within one of the recent directions of HSP studies, attempts are made to uncover mechanisms underlying the organisms' adaptation to various stresses. Promising model objects for this research are protists – lower eukaryotes that are at the same time a cell and a fully fledged organism.

In this paper, we present some results of our studies on HSP70 level in intact cells of several free-living protists and on the characteristics of its dynamics in the cells in response to the changes in natural environmental factors, salinity and temperature. The protists chosen for the study, the amoebae and the ciliates, possess an essentially different organization of the cell and belong to the most phylogenetically distant groups.

In many cases, a high constitutive level of HSP70 was recorded in intact cells under normal (non-stressful) conditions. It may be considered as a universal pre-adaptation of these protists to possible drastic environmental changes.

The strains of *Amoeba proteus* and several related species were very similar as to the level of HSP70 and the position of the stained zone on the blots, despite differences in geographic provenance, temperature conditions in natural habitats the strain was isolated from, and the strain age. Species of other genera of freshwater lobose amoebae studied, though close to *Amoeba*, differed considerably in the HSP70 level.

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Out of the seven strains of the facultative parasites *Acanthamoeba*, only two had a noticeable constitutive level of HSP70. Differences in the constitutive level of HSP70 in the cells of different *acanthamoebae* strains may reflect their potential pathogenicity.

The ciliates used in the study represented various ecological groups different as to their attitude to environmental salinity. They were shown to employ various strategies of the chaperone system response to increasing and decreasing salinity of the medium. Constitutive level of HSP70 in their cells correlated with the degree of the salinity tolerance.

Introduction

Living organisms respond to various stresses by activation of their defensive mechanisms. A basic defensive mechanism is the synthesis of stress proteins, including the heat shock proteins (HSP). They are represented by a number of families, differing in molecular weight, nucleotide sequences of the encoding genes, and functions. The best studied HSP are those with the molecular weight of 70 kDa (HSP70). HSP70 are highly conservative proteins with a low species-specificity and a recognized chaperone activity. They are present in the cytoplasm of animals and plants, including unicellular ones, in both inducible and constitutive forms [Lindquist, 1986; Feder & Hofmann, 1999; Margulis & Guzhova, 2000, 2009].

A high HSP70 level in cells under normal, that is, non-stressful conditions may indicate a better adaptive potential of the organism. HSP70 level in the cell was shown to correlate positively with the temperature conditions in the environment [Lyashko et al., 1994]. For poikilotherms, this regularity was elevated to the rank of a general rule: the higher the environmental temperature, the higher the level of HSP70-like proteins in the organisms' cells [Ulmasov et al., 1992].

One of the recent directions in HSP-related research is the study of their role in adaptation of organisms to stresses [Feder & Hofmann, 1999; Evgen'ev et al., 2005; Garbuz et al., 2008]. In these studies, the stress is usually represented by temperature, and the objects, by multicellular organisms, cultures of their tissues, or prokaryotic microorganisms.

In this paper, we would like to draw the researchers' attention to protists as promising objects for the study of adaptive mechanisms at the biochemical and the molecular level. The name "protists" is currently applied to diverse lower eukaryotes that have in common a simple cellular organization: they are mostly unicellular and sometimes multicellular but without true tissues [Whittaker, 1969; Corliss, 1984; Cavalier-Smith, 1999]. Their main merit as model objects for experimental research resides in the fact that a protist is at the same time a cell and an independent fully fledged organism. Therefore, the protists' response to, for instance, a stress is not mediated by the specific features of cell functioning in different tissues, and thus can be considered as a simplified model of the multicellular animals' response. Incidentally, HSP level in the cells of various tissues of an organism subjected to a salinity stress may be considerably different [Deane & Woo, 2004].

Despite the attractiveness of protists as research objects, their HSP70 are rather poorly studied in comparison to those of bacteria and multicellular animals. Moreover, most of the information available concerns HSP70 in protists parasitizing warm-blooded hosts [for

example see: Requena et al., 1992; Field et al., 2000; Pérez-Serrano et al., 2000; Bakatselou et al., 2003; Varadharajan et al., 2004, etc.]. Free-living protists have been paid little attention on this point. There are just a few studies of HSP70 in ciliates [for example see: McMullin & Hallberg, 1987; La Terza et al., 2001, 2004; Smurov et al., 2007; Podlipaeva et al., 2008], flagellates [Drzymalla et al., 1996; Barque et al., 2000], and amoebae [Kalinina et al., 1988; Podlipaeva, 2001; Podlipaeva et al., 2006].

The functions of HSP70 in unicellular organisms are usually investigated in the context of their adaptation to the changes in the environmental temperature. However, HSP and, in particular, HSP70 are involved in adaptation to various adverse factors. In this respect, a very interesting topic is the adaptation of protists to the changes in the water salinity. Salinity, alongside with temperature, is a key factor in the evolution of aquatic organisms. The dependence of the protists' chaperone system upon salinity may be detected in experiments recording the constitutive level of HSP70, its expenditure, and its induction in response to the salinity stress under constant temperature conditions in protists from various ecological groups. Comparative analysis of such data obtained from evolutionary distant protists would reveal the universal characteristics underlying the chaperone system's functioning. Moreover, the regularities thus discovered might also be expected to be true of the multicellular organisms.

In this paper, we report the results of our studies on HSP70 level in intact cells of free-living protists and on the characteristics of HSP70 induction in the cells in response to the changes in salinity and temperature. The protists chosen for the study, the amoebae and the ciliates, belong to phylogenetically distant groups with an essentially different organization of the cell.

Material and Methods

Organisms and Cultures

Lobose Amoebae

Freshwater lobose amoebae used in the study were represented by 15 strains (clones) of different provenance (Table 1) taken from the Collection of the Laboratory of Cytology of Unicellular Organisms (Institute for Cytology of the Russian Academy of Sciences). The strains belonged to the species *Amoeba proteus*, *A. amazonas*, *A. borokensis*, "*A. indica*", *Amoeba* sp. and *Trichamoeba* sp. The amoebae were cultivated in the modified Prescott and Carrier solution according to the standard procedure [Prescott & Carrier, 1964] at room temperature. They were fed on *Tetrahymena pyriformis* ciliates (strain GL).

Acanthamoebae

Seven strains of amphizoic (species capable of living as free-living or as parasites) acanthamoebae (*Acanthamoeba* spp.) used in the study were obtained from the collection of the Parasitology Institute of the Czech Republic Academy of Sciences (from Prof. I. Dykova) (Table 2). Strains 4465, 4337, 3668 [Dykova et al., 1999], 4774, 4628 and 4690 [I. Dykova, unpublished data] were isolated in the Czech Republic from the internal organs of roach (*Rutilus rutilus*), pikeperch (*Esox lucius*) and perch (*Perca fluviatilis*). Strains P121 and P128

were isolated in Peru in 2005 from the gills of fish *Calophrysus macropterus* and *Amblydoras hancockii* (I. Dykova, unpublished data). All the strains were cultured under laboratory conditions [Dykova et al., 1999].

Table 1. Free-Living Lobose Amoebae Species and Strains Studied

NN	Species and Strains	About the Strain
1	<i>Amoeba proteus</i> Da	Received from Southampton University (UK) in 1970, isolated from Floral Park (New York, USA), not later than 1950.
2	<i>Amoeba proteus</i> B	Received from Budapest (Hungary) in 1959, there from King's College (London, UK).
3	<i>Amoeba proteus</i> tD	Received from King's College (London, UK) in 1963, initially referred to separate species <i>A. discoides</i> (for systematic status see: Jeon & Lorch, 1973; Page, 1988).
4	<i>Amoeba proteus</i> tP	Received from Southampton University (UK) in 1975.
5	<i>Amoeba proteus</i> Val	Established in 1989, isolated from Lake Sys'jarvi, Valaam Archipelago (North-Western Russia).
6	<i>Amoeba proteus</i> Kan	Established in 1989, isolated from Lake Kanevskoe, Valaam Archipelago (North-Western Russia).
7	<i>Amoeba proteus</i> Binucl	Received not later than 1977, origination is obscure.
8	<i>Amoeba proteus</i> Lesch	Received not later than 1977, origination is obscure, initially referred to separate species <i>A. lescherae</i> (for systematic status see: Page, 1988; Sopina, 2000).
9	<i>Amoeba proteus</i> Neapol	Established in 2005, isolated from Naples region (Italy).
10	<i>Amoeba proteus</i> Obsc	Date of receiving and strain origination are obscure.
11	<i>Amoeba amazonas</i> Amaz	Received from D.M. Prescott in 1969, isolated from Amazon River in Brazil, initially referred to <i>A. proteus</i> (for systematic status see: Friz, 1992).
12	<i>Amoeba indica</i> Ind	Received from CCAP (Cambridge, UK) in 1985, isolated from local pond in Bombay (India) in 1971, initially referred to separate species, but formal species description is obscure (for systematic status see: Page, 1988; Friz, 1992; Сопина, 2000).
13	<i>Amoeba borokensis</i> Bor	Established in 1974, isolated from local pond in Borok (Yaroslavl' region, Russia), initially referred to <i>A. proteus</i> (for systematic status see: Kalinina et al., 1986).
14	<i>Amoeba</i> sp. Belomor	Established in 1986, isolated from freshwater lake, (White Sea region, North-Western Russia), presumably not a member of the genus <i>Amoeba</i> .
15	<i>Trichamoeba</i> sp. As102	Established in 1971, isolated from small river in Azerbaijan, initially referred to <i>A. proteus</i> , but considerably differs from this species (Sopina, 2000) and provisionally referred to <i>Trichamoeba</i> sp. (Ivanova et al., 2004).

Table 2. Amphizoic Strains of *Acanthamoeba* Studied

NN	Strains	Place and date of isolation	Notes
1	4465	Vltava River, South Bohemia, 1990	The perch (<i>Perca fluviatilis</i>), brain
2	3668	Skalice River, Central Bohemia, 1990	The catfish (<i>Silurus glanus</i>), spleen
3	4690	Czech Republic, 1991	The roach (<i>Rutilus rutilus</i>), brain
4	4774	Czech Republic, 1991	The pike (<i>Exox lucius</i>), kidney
5	4628	Czech Republic, 1991	The perch (<i>Perca fluviatilis</i>), brain
6	P121	Peru, 2004	The catfish (<i>Calophysus macropterus</i>), gills
7	P128	Peru, 2004	The catfish (<i>Amblydaras hancockii</i>), gills

Free-living acanthamoebae were represented by strain Am61, obtained from the Laboratory of Soil Cryology (Institute for Physical-Chemical and Biological Problems of Soil Sciences of the Russian Academy of Sciences, Puschino). This strain was isolated from the upper soil horizon of the tundra in the Eastern Arctic Sector (Russia). Accumulative cultures were established on the agarized Prescott and James medium in the field [Page, 1988]; in the laboratory, individual cysts were extracted and used to obtain monocultures. The amoebae were cloned and maintained in the laboratory in Petri dishes at room temperature.

The cysts of ancient acanthamoebae were extracted from samples of late Pleistocene and Holocene permafrost and soil buried in it in the Eastern Arctic Sector (Russia), the age of the samples being 32-35 thousand years [Shatilovich et al., 2005]. Viable cysts were found in accumulative cultures obtained after de-freezing the samples according to the previously described procedure [Shatilovich et al., 2005]. Trophozoites of the excysted amoebae were established in an independent culture (strain Am8) maintained in Petri dishes on the agarized Prescott and James medium under a liquid layer (Cerophyl-Prescott infusion) at room temperature [Page, 1988]; the Petri dishes were stored in a sterile box. Morphological characters of the cysts and the trophozoites correspond fully to the diagnosis of the genus *Acanthamoeba* [A. Goodkov, unpublished data].

Ciliates

The ciliates used in the study were two *Paramecium* species, *P. jenningsi* (strain SR1-10) and *P. nephridiatum* (strain SR98-1), both from the culture collection of the Laboratory of

Invertebrate Zoology (the Biological Research Institute of the St. Petersburg State University, Russia), and *Tetrahymena pyriformis* (amicronucleate strain GL) from the culture collection of the Laboratory of Cytology of Unicellular Organisms (Institute for Cytology of the Russian Academy of Sciences). The ciliates were cultured according to the standard procedures: on the lettuce medium inoculated with *Klebsiella aerogenes* [Sonneborn, 1970] at room temperature.

Temperature Studies

Lobose Amoebae

Amoebae *Amoeba proteus*, strain Val normally cultivated at 20 °C, were treated at 37 °C during 1h. The portions of cells were separated from the major culture volume immediately after the treatment (0h) and after some time, different in different experiments. Every portion, as well as the control, untreated amoebae, was precipitated in a low speed (1000 rpm) centrifuge and homogenized in buffer for extraction according to the previously described procedures [Podlipaeva, 2001; Plekhanov et al., 2006].

Acanthamoebae

Cells of strain Am61 cultured at room temperature were heat shocked at 40 °C for 1 h or cold shocked at 4 °C for 1 h.

Cells of the ancient strain Am8 cultured at room temperature were heat shocked at 40 °C for 1 h or cold shocked at 8 °C for 1 h.

Immediately after the shock, the acanthamoebae together with the medium were collected into 2 ml tubes and centrifuged for 5 min at 12 000 rpm. After centrifugation, the precipitated cells were stored for some time in the refrigerator at -20 °C and then treated further as previously described [Podlipaeva et al., 2006, 2008].

Ciliates

Strains of the ciliates (*P. jenningsi*, *P. nephridiatum* and *T. pyriformis*) cultivated both in freshwater and in salty water medium, were heat shocked (at 37 °C for 1 h) in order to confirm that the polypeptides revealed in salinity shocks experiments indeed belonged to HSP70.

Salinity Studies

Four ecological groups of protists, differing in their attitude to salinity, occur in aquatic habitats [Smurov & Fokin, 2001; Kudryavtseva et al., 2007]. The first group, steno-freshwater species, comprises protists that can live under salinity not exceeding 2.5-3 ‰. The second group comprises protists that can live in the salinity range from 0 to 6-8 ‰ (freshwater species). Species of the third group, meta-freshwater species, can live in more salty water, up to 12-16 ‰. The fourth group, truly euryhaline species, comprises protists that can tolerate direct transfer from salty seawater to freshwater.

The procedures used for determining the salinity tolerance ranges of the protists studied and the design of experiments on the impact of environmental salinity changes are discussed in detail elsewhere [Smurov & Fokin, 2001; Plekhanov et al., 2006; Smurov et al., 2007]. Below we present only brief schemes of the experiments.

P. jenningsi and *A. proteus* Val (*Steno-Freshwater Species*)

The upper limits of the salinity tolerance of the individuals of these species acclimated to freshwater conditions (0 ‰) were 3.25 ‰ for *P. jenningsi* and 2.5 ‰ for *A. proteus* strain Val. For the individuals acclimated to 2 ‰, the upper limits of salinity tolerance were 6 ‰ and 3.5 ‰, respectively.

Cells grown in freshwater medium (0 ‰) were placed for 2 h in a medium with 2 ‰ salinity. The cultures acclimated to 2 ‰ salinity were placed in fresh water also for 2 h. Protists cultivated in the media with the initial salinity values were used as control; the culture density was the same in the control and in the experiments.

Tetrahymena pyriformis (*Meta-Freshwater*)

The ciliates were acclimated to fresh water (0 ‰), to 2 ‰ salinity and to 10 ‰ salinity. The upper limits of their salinity tolerance were, respectively, 8 ‰, 12 ‰ and 15 ‰. Cells acclimated to 10 ‰ salinity tolerated direct transfer into fresh water (0 ‰), and could later adapt to it.

Some of the *T. pyriformis* cells were placed for 1 h into water with a different salinity: the cells acclimated to 2 ‰ were placed in 10 ‰, and the cells acclimated to 10 ‰, into 2 ‰. Then the ciliates were returned to the medium with the initial salinity. For control, ciliates were placed for the same time into water with the salinity usual for them.

Paramecium nehrdium (*Euryhaline*)

Ciliates were acclimated to freshwater medium (0 ‰) and to the 10 ‰ medium. The upper limit of the tolerance range for the cells acclimated to fresh water was set at 20 ‰.

Some of the *P. nehrdium* cells acclimated to fresh water (0 ‰) were placed for 1 h into water with a 10 ‰ salinity; some of the cells acclimated to 10 ‰ were placed for 1 h into fresh water (0 ‰); then in both cases the cells were returned to the medium with the initial salinity. These cells were considered as treated by salinity shock. For control, ciliates were placed into water with the salinity usual for them for the same time. Some of the cells were subjected to an analogous impact with the following difference: after the transfer into water with a different salinity, the cells were not returned to the medium with the initial salinity after an hour but were left in the water with a different salinity until the end of the experiment (up to 24 h). These cells were considered as subjected to adaptation.

Electrophoresis and Western Blotting

Samples of cells were prepared for SDS-electrophoresis [Laemmli, 1970] as previously described [Podlipaeva, 2001; Plekhanov et al., 2006]. Electrophoresis was immediately followed by electroblotting [Towbin et al., 1979] conducted overnight at 6V. HSP were

revealed after treatment of nitrocellulose by monoclonal antibodies SPA 822 against HSP70 (Stressgen Technologies, Canada), the antibodies being specific both to the constitutive and the inducible form of HSP70. Binding zones of proteins and anti-HSP70 antibodies were stained on nitrocellulose by means of the secondary biotin-conjugated antibodies conjugated with alkaline phosphatase (Sigma Chemical Company) as a result of enzymatic reaction. The molecular weight of the polypeptides revealed was determined using High Range Rainbow Molecular Weight Markers of 14–220 kDa (Amersham Biosciences, England) and bovine serum albumin (66 kDa).

Results and Discussion

Lobose Amoebae

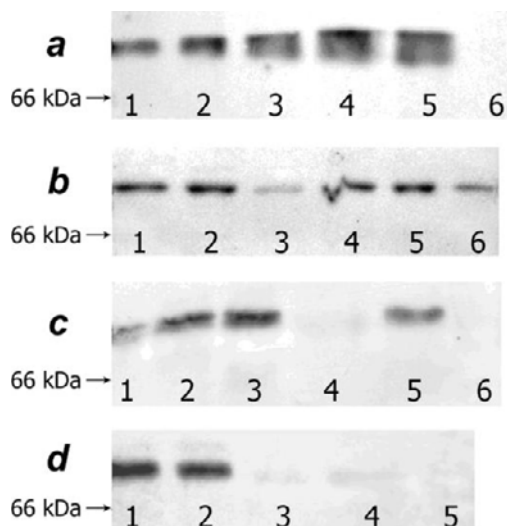
Constitutive Level of HSP70 in Cells

Almost all the lobose amoebae studied possessed constitutive HSP70, which was revealed at blots at rather low loads, 5–7 µg (Figure 1 a, b, d) and 11–12 µg (Figure 1 c) of total protein per starting gel. The *Amoeba proteus* strains and the related *Amoeba* species (*A. amazonas*, “*A. indica*” and *A. borokensis*) were very similar as to the level of HSP70 and the position of the stained zone on blots, which corresponded approximately to a polypeptide of 70–72 kDa. Only the “youngest” strain of the collection – the Neapol strain, isolated from nature in 2005 – had a somewhat lower level of HSP70 (Figure 1 b, lane 3). At the same time, in order to secure any noticeable staining of the 70 kDa zone in the amoebae of strain Belomor, the total protein load on the starting gel had to be increased first up to 12 µg (Figure 1 c, lane 4), and then up to 15 µg (Figure 1 d, lane 4). The weakly stained zone revealed occupied on the blot a slightly lower position than the stained zone of the other *Amoeba* strains studied. Finally, in strain AS102 (tentatively assigned to the genus *Trichamoeba*, see Table 1) no zone corresponding to 70 kDa could be revealed at all. At present, we cannot be sure whether the absence of this zone was associated with an insufficient protein load on the starting gel or with a characteristic feature of HSP of this strain. In the latter case, it may be supposed according to preliminary data, that the HSP of this strain is represented only by heavy isoforms about 97 kDa.

To sum up, most of the freshwater lobose amoebae strains studied possess a rather high HSP70 level under non-stress conditions. This characteristic did not correlate with any individual features of the strains, such as geographic provenance, temperature conditions in natural habitats the strain was isolated from, and the strain age (i.e. the time of maintenance in the laboratory). These results contradict the rule about a positive correlation between the temperature conditions in the habitat and the constitutive level of HSP70 in the cells of poikilotherms [Ulmasov et al., 1992].

There were no noticeable differences in the constitutive level of HSP70 in the cells of the *Amoeba proteus* strains and the related *Amoeba* species and in the distribution of the HSP isoforms revealed by anti-HSP70 antibodies. At the same time, the cells of the Belomor strain, traditionally denoted in the Collection as *Amoeba* sp. (Table 1), differed from all the strains and species of the “*proteus*” group in having a very low level of HSP70. Belomor

strain also differs from the other *Amoeba* strains in isozyme spectres of some enzymes, and probably does not belong to the genus *Amoeba* at all [Friz, 1992; Sopina, 2000]. Strain AS102, in which we failed to reveal any 70 kDa zone, is also not an *Amoeba* [Sopina, 2000] but a *Trichamoeba* species [Ivanova et al., 2004]. Interestingly, though all the lobose amoebae strains in the collection are cultured and fed under the same conditions, strains Belomor and AS102 are the most unstable and, one may say, capricious, demanding more frequent transfers and changes of the medium. One may suppose that such "petulance" reflects the connection between the low constitutive level of HSP70 and low adaptive capacities. One may further speculate that the differences in the level and the molecular characteristics of stress proteins are expressed in the lobose amoebae at the genus level.



- a:** 1 – *A. proteus* strain B, 2 – *A. proteus* strain Val, 3 – *A. proteus* strain Da, 4 – *A. amazonas* strain Amaz, 5 – *A. indica* strain Ind, 6 – *Amoeba* sp. strain Belomor (5-7 μ g of protein at each lane);
- b:** 1 – *A. proteus* strain Val, 2 – *A. proteus* strain tP, 3 – *A. proteus* strain Neapol, 4 – *A. proteus* strain Obsc, 5 – *A. proteus* strain Lesch, 6 – *A. proteus* strain Binucl (7 μ g of protein at each lane);
- c:** 1 – *A. proteus* strain Da, 2 – *A. proteus* strain Kan, 3 – *A. proteus* strain tD, 4 – *Amoeba* sp. strain Belomor, 5 – *A. borokensis* strain Bor, 6 – *Trichamoeba* sp. strain AS102 (11-12 μ g of protein at each lane);
- d:** 1 – *A. proteus* strain Da, 2 – *A. proteus* strain B, 3 and 4 – *Amoeba* sp. strain Belomor, 5 – *Trichamoeba* sp. strain AS102 (lanes 1-3 and 5 – 7 μ g of protein, lane 4 – 15 μ g of protein).

Figure 1. Heat shock protein of 70kDa family in intact cells of freshwater lobose amoebae of various strains.

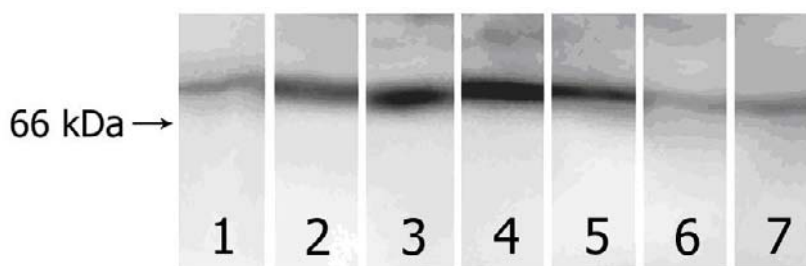
Salinity and Thermal Stresses

Cells of *A. proteus* strain Val were treated with thermal and salinity stresses in order to determine the dynamics of changes in the level of HSP70.

When the salinity of the medium increased from 0 to 2 ‰, HSP70 concentration increased markedly 3 h after the stress (Figure 2, lane 3). Thermal stress in the amoebae grown in freshwater medium resulted in an increase in the level of the same antigen as in the case of salinity shock (Figure 2, lane 2). The amoebae acclimated to 2 ‰ had a higher constitutive level of HSP70 (Figure 2, lane 4) than those adapted to fresh water (Figure 2,

lane 1). An increased HSP70 level at 2 ‰ salinity (the value close to the limit of this strain's tolerance range, 2.5 ‰) confirms that this salinity value is damaging for this strain. Additional stresses – transfer of cells acclimated to 2 ‰ into freshwater medium (Figure 2, lane 7) and heat shock (Figure 2, lanes 5 and 6) – result in a decrease in the HSP70 level.

The amoebae react to an increase in environmental temperature first by a decrease and then by an increase in the HSP70 concentration. The peak in the expenditure appears to fall on the time 3 h after the shock, after which the HSP70 level in the cells increases. The dynamics of expenditure-synthesis of HSP70 after salinity shock in the amoebae of this strain is similar to this dynamics after heat shock. Transfer of cells acclimated to 2 ‰ into freshwater medium results, similarly to the heat shock, in the protein expenditure.



1 – intact amoebae cells (0 ‰); 2 – amoebae from freshwater medium (0 ‰), treated by heat shock (37 °C, 1 h), in 3 hours after treatment; 3 – amoebae from freshwater medium (0 ‰), treated by salinity shock (2 ‰, 2 h), in 3 hours after treatment; 4 – amoebae acclimated to 2 ‰; 5 – amoebae acclimated to 2 ‰, treated by heat shock (37 °C, 1 h), immediately after treatment; 6 – amoebae acclimated to 2 ‰, treated by heat shock (37 °C, 1 h) in 3 hours after treatment; 7 – amoebae acclimated to 2 ‰, treated by salinity shock (0 ‰, 2 h), in 3 hours after treatment.

Figure 2. Heat shock protein of 70 kDa family in the cells of freshwater lobose amoeba *Amoeba proteus* strain Val, cultured in media of different salinity, and treated by salinity and heat shocks.

Acanthamoebae

Constitutive Level of HSP70 in the Cells and Thermal Stresses

Out of the seven strains of the amphizoic acanthamoebae studied, only two strains – 4465 and 4628 – were shown to possess constitutive HSP70 (Figure 3). In both these strains, the position of the stained zone on the blots was similar to that of the HSP70 zone in the *Amoeba* strains studied. Unfortunately, scarcity of the material prevented us from performing experiments with further increase in the load on the starting gel, which could reveal a stained zone in the acanthamoebae of the other amphizoic strains.

Contrary to the freshwater lobose amoebae, in *Acanthamoeba* differences in the HSP70 level were expressed within the genus; they may even be confined to strains within a species. The constitutive level of HSP70 is known to correlate positively with the degree of pathogenicity of the species and/or strain of acanthamoebae and with its higher tolerance of stresses, including thermal stresses [Pérez-Serrano et al., 2000]. Though all the amphizoic acanthamoebae strains in the present study were isolated from various internal organs of fish, they are not obligatory but facultative parasites [Dykova et al., 1999]. The differences in the

constitutive HSP70 level between the *acanthamoebae* strains studied may also reflect their potential pathogenicity.

Western blotting of trophozoites of the ancient *Acanthamoeba* strain Am8 revealed HSP in unstressed amoebae, in heat shocked amoebae and in cold shocked amoebae Figure 4. The stained binding zones with antibodies against HSP70 were situated in all the three cases somewhat lower than the molecular weight marker of 66 kDa (see also Figure 3 *lane* 11). Therefore, the protein found had a molecular weight of about 60 kDa and belonged to the family of HSP70.

Noteworthy, ancient *acanthamoebae* had a very high constitutive level of HSP (Figure 4, *lane* 3). The expenditure of the stress protein was somewhat less after cold shock (Figure 4, *lane* 1) than after heat shock (Figure 4, *lane* 2).

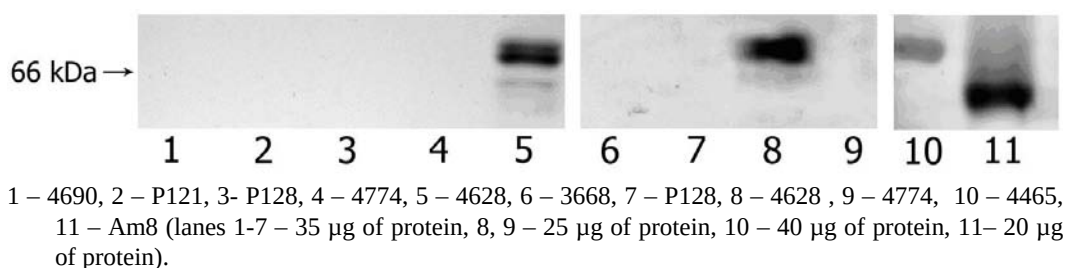
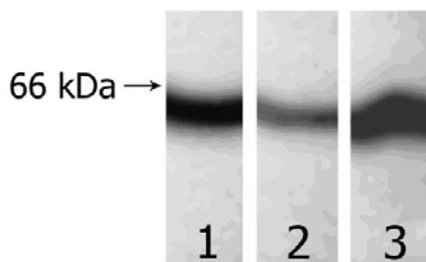
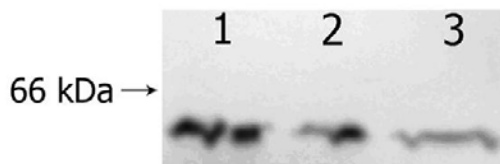


Figure 3. Heat shock protein of 70kDa family in intact cells of *Acanthamoeba* ssp. of various strains.



1 – cells after cold shock (8 °C), 2 – cells after heat shock (40 °C), 3 – intact cells.

Figure 4. Heat shock protein of 70kDa family in intact and stressed cells of ancient tundra *Acanthamoeba* sp. strain Am8.



1 – intact cells, 2 – cells after heat shock (40 °C), 3 – cells after cold shock (4 °C).

Figure 5. Heat shock protein of 70kDa family in intact and stressed cells of contemporary tundra *Acanthamoeba* sp. strain Am61.

Contemporary *acanthamoebae* from tundra (strain Am61) had a high constitutive level of HSP70 (Figure 5, *lane* 1). The cells of this strain were heat shocked at 40°C and cold

shocked at 4 °C. The expenditure of HSP70 was observed in both cases, but was higher after cold shock (Figure 5, *lane 3*) than after heat shock (Figure 5, *lane 2*). Noteworthy, the stained zone was situated on the blot somewhat lower than the molecular weight marker of 66 kDa. Thus, both ancient (Am8) and contemporary (Am61) strains of acanthamoebae from tundra had a high constitutive level of HSP70 and did not demonstrate typical heat shock response (HSR), that is, the induction of HSP70 in response to the shock. Their HSP, revealed by anti-HSP70 antibodies, were situated on the blot at the same position (lower than 66 kDa), differing to the amphizoic acanthamoebae (strains 4465 and 4628) and the freshwater lobose amoebae, whose HSP had a molecular weight of about 70 kDa. In both strains of acanthamoebae from tundra, HSP70 was expended after heat and cold shock, but in strain Am8 the expenditure was greater after heat shock, while in Am61 it was greater after cold shock. Lack of HSR may be associated with the initially high constitutive level of HSP70, as is the case, for instance, in some Antarctic fish [Place & Hoffman, 2005] and in embryos and larvae of the "living fossil", the horseshoe crab [Botton et al., 2006].

Ciliates, Salinity and Thermal Stresses

Paramecium jenningsi

In the total protein extract of the steno-freshwater ciliate *P. jenningsi* an antigen cross-reacting with anti-HSP70 antibodies was revealed. Its position on the blot was the same as that of the analogous polypeptide revealed in *A. proteus*. As shown by dot-blotting, the level of HSP70 in *P. jenningsi* cells grown in freshwater medium (Figure 6, dot 1) was very low, but increased after salinity shock (Figure 6, dot 3). On the contrary, ciliates acclimated to 2 ‰ had a high constitutive level of HSP70 (Figure 6, dot 2), which decreased after salinity shock (Figure 6, dot 4).

Paramecium nephridiatum

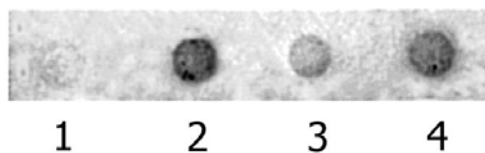
A polypeptide antigen (about 70 kDa) was revealed by western blotting in the total protein extract of *P. nephridiatum* cells, both in intact (control) cells after prolonged acclimation to freshwater medium or salty water medium, and in salinity shocked cells (Figure 7-9). Besides, *P. nephridiatum* cells acclimated to 10 ‰ were shown to possess an additional polypeptide with a molecular weight of about 60 kDa, which also cross-reacted with antibodies against HSP70 (Figure 7, *lane 2*; Figure 8, *lane 2*; Figure 9, *lane 2*).

The level of HSP70 in ciliates acclimated to freshwater conditions (Figure 7, *lane 1*; Figure 8, *lane 1*, Figure 9, *lane 3*) was much higher than in ciliates acclimated to 10 ‰ (Figure 9, *lane 2*; Figure 7, *lane 2*; Figure 8, *lane 2*).

In ciliates acclimated to fresh water and salty water, salinity shock elicited an asymmetric response as to the expenditure-synthesis of HSP70. The HSP70 concentration in the cells transferred from the 10 ‰ medium into fresh water (Figure 7, *lane 3*) was much higher than that in the cells transferred from fresh water into salty water (Figure 7, *lane 4*), the time after transfer being equal.

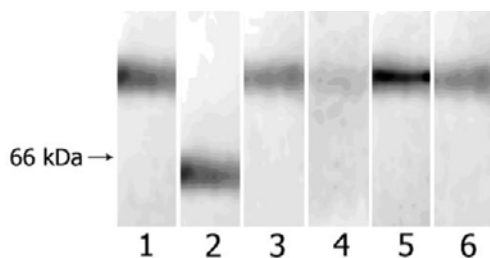
The level of 70 kDa protein after transfer from 10 ‰ medium to fresh water (10 → 0‰) was higher than that in the control (Figure 7, *lanes 2, 3*; Figure 8, *lanes 2, 3*). After transfer

from fresh to salty water (0 → 10‰), the HSP70 staining zone was weak, the protein concentration being much less than in the control (Figure 7, lanes 1, 4).



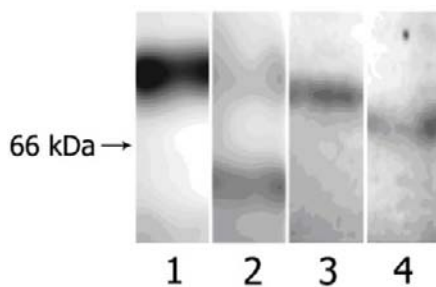
1 – intact cells from 0 ‰, 2 – cells acclimated to 2 ‰, 3 – cells from 0 ‰ treated by salinity stress (2 ‰), 4 – cells acclimated to 2 ‰ treated by salinity stress (0 ‰).

Figure 6. Heat shock protein of 70kDa family in the cells of freshwater ciliate *Paramecium jenningsi* strain SR1, cultured in the media of different salinities and treated by salinity stresses (Dot-Blotting).



1 – intact cells acclimated to freshwater medium (0‰); 2 – cells acclimated to 10‰; 3 – cells acclimated to 10‰, 2 h after salinity stress (0‰, 1 h – “shock”); 4 – cells acclimated to freshwater medium (0‰), 2 h after salinity stress (10‰, 1 h – “shock”); 5 – ciliates, acclimated to the freshwater medium (0‰), 3 h after remaining in the stress salinity of 10‰ (“adaptation”); 6 – cells acclimated to 10‰, 3 h after remaining in the stress freshwater (0‰) medium (“adaptation”).

Figure 7. Heat shock protein of 70kDa family in intact cells of euryhaline ciliate *Paramecium nephridiatum* and after two types of stresses – “shock” and “adaptation” (part 1).



1 – intact cells acclimated to the freshwater medium (0‰); 2 – cells acclimated to 10‰; 3 – cells acclimated to 10‰, 2 h after salinity stress (0‰, 1 h – “shock”); 4 – cells acclimated to 10‰, 3 h after remaining in the stress freshwater (0‰) medium (“adaptation”).

Figure 8. Heat shock protein of 70kDa family in intact cells of euryhaline ciliate *Paramecium nephridiatum* and after two types of stresses – “shock” and “adaptation” (part 2).

Adaptation to a new salinity (that is, a prolonged variant of the salinity shock) in the euryhaline ciliate *P. nephridiatum* resulted in HSP70 induction in experiments with both directions of salinity changes (Figure 7, lanes 1, 5; Figure 8, lanes 2, 4).

The protein with a molecular weight of about 60 kDa was present in a considerable quantity, higher than that of the protein of about 70 kDa, in intact *P. nephridiatum* cells

acclimated to 10 ‰ (Figure 7, *lane 2*; Figure 8, *lane 2*; Figure 9, *lane 2*) and was almost absent in intact cells acclimated to fresh water (Figure 7, *lane 1*; Figure 8, *lane 1*; Figure 9, *lane 3*).

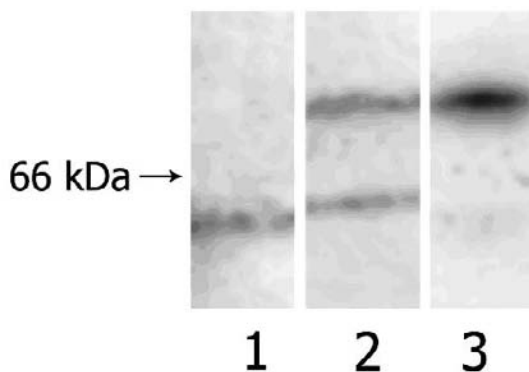
To sum up, steno-freshwater and euryhaline ciliates have different strategies of the chaperone system's response to increasing and decreasing salinity. In the steno-freshwater species *P. jenningsi*, acclimation results in an increase in the constitutive level of HSP70 in the cell, whereas in the euryhaline species *P. nephridiatum* it does not result in any change in the HSP70 level. Decreasing salinity of the medium is accompanied in euryhaline organisms by an increased level of HSP70 in the cell: in *P. nephridiatum* individuals acclimated to fresh water (0 ‰) the level of HSP70 was much higher than in *P. nephridiatum* individuals acclimated to salty water (10 ‰) (Figure 7-9). These results indicate that for euryhaline parametia a prolonged acclimation to fresh water is a stronger stress than acclimation to 10 ‰. Prolonged acclimation to fresh water appeared to result in the activation of their chaperone system, which was manifested in a higher constitutive level of HSP70.

The chaperone system of euryhaline ciliates, such as *P. nephridiatum*, is characterized by an asymmetric response as to the expenditure-synthesis of HSP70 after salinity shock depending on the direction of salinity change (Figure 7, 9).

Adaptation to new salinity values brings about HSP70 induction in *P. nephridiatum* in the case of salinity changes in both directions. This observation agrees with the reports that in some instances only a prolonged stress elicits HSP70 induction. For example, in the ciliate *Moneuplotes crassus* the level of HSP70 was the same as in intact cells after a short heat shock (5 min) but increased after a prolonged heat shock, reaching the maximum after 180 min [Ullmann et al., 2004].

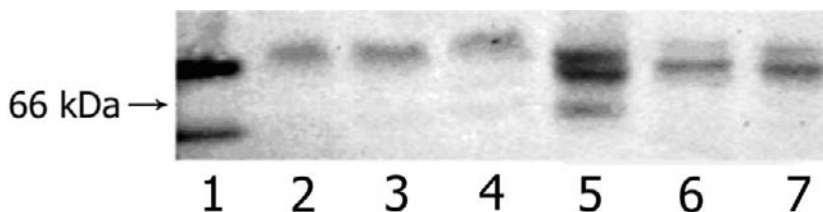
Tetrahymena pyriformis

In the meta-freshwater ciliate *T. pyriformis* two zones were revealed in control cells cultivated under optimal conditions (0 ‰): an intensely stained zone with a molecular weight of about 70 kDa (72 kDa) and a zone of about 60 kDa (Figure 10, *lane 1*). In intact *T. pyriformis* cells acclimated to 2 ‰, a stained zone of a somewhat larger molecular weight (73 kDa) was revealed (Figure 10, *lane 2*; Figure 11, *lane 1*), which remained almost unchanged 2, 4 and 24 hour after the salinity shock (1 h at 10 ‰) (Figure 10, *lanes 3, 4*; Figure 11, *lanes 2-4*). Besides, both in the control and in the "24 h after shock" sample there was a weakly stained zone corresponding to a protein with a molecular weight of about 65 kDa (Figure 10, *lanes 2*; Figure 11, *lanes 1, 4*).



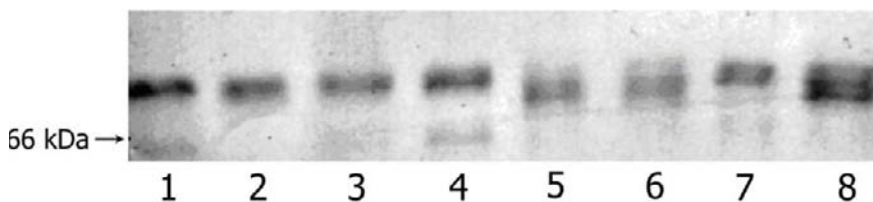
1 – cells acclimated to 10‰, after heat shock (37 °C, 20 min); 2 – cells acclimated to 10‰; 3 – intact cells acclimated to freshwater medium (0‰).

Figure 9. Heat shock protein of 70 kDa family in the cells of euryhaline ciliate *Paramaecium nephridiatum*, acclimated to 10 and 0‰, and treated by heat shock.



1 – intact cells acclimated to freshwater (0 ‰) medium; 2 – cells acclimated to 2 ‰; 3 – cells acclimated to 2 ‰ in 2 hours after salinity shock (10 ‰, 1 h); 4 – cells acclimated to 2 ‰ in 24 hours after salinity shock (10 ‰, 1 h); 5 – cells acclimated to 10 ‰; 6 – ciliates acclimated to 10 ‰, in 2 hours after salinity shock (2 ‰, 1 h); 7 – cells acclimated to 10 ‰, in 24 hours after salinity shock (2 ‰, 1 h).

Figure 10. Heat shock protein of 70 kDa family in intact cells of freshwater ciliate *Tetrahymena pyriformis* and after different periods of salinity stresses treatment (part 1).



1 – intact cells acclimated to 2 ‰; 2 – cells acclimated to 2 ‰ in 2 hours after salinity shock (10 ‰, 1 h); 3 – cells acclimated to 2 ‰ in 4 hours after salinity shock (10 ‰, 1 h); 4 – cells acclimated to 2 ‰ in 24 hours after salinity shock (10 ‰, 1 h); 5 – cells acclimated to 10 ‰; 6 – cells acclimated to 10 ‰, in 2 hours after salinity shock (2 ‰, 1 h); 7 – cells acclimated to 10 ‰, in 4 hours after salinity shock (2 ‰, 1 h); 8 – cells acclimated to 10 ‰, in 24 hours after salinity shock (2 ‰, 1 h).

Figure 11. Heat shock protein of 70 kDa family in intact cells of freshwater ciliate *Tetrahymena pyriformis* and after different periods of salinity stresses treatment (part 2).

T. pyriformis acclimated to 10 ‰ had a constitutive HSP with a molecular weight of 72 kDa, the same as in the ciliates from fresh water (Figure 10, lanes 1, 5; Figure 11, lane 5) and somewhat lower than in the ciliates acclimated to 2 ‰ (Figure 11, lanes 1, 5). The level of this protein decreased 2 h after salinity shock at 2 ‰ (Figure 10, lane 6; Figure 11, lane 6). After 4 h, the blot contained a protein with a slightly higher molecular weight (73 kDa), which substituted the protein found in the control and in the "2 h after shock" sample (Figure 11, lane 7). Finally, 24 h after the shock both zones, 72 and 73 kDa, were distinctly stained on the blot (Figure 10, lane 7; Figure 11, lane 8). No protein with a molecular weight about 65 kDa was found.

We failed to reveal any induction of HSP70 synthesis in *T. pyriformis* cells after salinity shocks in both directions of salinity change (Figure 10, 11). On the contrary, there was a noticeable expenditure of the 72 kDa protein, which substituted the 65 kDa protein 4 h after the shock. The levels of HSP70 in the cells acclimated to different salinities were similar, contrary to, for instance, the euryhaline *P. nephridiatum*. However, such a distribution of HSP in *T. pyriformis* may be associated not only with the ecological characteristics of this species but also with the peculiarities of the strain used. Strain GL is an amiconucleate one; it has been cultivated continuously for several decades under stable laboratory conditions, which could influence the reactivity of its chaperone system.

Freshwater *Tetrahymena* ciliates are known to possess a heat shock protein, called HSP90, with a molecular weight of 82-85 kDa. Its primary structure does not have a high homology with HSP70 of vertebrates [Frankel et al., 2001]. The protein found in our study has common antigen determinants with HSP70 of vertebrates, a fact that may indicate a considerable homology in primary structure.

As noted above, there are several ecological groups of aquatic protists different as to their attitude to environmental salinity [Smurov & Fokin, 2001; Kudryavtseva et al., 2007]. Similar groups are known in multicellular animals, for instance, mollusks and crustaceans [Aladin, 1996]. Since the emergence of such groups is an outcome of a long evolution, it may be suggested that they possess certain characteristic features of the chaperone system's functioning. Indeed, steno-freshwater and euryhaline protists have different strategies of the chaperone system response to an increase or a decrease in the salinity. In freshwater species, acclimation to an increased salinity results in an increase in the constitutive level of HSP70 in the cell, whereas in euryhaline species it does not change the constitutive level of HSP70. Euryhaline organisms respond to a decreased salinity by an increase in the HSP70 level in the cell. Therefore, in the process of acclimation these ciliates turn out to be, in a way, pre-adapted to abrupt changes in the environmental salinity and respond to them by using the pool of HSP accumulated in the cell. The meta-freshwater species *T. pyriformis*, intermediate as to its ecological characteristics, demonstrates a weak response to salinity changes. The level of HSP70 in *T. pyriformis* cells changes little, i.e. the chaperone system of this species is less reactive than in steno-freshwater or euryhaline species. To note, the chaperone system of the *Paramecium* species studied is more mobile than that of *Tetrahymena pyriformis* in response to both a prolonged salinity acclimation and a short stress.

Constitutive level of HSP70 in the cells of the ciliates studied correlates with the degree of their salinity tolerance. In the steno-freshwater species *P. jenningsi*, this level was lower in the fresh than in salty water. In the euryhaline ciliate *P. nephridiatum*, this level was higher

in fresh than in salty water, while in the meta-freshwater *T. pyriformis* it remained stable in both media. The constitutive level of HSP70 in the cells of these ciliates may reflect, in some respect, the conditions in the habitat of their origin: freshwater bodies in the case of *P. jenningsi*, freshwater or brackish-water bodies in the case of *T. pyriformis*, brackish-water estuaries or seas in the case of *P. nephridiatum*. Differences in salinity tolerance limits of the species studied suggest that, even though all of them may live in fresh water, they are pre-adapted to salinity changes within these limits.

Our results show that steno-freshwater and euryhaline ciliates possess a pronounced HSR, whereas the ciliate with intermediate adaptive capacities (*T. pyriformis*) lacks a pronounced HSR but has an initially high constitutive HSP level. An initially high level of HSP70 appears to correlate with the lack of HSR also in the tundra strains of acanthamoebae. These strains were isolated from habitats with extreme fluctuations of the environmental factors, first of all, temperature. So, a high constitutive level of HSP70 and a lack of HSR allow the species to exist under harsh conditions. However, in order to be able to colonize new habitats (e.g. salty water for freshwater species and vice versa) the presence of HSR is essential.

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Chapter IX

Quality Control in the Secretory Pathway

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Abstract

The endoplasmic reticulum is an essential cellular compartment with many diverse functions. It is the main Ca^{2+} storage site and is involved in maintaining Ca^{2+} homeostasis. The endoplasmic reticulum is the first compartment of the secretory pathways, making it the entry compartment for approximately one-third of all the proteins synthesized by the eukaryotic cell. The endoplasmic reticulum contains unique enzymes, maintaining an oxidative environment that allows co- and post-translational modifications such as glycosylation and disulfide bond formation, as well as molecular chaperones that assist in protein folding and quality control of newly synthesized membrane and secretory proteins. Protein folding in endoplasmic reticulum is controlled by endoplasmic reticulum quality control mechanisms. The primary players of endoplasmic reticulum quality control are the molecular chaperones like calnexin and calreticulin that reside in the endoplasmic reticulum. The mechanism of endoplasmic reticulum quality control assures that only correctly folded, functional proteins will exit the endoplasmic reticulum whereas non-native, misfolded proteins will be degraded via endoplasmic reticulum-associated degradation. ERAD pathway is closely connected to unfolded protein response pathway that is involved in its induction and regulation.

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Introduction

The endoplasmic reticulum (ER) is a large membrane-bound network that is continuous with the nuclear envelope and found throughout the cytoplasm of all eukaryotes (Bedard et al., 2005; Lin et al., 2008). This organelle is a major protein folding compartment of the eukaryotic cell as well as the entry site for the secretory pathway (Schroder and Kaufman, 2005a). The ER luminal milieu is distinctly different from the remainder of the cell, and accommodates and supports the diverse roles of the ER including regulation of Ca^{2+} homeostasis, synthesis and modification of membrane and secretory proteins, regulation of protein folding and trafficking, and cellular responses to stress (Bedard et al., 2005; Hebert and Molinari, 2007; Hendershot, 2004; Ma and Hendershot, 2004; Schroder and Kaufman, 2005a). Most importantly, many ER functions, including protein folding, posttranslational modification and intracellular trafficking are dependent on intraluminal ER Ca^{2+} concentrations (Lodish et al., 1992) (Figure 2). This review focuses on the ER compartment as the major site for quality control in the secretory pathway.

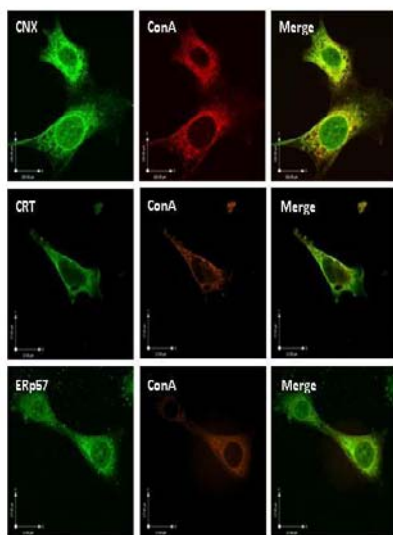


Figure 1.

Figure 1. The endoplasmic reticulum calnexin, calreticulin and ERp57 (green) colocalize (yellow) with the ER marker, concanavalin A (red) (Coe et al., 2008). Confocal images of ER network are shown. Cells were grown on cover slips, fixed with formaldehyde and immunostained for ER markers with primary antibody rabbit anti-calnexin (1:100) (Stressgen), goat anti-calreticulin (1:100) rabbit anti ERp57 (1:1000) and with Texas Red conjugate of concanavalin A (1:1000) (Invitrogen). Fluorescein isothiocyanate conjugate secondary antibodies were used at a dilution of 1:100.

Calreticulin, Calnexin and ERp57: Molecular Components of Protein Quality Control

Quality control is defined as a collection of activities designed to ensure adequate quality and is used for analysis of design and inspection for defects. Within the ER, molecular chaperones (BiP/Grp78, calreticulin, calnexin, Grp94), folding enzymes (thiol oxidoreductases [protein disulphide isomerase, (PDI) and ERp57]) and sensing enzymes (UDP-glucose glycoprotein:glucosyltransferase [UGGT]) are the key components of quality control as they are all involved in generating conformationally competent and functional proteins. Each of the molecular chaperones, folding enzymes and sensing enzymes have their own unique mechanism to prevent the transport of conformationally incompetent proteins out of the ER. BiP and Grp94 recognize exposed hydrophobic regions commonly found in misfolded proteins, assisting in the folding and assembly (Argon and Simen, 1999; Gething, 1999). In addition, calreticulin and calnexin interact with glycoproteins via their lectin binding ability (Helenius et al., 1997). PDI and ERp57, both thiol oxidoreductases, utilize the oxidizing environment of the ER to generate disulfide linkages (Molinari and Helenius, 1999; Noiva, 1999; Oliver et al., 1997). The formation of these intra- or interchain disulfide bonds is an integral part of the maturation of most secretory and membrane bound proteins in the ER. UGGT is the sensor of non-native partially folded glycoproteins. This enzyme re-glucosylates N-glycans attached to glycoproteins during late stages of folding, allowing incompletely folded substrates to enter another round through the folding machinery (Ritter and Helenius, 2000).

Calreticulin and Calnexin

Calreticulin and calnexin are two major lectin-like molecular chaperones in the ER (Michalak et al., 1999; Wada et al., 1991) (Figure 2). They efficiently suppress the aggregation of glycosylated and non-glycosylated proteins via oligosaccharide and protein-protein interactions (Ireland et al., 2008; Saito et al., 1999; Williams, 2006). In addition to this chaperone function, calreticulin regulates cellular Ca^{2+} homeostasis and intracellular Ca^{2+} signaling, most notably providing the Ca^{2+} necessary for the function of Ca^{2+} -dependent enzymes, including phosphatases and kinases (Groenendyk et al., 2004; Lynch et al., 2005; Lynch et al., 2006; Michalak et al., 2002a). Calreticulin is a classical ER resident protein, as it contains an N-terminal cleavable signal sequence that directs the protein to the ER lumen and an ER KDEL retrieval signal (Koch et al., 1989). The protein is composed of three structural and functional domains, a globular N-domain, an extended arm P-domain and an acidic C-domain. Calnexin is a 90-kDa type I integral membrane protein with C-terminal RKPRRE-ER retention signal (Bergeron et al., 1994). The protein binds ATP and Ca^{2+} with high affinity (Ellgaard and Frickel, 2003; Michalak et al., 2002b; Ou et al., 1995; Tjoelker et al., 1994). Similar to calreticulin, the protein also contains three domains, the globular N-domain, extended arm P-domain and cytoplasmic acidic C-domain. The N-domain of calreticulin and calnexin contains the carbohydrate binding site (Kapoor et al., 2004), the zinc binding site (Baksh et al., 1995), and a disulfide linkage (Andrin et al., 2000). The P-domain

is composed of a flexible extended finger-like region that interacts with ERp57 (Oliver et al., 1999) in a chaperone-dependent manner (Ellgaard et al., 2002; Frickel et al., 2002), and in conjunction with the N-domain, may form a functional protein-folding unit (Nakamura et al., 2001). The P-domain is very rich in proline residues and has several repeat sequences. The C-domain of calreticulin contains 19 pairs of acidic and negatively charged residues that bind ~50% of the ER luminal Ca^{2+} with high capacity (25 mol of Ca^{2+} per mol of protein) and low affinity ($K_d = 2 \text{ mM}$).

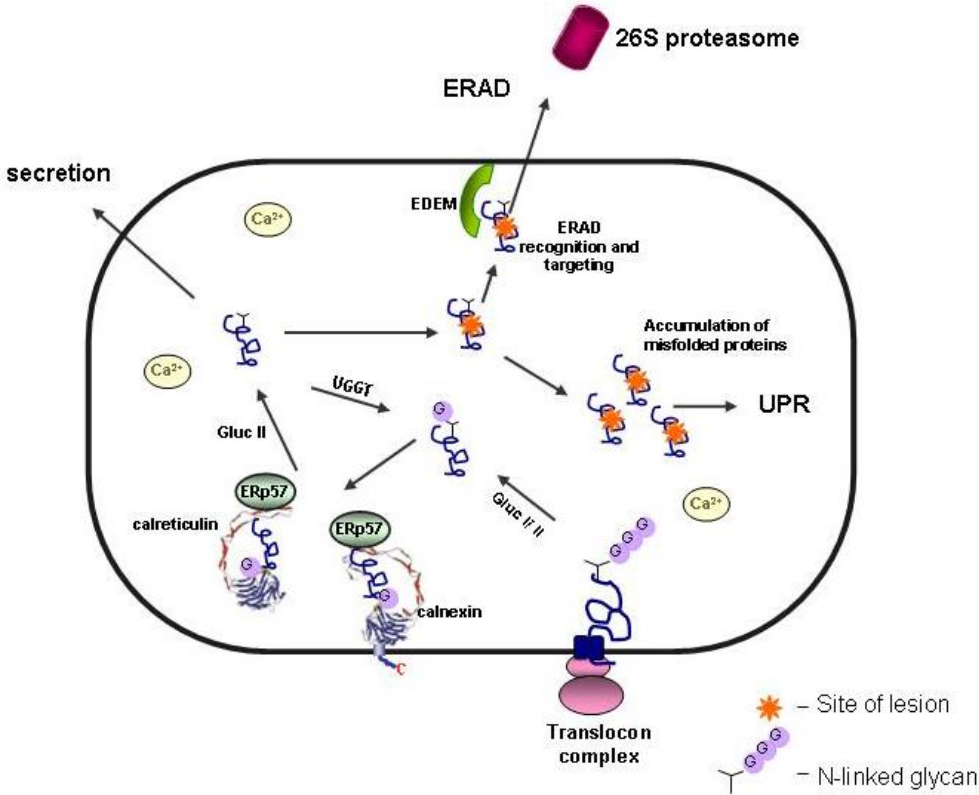


Figure 2. Maturation, post-translation modifications and quality control of the proteins in the ER Newly synthesized proteins (blue squiggle) entering the ER through translocon are often modified with N-glycan ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, where Glc is glucose [purple circle], Man is mannose and GlcNAc_2 is N-acetylglucosamine). Next, glucosidase I and II (Gluc I/II) remove the terminal glucose creating a substrate for lectin-like chaperones, calnexin and calreticulin, to facilitate folding. ERp57 interacts with calnexin/calreticulin and catalyzes formation and isomerization of disulfide bonds. Once the protein achieves its correct conformation, it is targeted to the secretory pathway. If folding requires additional association with calnexin/calreticulin, UGGT (UDP-glucose:glycoprotein glucosyltransferase) catalyzes re-glucosylation of non-native glycoproteins. If the protein is terminally misfolded (orange star), it is targeted for ER-associated degradation (ERAD). Misfolded proteins are recognized by ER residents - ER degradation-enhancing α -mannosidase-like lectins (EDEMs [yellow half moon]) that target substrates for ERAD. EDEMs associate with derlin-2 and derlin-3 (not shown) that are candidates for retro-translocation channel. After translocation to the cytoplasm, the protein is polyubiquitinated (not shown) and degraded via the proteasome.

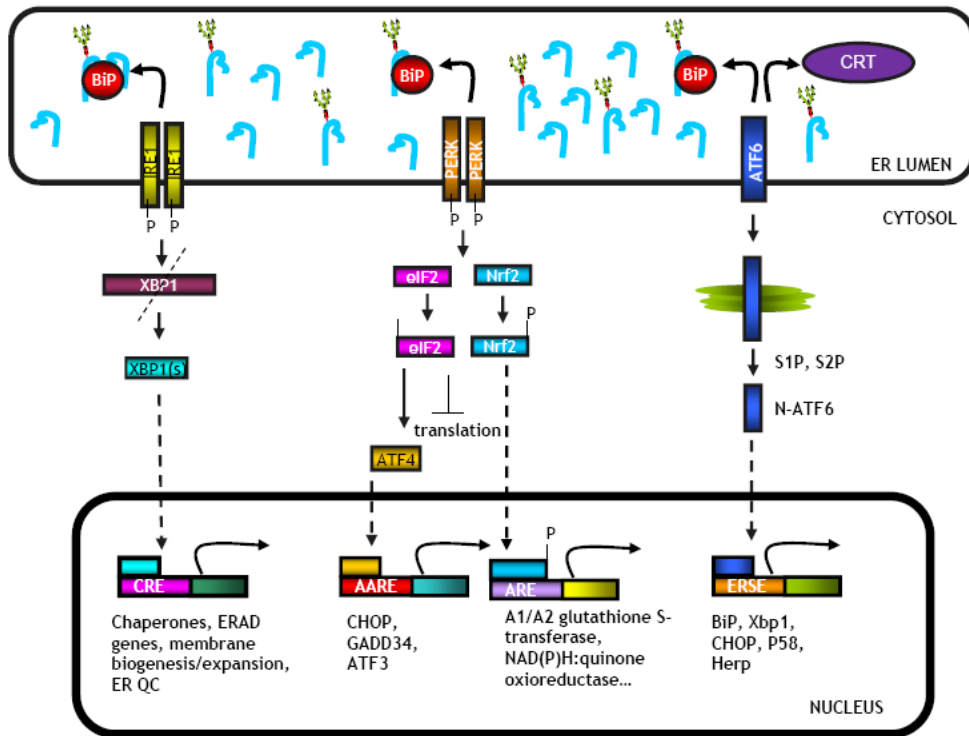


Figure 3. The Unfolded Protein Response. The activation of the UPR is a result of misfolded proteins in the ER. BiP is titrated off the transmembrane sensors inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor-6 (ATF6) to bind the misfolded proteins (both non-glycosylated and glycosylated) accumulating in the ER. IRE1 is free to homodimerize, then it autophosphorylates, activating its endoribonuclease activity. It is then free to splice 26-nucleotides from X-box binding protein 1 (Xbp1) mRNA which activates the active, spliced transcription factor, Xbp1(s). Xbp1(s) translocates to the nucleus where and binds cAMP response element (CRE) promoters resulting in activation of the transcription of chaperones, ER associated degradation (ERAD) genes, genes involved in membrane biogenesis and quality control. PERK is also free to homodimerize and autophosphorylate and this activates its phosphorylation activity. It phosphorylates eukaryotic elongation factor 2 (eIF2 α) thereby activating it to decrease overall translation but increasing the translation of activating transcription factor-4 (ATF4). Increased ATF4 protein translocates to the nucleus where it binds genes with amino acid response elements (AARE) causing an increase in transcription of CHOP, GADD34 and ATF3. PERK also phosphorylates nuclear respiratory factor 2 (NRF2) activating it as a transcription factor where, in the nucleus, it binds antioxidant response elements (ARE) to increase the transcription of genes involved in responding to oxidative stress. ATF6, when released by BiP and CRT, translocates to the Golgi apparatus where it is sequentially cleaved by site-1 and site-2 protease. This release the active transcription factor, ATF6-N that translocates to the nucleus to bind the ER-response element (ERSE) to activate the transcription of genes such as BiP, Xbp1, HERP, P58 and CHOP. Taken together, these three membrane sensors activate genes involved in decreasing ER stress

Calnexin and calreticulin, in conjunction with Erp57 and UGGT are an essential part of the ER protein quality control system (Bergeron et al., 1994; Ellgaard and Helenius, 2003; Johnson et al., 2001; Ware et al., 1995). This is supported by studies with calreticulin and calnexin-deficient cells. For example, in calreticulin-deficient mouse fibroblast cells, protein

folding was accelerated and there was significantly more partially folded proteins produced. Similarly, in calnexin-deficient cells, folding was severely impaired (Molinari et al., 2004).

Site specific mutational analysis of calreticulin demonstrates that single site mutations in calreticulin are sufficient to modify the structure and consequently the function of the protein (Guo et al., 2003; Martin et al., 2006). Two tryptophan residues are critical for the chaperone function of calreticulin: Trp³⁰², which is located in the carbohydrate binding pocket and Trp²⁴⁴, which is found at the tip of the “extended arm” in the P-domain (Martin et al., 2006). Interestingly, mutation of the cysteine residues disrupted the chaperone function only partially (Martin et al., 2006). His¹⁵³ is also essential for calreticulin chaperone function, significantly affecting the structure of calreticulin (Guo et al., 2003). Glu²³⁹, Asp²⁴¹, Glu²⁴³ and Trp²⁴⁴, located at the tip of the P-domain, are each important for the complex formation between calreticulin and ERp57 (Martin et al., 2006). These studies show that conformational changes in calreticulin induced by mutation of a single amino acid residue have negative consequences for chaperone function, demonstrating that mutations in chaperones may play a significant role in protein folding disorders.

Although calreticulin and calnexin share a high degree of structural similarity, the two chaperones demonstrate considerable differences in their substrates and specificities (Bedard et al., 2005; Denzel et al., 2002). Substrates can be divided into those which interact with calnexin exclusively, including vesicular stomatitis virus G protein (Hammond et al., 1994), the myelin protein PMP22 (Dickson et al., 2002) or the acetylcholine receptor (Keller et al., 1998), or those which interact with calreticulin only, such as coagulation factor V (Pipe et al., 1998). As evidenced from gene knockout studies in mice, the two proteins cannot compensate for one another (Denzel et al., 2002).

ERp57

ERp57 (also known as Grp58) is an ER localized glycoprotein specific thiol-disulfide oxidoreductase that is a member of the PDI family. ERp57 has an carboxy-terminal ER retention sequence, QEDL, and contains several modification sites: two thioredoxin-like domains, six protein kinase C phosphorylation sites, seven casein kinase II phosphorylation sites, three tyrosine phosphorylation sites, two sulfation sites and one N-myristoylation site (Khanal and Nemere, 2007). There are at least four domains in ERp57 named *a*, *b*, *a'* and *b'*. Two thioredoxin-like active sites (amino acid sequences WCGHCK) are found in the *a* and *a'* domains while the *b* and *b'* domains are redox inactive (Frickel et al., 2004; Grillo et al., 2007; Pollock et al., 2004). PDI and ERp57 share 33% overall amino acid sequence identity (Ferrari and Söling, 1999; Pollock et al., 2004), however differences are apparent in the C-terminus of the two proteins. The acidic C-terminal Ca²⁺ binding region of PDI is enriched in positively charged residues in the ERp57 orthologs (Pollock et al., 2004). These differences in the carboxy-termini are thought to modulate the substrate binding specificity of ERp57 and indicate that ERp57 might not play a role in Ca²⁺ buffering in the ER lumen (Pirneskoski et al., 2004).

ERp57, in association with calnexin or calreticulin, is important for the catalysis and isomerization of disulfide bonds in newly synthesized proteins. ERp57 has been most widely

studied with regards to its role in the immune system where the protein plays an important role in the early stages of assembly of the heavy chain of major histocompatibility complex (MHC) class I (Hughes and Cresswell, 1998; Morrice and Powis, 1998; Wearsch and Cresswell, 2008). Interestingly, ERp57 has been reported also to localize outside of the ER; to the nucleus, cytoplasm and cell surface (Altieri et al., 1993; Coppari et al., 2002; Grillo et al., 2002; Wearsch and Cresswell, 2008). The promiscuous localization of ERp57 suggests that it may have roles outside the ER. Indeed, with the use of DNA-protein cross-linking experiments, ERp57 has been reported to interact *in vivo* with DNA in mammalian cells (Coppari et al., 2002). ERp57 has also been postulated to sequester inactive and active signal transducer and activator of transcription 3 (STAT3) molecules and may be involved in the regulation of STAT3-dependent signaling (Coppari et al., 2002; Eufemi et al., 2004; Guo et al., 2002).

Expression of ERp57 may be regulated in disease states such as cancer, prion disorders, Alzheimer's disease and hepatitis (Erickson et al., 2005; Hetz et al., 2005; Khanal and Nemere, 2007; Martin et al., 1993; Muhlenkamp and Gill, 1998; Seliger et al., 2001; Tourkova et al., 2005). There is increased expression of ERp57 in transformed cells (Hirano et al., 1995). In cancer, ERp57 may be associated with oncogenic transformation by controlling intracellular and extracellular redox activities through its thiol-dependent reductase activity (Hirano et al., 1995). Another mechanism in which alteration in expression of ERp57 could be involved in cancer is through its regulation of STAT3 (Guo et al., 2002). ERp57 has increased expression in the early stages of prion disease suggesting that the protein may play a role in the cellular response to prion infection (Hetz et al., 2005). In Alzheimer's disease, ERp57 is reported to be a carrier protein that prevents the aggregation of β -amyloid and preventing plaques by keeping β -amyloids in solution (Erickson et al., 2005).

ER-Associated Degradation (ERAD)

Spontaneous errors during transcription or translation, genetic mutation and environmental factors may contribute to compromised protein folding efficiency. Therefore, ERAD ensures a balance between protein synthesis, folding and degradation which is essential for the homeostasis of every cell. Imbalance between these three processes leads to accumulation of misfolded proteins in the cytoplasm. To prevent accumulation of incorrectly folded peptides, terminally misfolded proteins are targeted for degradation (Bukau et al., 2006) by ERAD (McCracken and Brodsky, 1996). There are several steps of ERAD pathway: first, recognition of terminally misfolded protein; second, translocation the misfolded proteins to the cytoplasm across the ER membrane where ERAD substrates are ubiquitinated and targeted for proteasome-dependent degradation. It remains unclear as to how proteins are selected for ERAD pathway. It has been suggested that cytoplasmic molecular chaperone members of the Hsp70 (70-kDa heat shock protein) family may be involved in ERAD substrate recognition since they are known to bind hydrophobic patches exposed on misfolded proteins (Jahn and Radford, 2005). Furthermore, prolonged interactions between Hsp70 and ERAD substrates may be critical in the requirement of ubiquitin ligase and initiation of ubiquitination (Vembar and Brodsky, 2008). It has also been suggested, but is

still not clear, that BiP, an ER-luminal Hsp70-family member, may be involved in this process (Knittler et al., 1995; Schmitz et al., 1995). The mechanisms of retro-translocation of ERAD substrates to the cytosol are also not very well understood. Derlin-2 and derlin-3 translocation channels and/or Sec61 might be involved in retro-translocation (Lilley and Ploegh, 2004; Pilon et al., 1997; Plemper et al., 1997; Wiertz et al., 1996; Ye et al., 2004). These translocation channels have been found to associate with EDEMs (ER degradation enhancing α -mannosidase like lectins) ER proteins known to associate with ERAD substrates. This suggests that EDEMs may be involved in delivering ERAD substrates to translocation channels. Polyubiquitination of ERAD substrates occurs in the cytoplasm by a mechanism described for other cellular proteins (Goldberg, 2003) where ubiquitinated proteins are degraded by the proteasome (Vembar and Brodsky, 2008) (Figure 2).

The Unfolded Protein Response (UPR)

If newly synthesized proteins escape the quality control machinery and are not recognized as ERAD substrates there is a buildup of misfolded proteins within the ER lumen, resulting in ER stress (Ellgaard and Helenius, 2003; Eriksson et al., 2004; Imaizumi et al., 2001). In order to deal with the increased load of misfolded proteins, the ER has evolved the UPR. There are three main branches of UPR; inositol-1-requiring enzyme (IRE1), PKR-like endoplasmic reticulum kinase/pancreatic eukaryotic initiation factor2 α (PERK/PEK) and activating transcription factor 6 (ATF6) (Cox et al., 1993; Harding et al., 1999; Schroder and Kaufman, 2005a; Shenkman et al., 2007) (Figure 3). Collectively, these main branches carry out three specific cellular responses: to decrease misfolded protein load by activating transcription of ERAD encoding genes, inducing transcription of genes encoding chaperone proteins to increase the folding capacity of the ER and inhibiting general protein translation to decrease protein load on the ER (Brooks, 1997; Brostrom and Brostrom, 1998; Imaizumi et al., 2001; Meusser et al., 2005; Schroder and Kaufman, 2005b; Shen et al., 2005).

The first component of the UPR pathway is IRE1, an ER transmembrane protein kinase (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). IRE1 is a bifunctional protein that contains a cytoplasmic carboxy-terminal kinase and endoribonuclease (RNase) domain (Back et al., 2005). The receptor-like ER luminal domain of IRE1 binds BiP/Grp78 (Zhou et al., 2006). Under steady state conditions (the absence of stress), IRE1 is maintained as an inactive homodimer through interaction with BiP/Grp78. However, when misfolded proteins accumulate in the ER lumen, BiP/Grp78 dissociates from IRE1 resulting in IRE1 homodimer, *trans*-autophosphorylation and activation of its RNase activity (Malhotra and Kaufman, 2007). The RNase domain of IRE1 splices 26-nucleotides from the mRNA encoding the basic leucine zipper domain (bZIP) containing transcription factor X-box binding protein 1, Xbp1 (Malhotra and Kaufman, 2007). This cleavage generates a new species of mRNA with a translational frameshift now encoding a new transcription factor referred to as sXbp1 (Back et al., 2005; Malhotra and Kaufman, 2007). The sXbp1 translocates to the nucleus where it activates the transcription of genes encoding molecular chaperones (BiP, Grp94, calreticulin) and proteins involved in the ERAD in an effort to alleviate the build up of misfolded proteins in the ER lumen (Back et al., 2005).

PERK is an ER transmembrane kinase whose function is to transiently attenuate mRNA translation, decreasing the load of newly synthesized proteins on an already stressed ER (Lin et al., 2009; Malhotra and Kaufman, 2007). When there is an accumulation of misfolded proteins in the ER lumen, BiP dissociates from PERK leading to PERK dimerization and *trans*-autophosphorylation (Malhotra and Kaufman, 2007). Consequently, PERK's cytoplasmic kinase domain phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2 α) on Ser⁵¹ (Lin et al., 2009; Malhotra and Kaufman, 2007) (Figure 3). Phosphorylated eIF2 α interferes with the assembly of the 43S translation initiation complex, shutting down initiation of translation (Harding et al., 2000). Interestingly, phosphorylated eIF2 α is a key factor in the selective translation of a few specific mRNAs in response to ER stress. For example, activating transcription factor 4 (ATF4) mRNA is induced by phosphorylated eIF2 α . ATF4 acts as transcription factor which activates genes encoding proteins involved in apoptosis and the anti-oxidative stress response (see below) (Ameri and Harris, 2008). Activated PERK also affects the function of the nuclear respiratory transcription factor 2 (NRF2) (Cullinan et al., 2003) (Figure 3). Under conditions of no stress, NRF2 is cytoplasmic where it interacts with Kelch-like Echinoderm-associated protein 1, Keap1. However, under stressed conditions, activated PERK phosphorylates NRF2 resulting in its dissociation from Keap1, translocation of NRF2 to the nucleus, and binding to antioxidant response elements (ARE) to activate transcription of genes involved in detoxifying events (Cullinan et al., 2003; Nguyen et al., 2003). This role of PERK in regulation of NRF2 function supports the hypothesis that PERK may be involved in alleviating oxidative insult to the cell (Malhotra and Kaufman, 2007).

The third branch of the UPR involves ATF6, a 90-kDa ER transmembrane protein (Hong et al., 2004; Yoshida et al., 1998) (Figure 3). There are two isoforms of ATF6 (α and β), characterized by their divergent transcriptional activity domains (Thuerauf et al., 2004). ATF6- α and - β have similar domain structures with an amino-terminal transcriptional activation domain (TAD), a bZip cytoplasmic domain, a single transmembrane domain as well glycosylation sites within the carboxyl ER luminal domain (Kondo et al., 2005; Murakami et al., 2006). ATF6- α and - β , are cleaved/activated in response to ER stress (Kondo et al., 2005; Murakami et al., 2006). ER stress-dependent activation of ATF6 involves two ER resident chaperones, BiP and calreticulin. BiP and calreticulin bind to the ER luminal domain of ATF6 and mask ATF6 Golgi localization sites. Upon induction of ER stress, BiP/calreticulin dissociate from ATF6 revealing its non-consensus Golgi localization sites (Hong et al., 2004; Klumpp and Krieglstein, 2002; Shen et al., 2002; Shen et al., 2005). ATF6 then translocates to the Golgi apparatus where it is subject to proteolytic processing by intramembrane proteolysis (RIP) Site-1 protease (S1P) and Site-2 protease (S2P) (Hong et al., 2004; Shen et al., 2002). This releases nuclear-ATF6 (N-ATF6) transcription factor that subsequently translocates to the nucleus, combines with other transcription factors (NF-Y and YY1, for example) to bind to ERSE's and induce transcription of genes encoding ER stress proteins including BiP, calreticulin and Grp94 (Figure 3) (Thuerauf et al., 2007). Recent studies indicate that two isoforms of ATF6, α and β , may be expressed at temporally different levels in response to ER stress and may regulate responses to different types of stress. The divergence within the N-terminal domain of ATF6 isoforms may be responsible for different patterns of transcriptional activation and protein stability (Thuerauf et al., 2007). There

appears to be “fine tuning” of the ATF6 branch of the UPR so that the different isoforms of ATF6 regulate the strength and duration of the ER stress response (Thuerauf et al., 2007)

The molecular mechanisms of the UPR and protein quality control in the secretory pathway (protein folding, transport and degradation) must be in perfect balance (Hebert and Molinari, 2007). When the protein quality control cycle fails or missteps, the inappropriate retention or disposition of proteins may occur resulting in protein folding diseases (Bedard et al., 2005; Ellgaard and Helenius, 2003; Eriksson et al., 2004). ER stress-mediated signaling pathways have also been implicated in the pathologies of neurodegenerative and cardiovascular diseases, diabetes mellitus, and thyroid, bone and blood disorders (Kadowaki et al., 2004; Schroder and Kaufman, 2005a).

Protein Quality Control in the ER: Calreticulin/Calnexin Cycle

Quality control in the protein secretory pathway consists of a number of molecular chaperones, including calnexin and calreticulin. These molecular chaperones assist during the folding of nascent proteins and shuttling them through the ER as well as sensing misfolded proteins, attempting to refold them or targeting them for degradation (Ellgaard and Helenius, 2003). Proteins that traverse the eukaryotic secretory pathway are targeted to the ER where they are co-translationally inserted through the membrane via the translocon (Rapoport et al., 1996). As the protein emerges in the ER lumen, maturation and processing of the polypeptide chain occurs. This includes cleavage of the signal sequence (Johnson and van Waes, 1999), transfer and modification of N-linked glycans (Figure 2) and disulfide bond formation. In the lumen of the ER, newly synthesized proteins associate with a number of Ca^{2+} -dependent molecular chaperones (Helenius and Aebi, 2004) responsible for their folding (Molinari and Helenius, 2000). Molecular chaperones perform their function by slowing down the folding rate of nascent proteins, thereby allowing the protein to find its lowest energy conformation and preventing aggregation. This results in an increased yield of correctly folded proteins and their multi protein complexes, and also an increase in the rate of correctly folding intermediates (Molinari et al., 2005).

The discovery of the lectin-like chaperones, calreticulin and calnexin, led to the establishment of an elegant carbohydrate-dependent protein folding cycle, so called the calreticulin/calnexin cycle (Bergeron et al., 1994, Helenius and Aebi, 2001). As the nascent protein traverses the translocon as an extended chain (Whitley et al., 1996), and emerges in the lumen of the ER, an enzyme, OST (oligosaccharyl transferase), closely associated with the translocon (Knauer and Lehle, 1999; Shibatani et al., 2005; Silberstein and Gilmore, 1996) recognizes a specific amino acid sequence (NXS/T) (Breuer et al., 2001) and assists in the linkage of oligosaccharide to asparagine residues via an amide linkage. ER luminal enzymes carry out further modification of the oligosaccharide by sequential cleavage of the terminal glucose residues. Glucosidase I removes an initial glucose residue (Kalz-Fuller et al., 1995), while glucosidase II cleaves two further glucose residues (Hammond et al., 1994; Ray et al., 1991). The monoglucosylated intermediate is recognized by the components of the calreticulin/calnexin cycle, which manages the proper processing of glycoproteins that shuttle

through the ER (Helenius and Aebi, 2004). Once the protein has been properly folded, the third glucose is removed by glucosidase II, the protein is released from the quality control cycle and is transported out of the ER. In some cases, this third glucose is prematurely removed and this misfolded or unfolded protein is recognized by another member of the quality control system, UGGT (Fernandez et al., 1998; Parodi, 2000). Incompletely folded glycoproteins can be re-glucosylated by the folding sensor UGGT (Trombetta and Helenius, 2000), thereby promoting further association between incompletely folded glycoproteins and calreticulin/calnexin cycle. This quality control cycle serves as an efficient retention method as unfolded glycoproteins cannot be released from these chaperones unless they are de-glucosylated by glucosidase II and not re-glucosylated by the folding sensor UGGT (Trombetta and Helenius, 2000). This cycle may repeat numerous times until the protein is folded correctly. Alternatively, if the quality control cycle is unable to fold the protein, these misfolded and unfolded proteins accumulate and result in a variety of signaling pathways being activated to control the ensuing ER stress. These include ER assisted protein degradation and the unfolded protein response (UPR). If the outcome of impaired ER protein quality control can not be relieved by ERAD or UPR, then apoptotic pathways are activated. However, in many cases misfolded proteins may aggregate, leading to serious physiological consequences. Accumulation of misfolded proteins is a well-studied feature of various human neurodegenerative diseases. Misfolded proteins can contribute to pathological conditions through diverse mechanisms. For example, if folding or trafficking of proteins is reduced by accumulating misfolded protein and insufficient protein is delivered to its final localization, loss of function will be observed (Grandis et al., 2008). Alternatively, gain of function occurs when the misfolded protein interacts with functional protein, causing conformational changes, or when misfolded protein accumulates and forms toxic aggregates (Yang et al., 2005). Moreover, misfolded proteins may overwhelm the ERAD pathway and significantly decelerate protein degradation causing changes in cell physiology (Fortun et al., 2005).

Although the link between ER protein quality control, ER molecular chaperones and human diseases in many cases still needs to be proven, recent research using developing mouse models where the genes encoding ER chaperones were deleted provides fascinating insights on physiological contribution of ER chaperones *in vivo*.

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Chapter X

Structure, Properties and Multiple Functions of Human Small Heat Shock Protein HspB8 (Hsp22, H11 Protein Kinase or E2IG1)

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Abstract

HspB8 is one of the recently described members of a large family of human small heat shock proteins. This family is presented by ten members that are characterized by a rather small molecular mass (16–28 kDa) and by the presence of a short (80-100 residues) α -crystallin domain. HspB8 is widely expressed in different human tissues and its content is especially high in muscles and nerves. Expression of HspB8 is dependent on many factors and is induced under certain unfavorable conditions. HspB8 tends to form small homooligomers and seems to interact with other members of the family of small heat shock proteins forming different heterooligomers. HspB8 is phosphorylated by a number of different protein kinases and phosphorylation might affect its structure and chaperone-like activity. HspB8 prevents aggregation of partially denatured proteins both *in vitro* and *in vivo*. The detailed mechanism of chaperone-like activity of HspB8 in the cell remains unclear, however the data of literature indicate that HspB8 is able to directly interact with denatured proteins, activates elimination of denatured proteins by enzymes, amyloid proteins, different heat shock proteins, protein kinases, RNA-binding protein SAM68 and biomembranes. The molecular basis underlying interaction of HspB8

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with so many diverse substrates remains unknown; however it is supposed that having an intrinsically disordered structure HspB8 can adopt different conformations suitable for interaction with different ligands. Interacting with so many substrates HspB8 seems to be involved in regulation of apoptosis, cell differentiation and proliferation and protects the cell from accumulation of denatured and aggregated proteins. Therefore point mutations of HspB8 correlate with development of certain neurodegenerative diseases and the level of HspB8 expression might affect cardiac hypertrophy and carcinogenesis.

Introduction

Small heat shock proteins (sHsp) are widely expressed in practically all kingdoms except of certain pathogenic bacteria (Haslbeck et al., 2005, Franck et al., 2004, Narberhaus, 2002). Multiple members of this protein superfamily contain a rather conservative α -crystallin domain consisting of 80-100 amino acid residues and located in the C-terminal part of the protein molecule and usually have a rather small molecular mass that varies in the range of 12-43 kDa (Haslbeck et al., 2005, Sun, MacRae, 2005). The primary structure of sHsp consists of a variable in length N-terminal part, that sometimes contains several sites of phosphorylation (Vos et al., 2008) and that might contain several short stretches of α -helices and β -structures (Kim et al., 1998, van Monfort et al., 2001, Stamler et al., 2005). This variable N-terminal part is followed by conservative α -crystallin domain consisting of seven or eight β -strands packed into two β -sheets forming immunoglobulin-like β -barrel (Kim et al., 1998, van Monfort et al., 2001, Stamler et al., 2005). The very C-terminal part of the small heat shock proteins has variable length and might contain short stretches of α -helices and β -structures. As a rule, the small heat shock proteins tend to form oligomers (Haslbeck et al., 2005, Sun, MacRae, 2005). The size of these oligomers is variable and starts from dimers up to large oligomers consisting of 12-24 monomers (Kim et al., 1998, van Monfort et al., 2001, Stamler et al., 2005). The quaternary structure of sHsp oligomers is very labile and the size and stability of these oligomers is dependent on many factors such as protein concentration, posttranslational modifications, pH, temperature or presence of protein targets. This makes crystallization of sHsp very complicated and until now the three-dimensional structure of only three members of this family, namely Hsp16.5 of *Methanococcus jannaschii* (Kim et al., 1998), wheat Hsp16.9 (van Monfort et al., 2001) and Tsp36 of parasitic worm *Taenia saginata* (Stamler et al., 2005) was described in the literature. All attempts to crystallize mammalian sHsp were unsuccessful and the structure of these proteins was analyzed by means of cryo-electron microscopy (Haley et al., 2000), small angle X-ray scattering (SAXS) (Feil et al., 2001, Michiel et al., 2009), nuclear magnetic resonance (Jehle et al., 2009), electron spin resonance (Berengian et al., 1999) or by crystallization of short fragments of sHsp (Bagneris et al., 2009). These approaches indicate that many structural properties of mammalian sHsp are similar to those of already crystallized proteins and this makes possible molecular modeling of the structure of mammalian sHsp based on the determined structure of *M. jannaschii* Hsp 16.5 or wheat Hsp16.9 (Ghosh, Clark, 2005, Michiel, 2009, Theriault et al., 2004). However, there are important differences in the primary structures of the crystallin domain as well as differences in the length and

structure of the variable N- and C-terminal parts of molecule of different sHsp. This makes it difficult to predict the exact spatial structure of mammalian small heat shock proteins.

Table 1. Classification and some properties of human small heat shock proteins (after Vos et al., 2009, Kappe et al., 2003, Fontaine et al., 2003)

New classification	Old classification	Protein accession number ^a	Number of amino acid residues	Calculated molecular mass, kDa	Calculated isoelectric point, pI	Chromosome location
HspB1	Hsp25 or Hsp27	P04792	205	22.8	6.4	7q11.23
HspB2	MKBP ^b	Q16082	182	20.2	4.8	11q22-q23
HspB3		Q12988	150	17.0	5.9	5q11.2
HspB4	α A-crystallin	P02489	173	19.9	6.2	21q22.3
HspB5	α B-crystallin	P02511	175	20.2	7.4	11q22.3-q23.1
HspB6	Hsp20	O14558	157	17.1	6.4	19q13.12
HspB7	cvHsp ^c	Q9UBY9	170	18.6	6.5	1p36.23-p34.3
HspB8	Hsp22, H11 protein kinase, E2IG1	Q9UKS3	196	21.6	4.7	12q24.23
HspB9		Q9BQS6	159	17.5	9.16	17q21.2
HspB10	ODF1 ^d	Q14990	250	28.4	8.46	8q22.3
HspB11		Q9Y547	144	16.3	4.93	1p32.1-p33

^aProtein accession numbers are from UniProtKB database

^bHspB2 was identified as a myotonic dystrophy kinase – binding protein (MKBP)

^cHspB7 was identified as cardio-vascular small heat shock protein (cvHsp)

^dHspB10 was identified as outer dense fiber protein (ODF1)

The human genome contains ten genes encoding small heat shock proteins (Table 1) (Vos et al., 2008, Kappe et al., 2003, Fontaine et al., 2003). Recently described 16.2 kDa heat shock protein (C1orf41) having distantly similar primary structure was denoted as HspB11 (Bellyei et al., 2007, Vos et al. 2008). The proteins belonging to the family of human small heat shock proteins contain conservative α -crystallin domain, have small subunit molecular mass (16-28 kDa) and as a rule neutral or slightly acidic isoelectric point (except of HspB9 and HspB10). Some of these proteins (such as HspB1, HspB5, HspB6 and HspB8) are ubiquitous, whereas others are predominantly expressed in muscles (HspB2, HspB3, HspB7), eye lens (HspB4), or testis (HspB9 and HspB10) (Taylor, Benjamin, 2005). The human small heat shock proteins seem to be involved in regulation of many different cell processes such as protection from unfavorable conditions, protein quality control, maintenance of cytoskeleton, cell motility, proliferation, apoptosis and many others. Mutation of human small heat shock proteins correlates with development of cataract, certain types of neurodegenerative diseases and myopathies, in addition the level of expression of certain sHsp correlates with tumorigenesis (Vos et al., 2008). A number of reviews dealing with the structure and properties of HspB1 (Arrigo et al., 2007, Salinthon et al., 2008), HspB2 (Hu et al., 2008), HspB4 and HspB5 (Horwitz et al., 2008, Ecroyd, Carver, 2009), HspB6 (Gusev et al., 2005,

Salinthone et al., 2007) were published recently. This review deals with description of structure and properties of HspB8 (Hsp22), a member of a superfamily of human small heat shock proteins.

Discovery of HspB8, Tissue Distribution and Regulation of Expression

Initially HspB8 was described as a putative protein kinase domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (Smith et al., 2000). Analysis of the primary structure of this newly described protein revealed seven motifs characteristic for protein kinases, and immunoprecipitate of this protein was able for autophosphorylation (Smith et al., 2000). Therefore this protein was designated as H11 protein kinase. A year later HspB8 was rediscovered by Benndorf et al. (Benndorf et al., 2001). It has been shown that this protein contains a typical α -crystallin domain and is closely related to other human small heat shock proteins. Since calculated molecular mass of this protein (21.6 kDa) was close to 22 kDa it was designated as Hsp22 (Benndorf et al., 2001). mRNA of HspB8 was detected in practically all human tissues and its content was especially high in smooth, heart and skeletal muscles, placenta, brain, spinal cord, prostate and lung (Benndorf et al., 2001, Gober et al., 2003). This conclusion was confirmed by Verschuure et al. (2003) who using anti-HspB8 antibodies have shown that HspB8 is highly expressed in brain, heart and different muscles of perinatal developing pig and the level of expression is dependent on the stage of development.

The mechanisms underlying regulation of HspB8 expression remain poorly understood. Chowdary et al. (2004) have identified a putative heat-shock factor 1 binding site upstream of the HspB8 transcription start site and found that expression of HspB8 was heat-inducible in the case of MCF-1 cell, but not in the case of HeLa cells. Upregulation of HspB8 gene was detected in the case of amyotrophic lateral sclerosis (Anagnostou et al., 2008), experimental hibernating myocardium (Depre et al., 2004) and transient ischemia in conscious pigs (Depre et al., 2001). At the same time hypoxia induced a dramatic increase of expression of HspB5 (α B-crystallin) and did not affect expression of HspB8 in piglet heart at birth (Louapre et al., 2005). The data presented mean that the mechanism of regulation of HspB8 expression is very complex and is dependent on many different poorly understood factors (such as cell type and/or age).

Intracellular localization of HspB8 is not well-documented, but it is assumed that HspB8 has predominantly cytosolic localization. At the same time the data of Chowdary et al. (2007) indicate that HspB8 effectively interacts with lipid membranes especially with membranes enriched in phosphatidic acid, phosphatidylserine and phosphatidylinositol, i.e. with lipids located in the intracellular leaflet of biological membranes. The pool of HspB8 remaining in cytosol are often co-localized with aggregates formed by partially denatured or improperly folded proteins. For instance, HspB8 became trapped within inclusion bodies formed by polyglutamine protein Huntingtin (Carra et al., 2005). HspB8 is associated with severe cerebral amyloid angiopathy in hereditary cerebral hemorrhage with amyloidosis and with senile plaques in brains with Alzheimer disease (Wilhelmus et al., 2006, 2007). Finally, HspB8 was co-localized with protein aggregates formed by certain myopathy-associated α B-

crystallin mutants (Simon et al., 2007). This type of localization might be connected with chaperone-like activity of HspB8 and its participation in different processes protecting the cell from accumulation of protein aggregates. The mechanism of these processes will be discussed later.

Structure of HspB8 and Its Interaction with Other Small Heat Shock Proteins

The primary structure of HspB8 from different sources is described in the literature (Benndorf et al., 2001, Chowdary et al., 2004) and prediction of the secondary structure performed by different methods (Chowdary et al., 2004, Kasakov et al., 2007) indicates that the C-terminal part of this protein contains a well-conserved α -crystallin domain presented by six β -strands. At the same time the data of circular dichroism (Kim et al., 2004b, Chowdary et al., 2004, Kim et al., 2006) and theoretical predictions indicate that a rather large portion of HspB8 structure is unordered and the estimated random coil content varies between 60 and 80% (Chowdary et al., 2004, Kim et al., 2006). Predictions of disordered regions performed by different programs (Kasakov et al., 2007) indicate that HspB8 belongs to the group of so-called intrinsically disordered proteins (Uversky et al., 2008). This conclusion is supported by the data of circular dichroism, resistance to thermal denaturation and extra high susceptibility of HspB8 to proteolysis (Kasakov et al., 2007, Kazakov et al., 2009). We suppose that the intrinsically disordered structure is of great importance and provides for “chameleon-like” behavior of HspB8 and its ability to interact with a wide number of different proteins.

The quaternary structure of HspB8 remains a controversial issue. Chowdary et al. (2004) postulated that HspB8 is presented in the form of an extended (unordered) monomer. The same viewpoint is presented by Hu et al. (2007). Moreover, the database UniProtKB (access number Q9UKS3) also postulates a monomeric nature for HspB8. If this viewpoint is correct then HspB8 is absolutely unique and is the only one small heat shock protein being monomer. On the other hand on size-exclusion chromatography the elution volume of HspB8 is dependent on the quantity of protein loaded on the column (Kim et al., 2006, Kasakov et al., 2007), chemical cross-linking leads to formation of dimers (and high molecular mass oligomers) (Kim et al., 2004b, Kasakov et al., 2007), the yeast two-hybrid system indicates that HspB8 is able to form dimers (Sun et al., 2004), in cell extracts HspB8 was sedimented with apparent size distribution consistent with the formation of dimers (or even tetramers) (Chavez Zobel et al., 2003) and the two-dimensional electrophoresis of cell extracts indicates the presence of both monomer and dimer of HspB8 (Benndorf et al., 2001). We suppose that these data indicate that both *in vivo* and *in vitro* HspB8 is presented in the form of a dimer that is in equilibrium with the monomer and equilibrium between these forms is dependent on protein concentration, posttranslational modifications and presence (and concentration) of other protein partners.

At the very beginning of investigation it has been shown that HspB8 can be phosphorylated (Smith et al., 2000, Benndorf et al., 2001). Moreover, it was postulated that HspB8 possesses intrinsic protein kinase activity and undergoes autophosphorylation (Smith

et al., 2000, Depre et al., 2002, Gober et al., 2003). Further investigations confirmed that isolated HspB8 can undergo autophosphorylation (Chowdary et al., 2004), however, the extent and the rate of autophosphorylation was extremely low (Kim et al., 2004b). Therefore we concluded that the autokinase activity ascribed to HspB8 is not an intrinsic property of this protein, but is due to contamination by trace amounts of protein kinases that are co-purified with HspB8 (Kim et al., 2004b). This viewpoint was criticized by Gober et al. (2004), who advocated the protein kinase activity of HspB8. It is worthwhile to mention that initially it was postulated that the structure of HspB8 is similar to that of the so-called protein kinase domain of herpes simplex virus type II ribonucleotide reductase (Smith et al., 2000). However, the data of literature indicate that highly purified ribonucleotide reductase lacks intrinsic protein kinase activity and that the protein kinase activity earlier ascribed to this enzyme is due to contamination by exogenous protein kinases (probably casein kinase 2) (Langelier et al., 1998). Moreover, the similarity between HspB8 and the N-terminal part of herpes simplex virus type 1 and 2 ribonucleotide reductase subunit R1 was confirmed (Chabaud et al., 2003). However, this similarity is due not to the presence of a putative protein kinase domain, but to the presence of a conservative α -crystallin domain presented in both proteins (Chabaud et al., 2003). Phylogenetic investigations failed to detect any relationship between HspB8 (or any other small heat shock proteins) with protein kinases (Franck et al., 2004, Sun et al., 2004). Therefore, at present the intrinsic protein kinase activity of HspB8 seems to be very unlikely (Kim et al., 2004b, Shemetov et al., 2008b).

Isolated HspB8 is phosphorylated by a number of protein kinases *in vitro*. For instance protein kinase C phosphorylates Ser14 and Thr63, p44 mitogen activated protein kinase phosphorylates Ser27 and Thr87, whereas casein kinase 2 phosphorylates unidentified sites of HspB8 (Benndorf et al., 2001). Two serine residues of HspB8, namely Ser24 and Ser57, are located in consensus sequences RXS recognized by cAMP-dependent protein kinase, and this enzyme effectively phosphorylates Ser57 of HspB8 *in vitro* (Shemetov et al., 2008a). Mutations mimicking phosphorylation (S24D, S57D or S24,57D) decreased stability of HspB8 to proteolysis, affected its quaternary structure and decreased chaperone-like activity of HspB8 measured with insulin as a model protein substrate (Shemetov et al., 2008a). Recently introduced powerful proteomic methods have shown that *in vivo* HspB8 is phosphorylated at Ser24 (Dephoure et al., 2008, Cantin et al., 2008) and at Ser(Thr)87 (Villen et al., 2007). It was also found that in non-small cell lung cancer cells HspB8 can be phosphorylated at Tyr118 (Rikova et al., 2007). At present not all sites of phosphorylation detected *in vitro* coincide with the sites determined *in vivo* and physiological significance of phosphorylation of many sites of HspB8 remains enigmatic. However, we might conclude that Ser24 is phosphorylated *in vivo* by Pro-directed protein kinases (Dephoure et al., 2008, Cantin et al., 2008) (but not by cAMP-dependent protein kinase) and that phosphorylation of this site might affect the structure and chaperone-like activity of HspB8 (Shemetov et al., 2008a). We suppose that it is of great importance to analyze effect of phosphorylation of Thr87 (that seems to be phosphorylated both *in vivo* and *in vitro*) on the structure and properties of HspB8. Except of Tyr118 all potential sites of phosphorylation are located in the poorly ordered N-terminal end of HspB8 (Figure 1) and might play an important role both in intersubunit interaction and in interaction of HspB8 with different protein substrates. This makes further investigation of HspB8 phosphorylation very important.

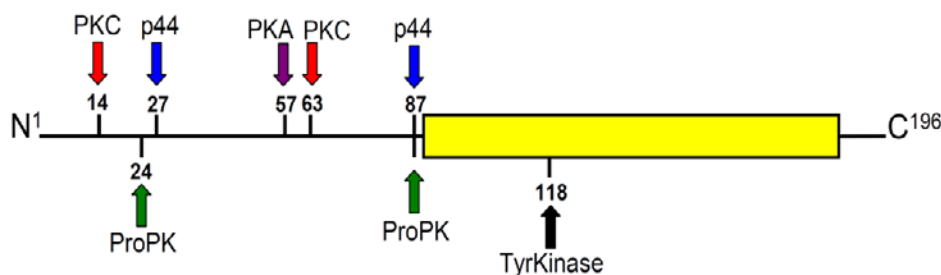


Figure 1. Location of potential sites of HspB8 phosphorylation. Location of the sites of phosphorylation and potential protein kinases involved in phosphorylation are marked by differently colored arrows. Crystallin domain is shown as a yellow rectangle. The sites marked above the scheme were detected *in vitro*, the sites indicated below the scheme were detected *in vivo*. PKC, protein kinase C; PKA, protein kinase A; ProPK, Pro-directed protein kinases; p44, p44 MAP-kinase; TyrKinase, protein kinase phosphorylating Tyr residues.

Interaction of HspB8 with other small heat shock proteins was analyzed in a number of publications. At first HspB8 was described as a protein specifically interacting with phosphomimicking mutant of HspB1 (Hsp27) (Benndorf et al., 2001). Later using the yeast two hybrid system, fluorescence resonance energy transfer (FRET), and size exclusion chromatography (SEC) it has been shown that HspB8 interacts with itself as well as with the wild type HspB1, HspB2 (MKBP), HspB7 (cvHsp) (Sun et al., 2004). Later interaction of HspB8 with HspB7 was confirmed by modern proteomic methods (Rual et al., 2005). Analysis of interaction of HspB8 with other small heat shock protein was extended and by using the yeast two-hybrid system, FRET and SEC it has been shown that HspB8 interacts with HspB3, HspB5 (α B-crystallin) and HspB6 (Hsp20) (Fontaine et al., 2005). Mutations mimicking phosphorylation of Ser15 of HspB1 or homologous Ser16 of HspB6 affects their interaction with HspB8, whereas mutation mimicking phosphorylation of Ser19 of HspB5 had no significant effect on its interaction with HspB8 (Sun et al., 2006) (Table 2). It is important to mention that HspB8 interrupts formation of amyloid oligomers (Sanbe et al., 2007) and delay formation of aggresomes formed by myopathy-causing R120G mutant of α B-crystallin (HspB5) (Chavez Zobel et al., 2003). Summing up we may conclude that HspB8 interacts with practically all sHsp (except of HspB9-HspB11) and this interaction is dependent on mutations (and probably posttranslational modifications) of a protein partner (Table 2). At the same time it is worthwhile to mention that stability of complexes formed by HspB8 seems to be quite variable. For instance, all attempts to detect interaction of HspB8 with wild type HspB1 by means of immunoprecipitation or chemical crosslinking were unsuccessful (Sun et al., 2004) (Table 2). HspB8 did not form stable heterooligomeric complexes and only weakly co-immunoprecipitated with the wild type HspB1 or HspB5 (Chavez Zobel et al., 2003). Thus, although HspB8 seems to be able to interact with practically all small heat shock proteins the stoichiometry and stability of the complexes formed and the significance of this interaction need further detailed investigation.

Table 2. Protein partners interacting with HspB8

Proteins	Methods used for elucidation of interaction	Reference	Potential role	Comments
HspB1	Yeast two-hybrid system, FRET; <i>co-immunoprecipitation and cross-linking were negative</i>	Sun et al., 2004 Sun et al., 2006	Chaperone-like activity	Under certain conditions interaction is dependent on phosphorylation mimicking mutation S15D of HspB1
Phosphomimicking 3D mutant of HspB1	Yeast two-hybrid system	Benndorf et al., 2001	Chaperone-like activity	
HspB2	Yeast two-hybrid system, co-immunoprecipitation, FRET	Sun et al., 2004		
HspB3	Yeast two-hybrid system, <i>data of FRET were negative</i>	Fontaine et al., 2005	Chaperone-like activity	
HspB5	Yeast two-hybrid system, FRET, size-exclusion chromatography	Fontaine et al., 2005 Sun et al., 2006	Chaperone-like activity	Interaction is independent on phosphorylation mimicking mutation S19D of HspB5
Myopathy and cataract causing mutants of HspB5	Co-immunoprecipitation FRET, yeast two-hybrid system	Sanbe et al., 2007 Simon et al., 2007 Chavez Zobel et al., 2003	Chaperone-like activity	
HspB6	Yeast two-hybrid system, FRET, size-exclusion chromatography	Fontaine et al., 2005, Sun et al., 2006	Chaperone-like activity	Under certain conditions interaction is dependent on phosphorylation mimicking mutation S16D of HspB6
HspB7	Yeast two-hybrid system, co-immunoprecipitation, FRET	Sun et al., 2004	Chaperone-like activity	
HspB8	Yeast two-hybrid system, co-immunoprecipitation, cross-linking	Sun et al., 2004	Chaperone-like activity	
Bag3	Co-immunoprecipitation, yeast two-hybrid system	Carra et al., 2008a, 2008b, 2009 Rual et al., 2005	Regulation of macroautophagy	
Proteasome	Co-immunoprecipitation	Hedhli et al., 2008	Regulation of proteolysis and proteasome localization	
Phosphoglucosyltransferase	Yeast-two hybrid system, co-immunoprecipitation	Wang et al., 2004	Regulation of metabolism	
Sam68	Yeast two-hybrid system, pull-down assay, co-immunoprecipitation	Badri et al., 2006	Gene expression	
Casein kinase 2	Co-immunoprecipitation	Hase et al., 2005	Inhibition of casein kinase activity	

Putative Functions of HspB8

Chaperone-like activity of HspB8

As with other small heat shock proteins, HspB8 prevents aggregation of partially denatured or unfolded proteins, i.e. possesses chaperone-like activity. For instance, *in vitro* HspB8 prevents heat-induced aggregation of yeast alcohol dehydrogenase, bovine liver rhodanase (Kim et al., 2004b, Kasakov et al., 2007) and citrate synthase (Chowdary et al., 2004) as well as reduction-induced aggregation of insulin (Kim et al., 2004b, Chowdary et al., 2004, Kasakov et al., 2007). The chaperone-like activity of HspB8 was also detected *in vivo*. As already mentioned, HspB8 effectively prevents formation of amyloid oligomers formed by R120G mutant of α B-crystallin (HspB5) expression of which correlates with desmin-related cardiomyopathy (Sanbe et al., 2007). Acting as a true chaperone, HspB8 seems to assist proper folding of R120G mutant of α B-crystallin and thus prevents formation of aggresomes (Chavez Zobel et al., 2003). Other myopathy-associated α B-crystallin mutants, i.e. Q151X (deletion of the C-terminal amino acid residues 151-175) and 464delCT (deletion of two nucleotides causing frame shifting, change in the primary structure and length of the C-terminal end) were analyzed for their interaction with HspB8. It has been found that the mutant Q151X of α B-crystallin interacts with HspB8 weaker than the wild type protein, whereas mutation 464delCT does not affect interaction of mutated α B-crystallin with HspB8 (Simon et al., 2007).

In the cells expressing proteins with polyglutamine tails (fragments of Huntingtin (Httg43Q) and androgen receptor containing 65 Gln residues) HspB8 was occasionally trapped within inclusions, however in many cells it effectively prevented formation of inclusions and effect of HspB8 was more pronounced than the effect of HspB1 (Carra et al., 2005). HspB8 was detected in senile plaques of Alzheimer's disease brains. Moreover it was shown that HspB8 directly interacts with amyloid β -peptides ($A\beta_{1-42}$, and $A\beta_{1-40}$) and inhibited death of cerebrovascular cells mediated by Dutch type $A\beta_{1-40}$ by reduction in both β -sheet formation and accumulation of amyloid peptide on the cell surface (Wilhelmus et al., 2006).

Two mutations (K141N and K141E) of HspB8 were described in the literature (Irobi et al., 2004, Tang et al., 2005). Both these mutations correlate with development of distal hereditary motor neuropathy type II (dHMN II) (Irobi et al., 2004), whereas mutation K141N correlates with development of common inherited motor and sensory neuropathy, Charcot-Marie-Tooth type 2 disease (CMT2L) (Tang et al., 2005). Later these finding were confirmed (Dierick et al., 2008) and it has been found that point mutations of HspB1 and HspB8 correlate with development of dHMN II and CMT2L (Dierick et al., 2008) and that the mutation of the HspB8 gene is rather rare (Houlden et al., 2008). It is worthwhile to mention that certain mutations of HspB1 and HspB8 correlating with development of different forms of neuropathies are located in homologous positions or very close in the primary structures of two small heat shock proteins (K141 of HspB8 and homologous to this residue R136 of HspB1, or residue S135 of HspB1, Table 3) (Benndorf, Welsh, 2004, Houlden et al., 2008, Ikeda et al., 2009). The above mentioned mutants of HspB8 tend to aggregate inside of the cell and showed greater binding to the wild type HspB1 (Irobi et al., 2004). Both mutations of HspB8 lead to increased interaction of mutated HspB8 with itself, HspB1 and α B-crystallin,

and it was supposed that aggregation of mutated HspB8 inside of the cell can be at least partially explained by increased association with other small heat shock proteins (Fontaine et al., 2006). Mutations K141E, K137E or a double mutation K137,141E induce disordering of the secondary structure and probably therefore increases the susceptibility to proteolysis of HspB8, decrease the probability of dissociation of small oligomers of HspB8 and decrease the chaperone-like activity of HspB8 measured *in vitro* (Kim et al., 2006, Kasakov et al., 2007). Mutations K141E and K141N decreased the chaperone-like activity of HspB8 with polyglutamine proteins measured *in vivo* (Carra et al., 2005).

Table 3. Hot spots located in $\beta 5$ - $\beta 7$ strands of α -crystalline domain of small heat shock proteins mutations of which correlate with certain congenital diseases. Mutated residues are marked red and underlined.

Protein	Primary structure	Congenital diseases
HspB1 (Hsp27)	¹²⁴ HEE <u>R</u> Q DEHGY I <u>S</u> R <u>C</u> F <u>T</u> R <u>K</u> Y <u>T</u> L ¹⁴⁴	Distal hereditary motor neuropathy, Charcot-Marie-Tooth disease type 2
HspB4 (α A-crystallin)	¹⁰⁰ HNERQ DDHGY ISREF H <u>R</u> R <u>Y</u> R L ¹²⁰	Dominant cataract
HspB5 (α B-crystallin)	¹⁰⁴ HEE <u>R</u> Q DEHGF ISREF H <u>R</u> KYR I ¹²⁴	Desmin-related myopathy, cataract
HspB8 (Hsp22)	¹²⁵ HEEKQ QEGGI VSKNF <u>T</u> K <u>K</u> IQ L ¹⁴⁵	Distal hereditary motor neuropathy, Charcot-Marie-Tooth disease type 2

Summing up, we might conclude that HspB8 possesses chaperone-like activity and therefore prevents aggregation of partially unfolded or denatured proteins both *in vivo* and *in vitro*. This property of HspB8 is especially important for nervous and muscle tissues where accumulation of aggregated proteins might lead to different diseases such as Alzheimer's disease, distal hereditary motor neuropathies, Charcot-Tooth-Marie type 2 disease or different forms of myopathies. Mutations inside of α -crystallin domain destabilize the structure of HspB8, affect its interaction with other small heat shock proteins and decrease its chaperone-like activity. All these factors might contribute to development of different inherited neuromuscular diseases.

Probable involvement of HspB8 in regulation of protein degradation

Accumulation of aggregates of partially denatured or unfolded proteins inside of the cell can be prevented in different ways. True chaperones interact with unfolded proteins, form highly soluble complexes and in this way prevent formation of insoluble protein aggregates. Formation of aggregates can also be achieved by selective proteolytic degradation of damaged proteins. The data of literature indicate that HspB8 might play an important role in intracellular degradation of misfolded proteins. For instance, cardiac hypertrophy induced by overexpression of HspB8 is accompanied by an increase of proteasomic activity and in translocation of proteasomes to the nuclear periphery. Perinuclear located proteasomes were co-localized and interact with HspB8 (Hedhli et al., 2008). Moreover inhibition of

proteasomes reverses cardiac hypertrophy induced by HspB8 overexpression. These data may indicate that HspB8 interacts with damaged proteins and addresses them to proteasome for proteolytic degradation.

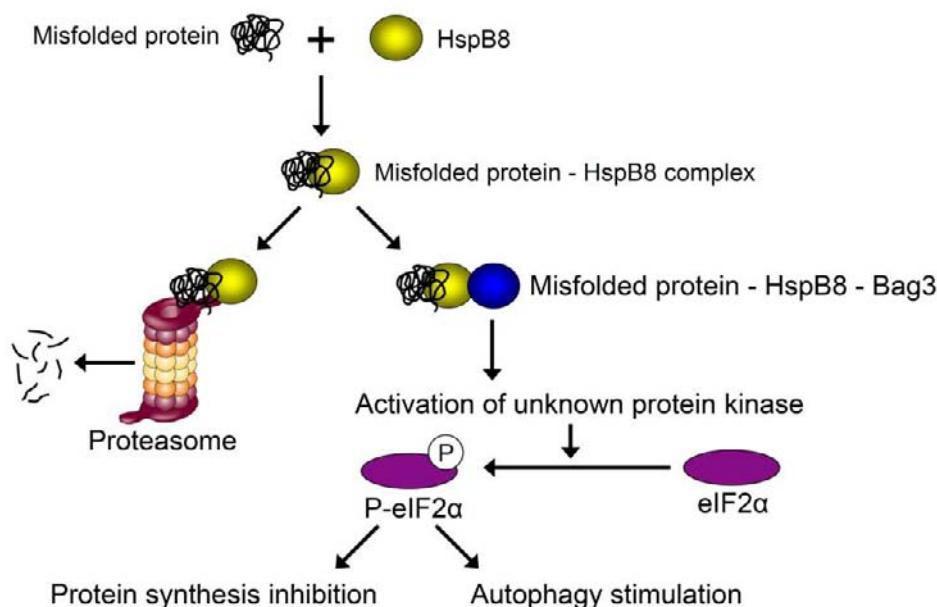


Figure 2. Hypothetical scheme of HspB8 participation in misfolded protein degradation.

Recently published data indicate that HspB8 specifically interacts with an important adapter protein Bag3 (Carra et al., 2008a, Rual et al., 2005). Bag3 contains three domains: WW domain responsible for recognition of proline-rich domains, so-called PXXP domain, that is rich in Pro and can interact with WW- or SH3 domains of target proteins, and finally BAG domain mediating interaction with Bcl2 and Hsp70 (Takayama, Reed, 2001). It was supposed that HspB8 interacts with misfolded proteins and binds to Bag3. WW- and PXXP domains of Bag3 somehow stimulate a signaling cascade leading to formation of autophagic vacuoles and activate macroautophagy providing degradation of misfolded proteins (Carra et al., 2008a, Carra et al., 2008b). This mechanism seems to be correct, at least in the case of polyglutamine containing protein Httg43Q since inhibition of macroautophagy or knockdown of Bag3 prevented HspB8-dependent degradation of Httg43Q (Carra et al., 2008a). New details of this process were described recently (Carra et al., 2009, Carra, 2009). The complex of HspB8-Bag3 activates still unknown protein kinases that phosphorylate translation initiation factor eIF2 α . Phosphorylation of eIF2 α shuts down translation of mutated or misfolded proteins and at the same time stimulates transcription/translation of specific transcription factors (such as ATF4 and consequently Atg12). Atg12 stimulates autophagy and by this means provides degradation of misfolded proteins (Figure 2). If this scheme is correct, then HspB8 controls accumulation of misfolded proteins by two different

mechanisms, inhibiting synthesis of this protein and by simultaneous stimulation of its macroautophagy and proteolytic degradation.

Effects of HspB8 on gene expression, carcinogenesis and apoptosis

HspB8 seems to be involved in a number of different processes underlying gene expression, myocardial hypertrophy, cell proliferation, carcinogenesis and apoptosis. Recently published data (Badri et al., 2006) indicate that HspB8 can interact with Sam68 both *in vivo* and *in vitro*. Sam68 is an RNA-binding protein that might be implicated in cell proliferation and tumorigenesis. Overexpression of HspB8 was accompanied by inhibition of Sam68-dependent expression of two independent reporter genes and this inhibition seems to be induced by direct interaction of HspB8 with Sam68 (Badri et al., 2006). It is worthwhile to mention that Sam68 associates with cell Src-kinase (Badri et al., 2006). Therefore if HspB8 indeed interacts and competes with Src-kinase for interaction with Sam68, then HspB8 will be able to regulate localization and/or activity of this important protein kinase.

Treatment of human breast cancer cells with 17β -estradiol (E_2) is accompanied by increased expression of certain genes and among them gene E2IG1 coding for HspB8 (Charpentier et al., 2000). Further investigations have shown that expression of HspB8 was increased by estrogen E_2 in receptor-positive MCF-7 breast cancer cells (but not in estrogen negative MDA-MB-231 cells) and that expression of HspB8 can also be increased after treatment with cadmium, an environmental pollutant with estrogen activity (Sun et al., 2007). Overexpression of cyclin D1 has dual effect on breast cancer cells. On the one hand increased expression of cyclin D1 correlates with tumor invasiveness, on the other hand cyclin D1 is known to enhance radiation sensitivity of cancer cells. The detailed mechanism of this action remains unknown, however, overexpression of HspB8 increased radiation sensitivity, whereas HspB8 small interfering RNA prevented cyclin D1-induced enhancement of radiation sensitivity (Trent et al., 2007). Increased expression of HspB8 was detected in invasive human cancer (ductal carcinoma) compared with preinvasive or normal samples and expression of HspB8 resulted in anchorage independence, increased cell proliferation and protection against apoptosis (Yang et al., 2006).

Stable transfection of HEK293 cells with HspB8 also induced anchorage-independent growth (Smith et al., 2000). Moreover, like other small heat shock proteins (such as HspB1 and HspB5) HspB8 demonstrated antiapoptotic activity in stomach tumors and rat pheochromocytoma (PC12) cells (Gober et al., 2005). However, this effect is cell-dependent. For instance, in SK-MEL-2 (melanoma), PC-3 (prostate cancer), and TC32 (Ewing's sarcoma family tumor) cells the level of HspB8 expression was reduced relative to the normal cell counterparts. DNA demethylation leading to increased expression of HspB8 or transient transfection with HspB8 expression vector induces caspase- and p38 MAPK-dependent apoptosis in these cell lines (Gober et al., 2003). These data indicate that depending on the cell line and on the level of expression HspB8 can possess both pro- and antiapoptotic activity.

A large series of investigations were undertaken in order to analyze effect of HspB8 in cardiomyocytes. Expression of HspB8 was increased in response to transient ischemia and this was interpreted as a sign of probable involvement of this protein in cell survival (Depre et al., 2001). Chronically dysfunctional myocardium in patients with coronary artery disease

that is designated as hibernating myocardium was also characterized by increased levels of both HspB8 RNA and expressed protein (Depre et al., 2004). At the same time in infarcted regions of myocardium the level of HspB8 was dramatically decreased compared with unaffected regions. This means that HspB8 plays an important housekeeping role in survival program, but seems to be ineffective in the case of irreversible injury (Depre et al., 2004). Increased (2-7 fold) expression of HspB8 in cardiac specific transgene mouse was accompanied by 30% increase of the heart weight/body weight ratio and concentric heart hypertrophy (Depre et al., 2002). In this case increased expression of HspB8 correlated with increased expression of glucose transporter GLUT1 in plasma membrane, increased glycogen content and activation of phosphoglucosyltransferase, that was able to directly interact with HspB8 (Wang et al., 2004). It was concluded that HspB8 is an integrative sensor playing an important role in cardiac adaptation by coordinating cell survival, growth and energetic metabolism (Wang et al., 2004). The effect of HspB8 on cardiomyocytes seems to be very complicated. At low doses adenovirus-mediated expression of HspB8 was accompanied by hypertrophy of cardiomyocytes and this effect seems to be dependent on the direct interaction with, and activation of Akt by HspB8 (Hase et al., 2005). On the contrary, expression of high doses of HspB8 decreased or prevented hypertrophy and induced apoptosis of myocytes. Point mutation K113G switching off putative protein kinase activity of HspB8 prevented apoptosis induced by increased expression of this protein (Hase et al., 2005). It is worthwhile to mention that this mutation will affect and will probably completely destroy β 4 of HspB8 and therefore might change not only putative protein kinase activity, but the whole structure of HspB8. According to Hase et al. (2005) the proapoptotic effect of HspB8 is dependent on its interaction and inhibition of casein kinase 2 and both these effects depend on HspB8 structure, since mutation K113G abrogates both interaction and inhibition of casein kinase.

Summing up, we might conclude that depending on the cell type and on the level of expression HspB8 can possess either anti- or proapoptotic activity. It is supposed that hypertrophic and probably antiapoptotic effect of HspB8 is at least partly dependent on its interaction with Akt protein kinase (Hase et al., 2005), activation and redistribution of proteasomes (Headly et al., 2008) and its chaperone-like effect. Proapoptotic effect of HspB8 seems to be caspase- and p38 MAPK-dependent (Gober et al., 2003) and can be induced by interaction with and inhibition of casein kinase 2 by HspB8 (Hase et al., 2005). The data of literature (Takayama, Reed 2001, Carra et al., 2008a, 2008b) indicate that both HspB8 and the antiapoptotic protein Bcl2 can interact with Bag3. If these proteins compete with each other for interaction with Bag3, than HspB8 indirectly participates in regulation of apoptosis.

Probable involvement of HspB8 in immune response

The data of literature (Srivastava, 2002) indicate that HspA (Hsp70), HspC (Hsp90) and HspD (Hsp60) can be directly involved in immune response. It is postulated that these heat shock proteins bind damaged or foreign (viral, bacterial or tumor) proteins or their fragments and after necrosis are liberated in the blood. The complexes of heat shock proteins with corresponding peptides interact with Hsp-receptors (CD91) on antigen presenting cells (APC) or on mature dendritic cells (DC). The peptides initially bound to the heat shock proteins undergo complex processing with involvement of proteasome and transporter associated with

antigen processing followed by secretion and binding to major histocompatibility complex I molecules on the surface of antigen presenting cells (Srivastava, 2002). Moreover, binding of heat shock proteins or their complexes with peptides to other unidentified receptors (probably Toll-receptors) might induce synthesis of interleukin-12 (IL 12), interleukin 1 β (IL 1 β), tumor necrosis factor α (TNF α) and nitrogen oxide (NO). Recently published data indicate that HspB8 can also be involved in such processes. For instance, HspB8 was abundantly expressed in synovial tissue of patients with rheumatoid arthritis and interacting with toll-like receptor 4 induced increased production of interleukins 6, 10 and 12 as well as TNF α (Roelofs et al., 2006). It was suggested that HspB8 (and, probably, α A-crystallin) might play an important role during the inflammatory processes in autoimmune diseases. This suggestion correlates with recent findings that HspB8 associated with cerebral amyloid angiopathy in hereditary cerebral hemorrhage with amyloidosis of the Dutch type and with senile plaques in Alzheimer's disease (Wilhelmus et al., 2006) increases secretion of interleukin-6 by cultured human brain pinocytes, astrocytes and microglia and by this means provokes inflammation accompanying cerebral hemorrhage and Alzheimer's disease (Wilhelmus et al., 2009).

Conclusions

HspB8 is one of the members of a large family of human small heat shock proteins. HspB8 is ubiquitously expressed in different human tissues and is involved in many important processes, such as quality control of proteins inside of the cell, gene expression, cell proliferation and apoptosis. Point mutations of HspB8 correlate with development of different inherited predominantly neuromuscular diseases. HspB8 interacts with many different protein kinases and undergoes multisite phosphorylation, however the exact role of HspB8 phosphorylation remains poorly understood. Unusual ability to interact with a huge number of different proteins (Table 2) is at least partially due to intrinsically disordered structure of HspB8. All of these unique properties suggest that HspB8 (as other proteins such as p53, sinuclein, certain DNA-binding proteins etc.) is located in the node of network underlying regulation of a number of different and important processes in the cell.

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Chapter XI

Small Stress Proteins and Their Therapeutic Potential

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Abstract

Small stress or heat shock proteins (sHSP) are involved in protective activity against some of the most important pathologies affecting human health including cancer and neurodegeneration. The fundamental molecular mechanism for the function(s) of small stress proteins (sHSPs or small heat shock proteins) are just beginning to be understood. The archetype for small stress proteins is human alphaB crystallin where multiple interactive sequences were identified using protein pin arrays and confirmed using site directed mutagenesis. A dynamic equilibrium between subunits of alphaB crystallin and with large polydisperse complexes has been described. The interactive domains were mapped to a homology model of the surface of the small stress proteins. The surface exposure of the interactive domains varies with unfolding and self assembling proteins. Peptides were synthesized on the basis of the interactive sequences and were found to be active in assays for protection against proteins associated with protein unfolding diseases. The equilibrium between sHSP subunits and assembled complexes may be a dynamic mechanism for regulation of stress protein function. Characterization of the functional mechanism of small stress protein action is expected to lead to novel therapies for diseases of aging.

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Introduction

Small heat shock proteins, sHSPs, are transforming our ideas on endogenous protective mechanisms against molecular aging in cells. sHSPs are a family of stress proteins of molecular weight less than 43kD that are upregulated in response to pathologies characterized by protein unfolding and aggregation, the molecular phenotypes of neurodegeneration, cardiovascular disease, age related macular degeneration (AMD) and cataract, well known threats to the quality of life for aging humans (Head et al 1993; Vicart et al 1998; Vleminckx et al 2002; Arrigo & Muller 2002; Crabb et al. 2002; Bloemendahl et al 2004; Dabir et al 2004; Nakata et al 2005, Benjamin et al 2007; Kampinga et al 2008; Quraishie et al 2008; Vos et al 2008). In protein unfolding diseases, modest alterations in the distribution of surface charge or hydrophobicity can influence molecular conformation and protein – protein interactions to favor aggregation (He et al 2005, Muchowski & Wacker 2005; Duennwald et al 2006; Roberson & Mucke 2006; Skovronsky et al 2006; Teplow et al 2006; Guo & Eisenberg 2007; Ono et al 2008; Saibil 2008; Cisse & Mucke 2009; Ecroyd & Carver 2009). While not recognized as protein unfolding diseases, tumorigenesis and cancer are known to increase in prevalence with aging and are associated with the upregulation of sHSPs (Aoyama et al 1993; Takashi et al 1998; Calderwood 2006; Moyano et al 2006; Pozsgai 2007; So 2007;), an association which may account for the interactions between stress proteins and regulatory proteins involved in maintaining cellular homeostasis (Ghosh et al 2007a). In the absence of pathological stress, protein – protein interactions are regulated by post translational modifications that can influence normal cell proliferation, cell shape, adhesion, signaling, migration or cell death (apoptosis), which are important dynamic processes in cells and tissues (Cherian & Abraham 1995; Liu et al 2004; Wyttenbach 2004; Gaestel 2006; Bellyei et al 2007; Lanneau et al 2008; Nicolau et al 2008). The special and unique activity of stress proteins in normal cell functions and in protective cell mechanism(s) makes the sHSPs ideal candidates for novel therapeutics against aggregation diseases of cell and molecular aging (Haslbeck et al 2005; Nakamoto & Vigh 2007; Arrigo et al 2007).

Important clues to the protective mechanism(s) of sHSPs and stress proteins can be found through a thorough analysis of the primary structure of alphaB crystallin, a prominent lens protein, discovered to be a sHSP upregulated to protect against protein unfolding and aggregation diseases in aging. Our approach is to identify the specific interactive sequences responsible for the protective activity of alphaB crystallin. We seek to characterize the recognition and response functions against protein unfolding that are necessary for protection against aggregate formation, for stabilization of the assembly of filaments and formation of protective protein complexes. The proteins that interact with alphaB crystallin come from diverse families of proteins involved in dynamic cellular activities that may require functional stability for long time periods in living cells (Haslbeck et al 2005; Nakamoto & Vigh 2007). The time periods can be as long as the lifetime of the individual organism for neurons, cardiomyocytes and transparent lens cells, where long term stability is required to protect against the abnormal effects of aging on filamentous cytoskeletal structures. The protective function of human alphaB crystallin depends on high sensitivity for recognition of protein unfolding and dynamic responses both to native and unfolding proteins. This review summarizes recent studies of the identification of interactive sequences in human alphaB

crystallin, mapping of their interactive surface domains, and modeling of their functions in the dynamic molecular mechanisms that regulate the self-assembly of alphaB crystallin subunits, cytoskeletal proteins, and unfolding protein in protein aggregation diseases. We expect that defining the structural basis for the protective interactions responsible for the stress response of alphaB crystallin to dysfunctional unfolding proteins will provide new insights for the design of novel therapeutics against protein aggregation disorders.

Human Disease and Stress Proteins

There is compelling experimental support for the importance of protein – protein interactions in the protective cellular response of stress proteins (sHSPs) to diseases of aging from neurodegeneration to cancer (Table 1). Common protein targets for the protective response of the sHSPs are self assembling proteins in the cytoskeleton, amyloid fibrils, unfolding proteins, and regulatory molecules, such as β -catenin and growth factors (Arai & Atomi 1997; Ghosh et al 2007a; Liang 2000; Perng et al 1999; Xi et al 2006). sHSP – unfolding protein interactions can contribute to the formation of disease causing protein aggregates. For example, in the ocular lens, mutations and post-translational modifications of α A and α B crystallin lead to the aggregation of lens crystallins and cytoskeleton causing cataract (Devi et al 2008; Litt et al 1998; Perng et al 2007; Santhiya et al 2006; Simon et al 2007). In the retina, upregulation of α B crystallin has been linked to age-related macular degeneration (AMD) and α B crystallin is a component of Drusen (Crabb et al 2002; De et al 2007; Johnson et al 2005; Nakata et al 2005). sHSP27 and α B crystallin are upregulated in the brain where they were associated with amyloid aggregates in Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob disease, and familial amyloidotic polyneuropathy (Renkawek et al 1994; Renkawek et al 1992; Renkawek et al 1999; Wang et al 2008; Wilhelmus et al 2006). In amyotrophic lateral sclerosis (ALS), sHSP27 and α B crystallin are upregulated and have been found in aggregates (Vlemminckx et al 2002; Wang et al 2008). Mutations in sHSP27 and sHSP22 are linked to Charcot-Marie-Tooth disease and distal hereditary motor neuropathy, possibly due to a defective interaction with neurofilaments (Evgrafov et al 2004; Irobi et al 2004; Tang et al 2005). Mutations in GFAP cause sHSP27 and α B crystallin to co-aggregate in Alexander's disease and mutations in α B crystallin produce similar co-aggregation with desmin leading to cardiomyopathy and desmin-related myopathy (Evgrafov et al 2004; Irobi et al 2004; Tang et al 2005; Simon et al 2007). Our interest is in identifying the primary sequences of the interactive surface domains on human alphaB crystallin, the archetype sHSP, and mapping the exposed interactive domains to the surface of the sHSP (Ghosh et al 2005a,2005b; 2006a;2006b;2006c; 2007a; 2007b;2007c; 2008a). Because the interactions with unfolding target proteins are dynamic they are expected to involve weak, non-covalent interactions. For this reason the mechanism of protective action can be expected to be unique to sHSPs and very different from the families of large HSPs. Advances in understanding the interactions between sHSPs and their target proteins under stress will provide opportunities for development of innovative therapies against protein unfolding and aggregation during aging.

Table 1. Small Heat-Shock Protein and Stress Protein Associated Diseases

Disease	Organ	sHSP	Aggregate proteins	Type	Notes
Alexander disease	Brain	HSP27, α B	GFAP	filament	HSP27 and α B crystallin coaggregate with GFAP filaments
Alzheimer's disease	Brain	HSP27, α B	β amyloid	amyloid	α B crystallin and HSP27 present in neurofibrillary tangles
Amyotrophic lateral sclerosis (ALS)	Brain	HSP27, α B	neurofilaments	filament	Presence of α B crystallin and HSP27 positive aggregates
Charcot-Marie-Tooth disease	Brain	HSP27, HSP22	neurofilaments	filament	Linked to mutations in HSP27 and HSP22
Creutzfeldt-Jakob disease	Brain	HSP27, α B	prion protein	amyloid	α B crystallin and HSP27 upregulated in disease
distal hereditary motor neuropathy	Brain	HSP27	neurofilaments	filament	Linked to mutations in HSP27
familial amyloidotic polyneuropathy	Brain	HSP27	transthyretin	amyloid	HSP27 upregulated in presence of aggregates
Parkinson's disease	Brain	HSP27, α B	α synuclein	amyloid	α B crystallin and HSP27 present in Lewy bodies
Age-related macular degeneration	Eye	α B	Drusen	unfolding proteins	α B crystallin present in Drusen aggregates
Cataract	Eye	α A, α B	β/γ crystallin, CP49, filensin, vimentin	unfolding proteins, filament	
Cardiomyopathy	Heart	α B	desmin	filament	α B crystallin coaggregates with desmin filaments
Desmin-related myopathy	skeletal muscle	α B	desmin	filament	α B crystallin coaggregates with desmin filaments
Cancer	prostate and mammary glands, brain, kidney, colon	HSP27, α B	-	-	α B crystallin and/or HSP27 are upregulated in disease. Some sHSP-positive tumors are drug resistant.

Note: Column 1 lists the name of the disease. Column 2 lists the organs affected. Column 3 lists the small heat-shock protein involved. Column 4 lists the names of the associated aggregated protein. Column 5 lists other details. Many of the diseases linked to sHSP are age related and most involve aggregate and filament formation. While cancer and tumors are not directly linked with protein unfolding and aggregation, modulation of cytoskeleton assembly or filament formation could account for the relationship between sHSP and these diseases which are increased in the aging populations.

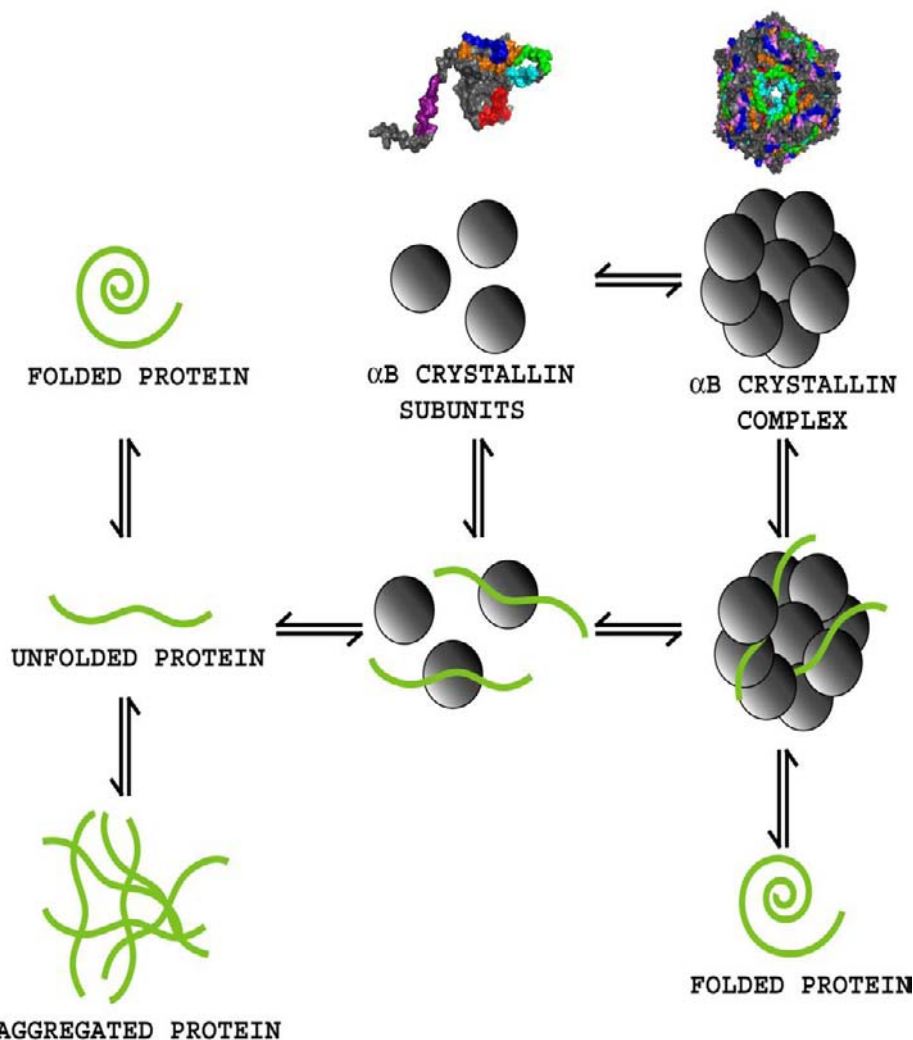
While the list of diseases in Table 1 could suggest that sHSPs are involved nonspecifically in a wide range of diverse pathologies, the common elements of nearly all the

diseases listed, are that they are the result of protein unfolding, and dynamic molecular mechanisms mediated by weak, non-covalent protein – protein interactions. Our approach to the development of innovative therapies in protein unfolding and aggregation diseases is to define the nature and mechanisms of the interactions between sHSPs and their target proteins. The first step was the identification of the interactive sequences. The next step was to observe the 3D structure of the interactive domains on the surface of the alphaB crystallin molecule. Based on the 3D molecular structure of the interactive domains on the surface of human alphaB crystallin, it is expected that novel protective reagents will be designed.

Molecular Structure of Human AlphaB Crystallin – Archetype for sHSP and Stress Proteins

The archetype for eleven sHSPs found in humans, is human alphaB crystallin (Kappe et al. 2003; Ghosh & Clark, 2005; Bellyei et al 2007), a highly interactive stress response protein characterized by three structural domains: the N-terminal domain, the α crystallin core domain, and the C-terminal domain (Figure1) (Kim et al 1998; van Montfort et al 2001; Bloemendahl 2005; Stamler et al 2005). The N-terminal and C-terminal domains are variable in sequence (Figure 1) and, in the crystal structures of *Methanococcus jannaschii* (Mj) sHSP16.3 and wheat sHSP16.9, the N-terminal domains can contain α helical content but are largely unstructured. In sHSPs, the C-terminal extension protrudes from the alpha crystallin core domain as an unstructured, flexible sequence. In contrast to the unstructured N- and C-terminal domains, the alpha crystallin core domain is an immunoglobulin-like sandwich of beta strands that are stabilized by two anti-parallel beta sheets formed from six to nine beta strands connected by loops of variable lengths. The formation of dimers in wheat sHSP16.9 is due to interactions between the beta2 and beta3 strands of one monomer with the beta6 strand contained in the loop connecting beta5 and beta7 of another monomer. Extensive hydrogen bonding accounts for the stability of the alpha crystallin core domain in sHSPs. The C-terminal extension contains a conserved I-X-I/V motif, where I is isoleucine, V is valine, and X is any natural amino acid (Pasta et al 2004). In wheat sHSP16.9, the I-X-I/V motif of one monomer interacts with residues of the beta4 and beta8 strands of another monomer to form the dodecameric quaternary structure observed using X-ray diffraction of the crystal structure. While human alphaB crystallin contains the same three structural domains found in Mj sHSP16.5 and wheat sHSP16.9, alphaB crystallin forms large and polydisperse complexes. The heterogeneity in the size of the complex formed reflects the variation in the multiple interactive domains that respond to unfolding proteins and accounts for the fact that alphaB crystallin has not been crystallized for X-ray diffraction while HSP16.5 and HSP16.9 have (Haley et al 1998, 2000; Salerno 2003; Horwitz 2009). This heterogeneity of alphaB crystallin in complex size and in interactions with unfolding proteins is consistent with the fact that the multiple interactive domains in human alphaB crystallin are more favorable for interactions with multiple unfolding proteins than the large HSPs. Proteolysis, deletion mutagenesis, single residue site-directed mutagenesis, and domain-swapping chimeric mutagenesis of alphaB crystallin and *Caenorhabditis elegans* sHSP12.2 were used to determine the sequences and specific residues in all three structural domains of sHSPs that

were important for complex assembly and protective activity against protein unfolding and aggregation (Ghosh et al 2006a; Ghosh et al 2006b). While the homology model in Figure 1 is a computer simulation, the results of mutation studies and the use of synthetic peptides were consistent with the expected molecular structure of α B crystallin and the interactive domains that respond to unfolding proteins. As will be described later, the structure of the α B crystallin provides a model for the design of novel therapeutics.



Note: The structure of human α B crystallin was modeled as previously described (Ghosh & Clark, 2005). The amino acid sequence of human α B crystallin is at the bottom of the figure. The secondary structure is indicated with squares (■) for β -strand and tildas (~) for α -helix. The color indicates locations of the interactive peptides; 41STSLSPFYLRPPSFLRAP58 (red), 73DRFSVNLDVKHFS85 (orange), 101HGKHEERQDE110 (light green), 113FISREFHR120 (dark green), 131LTITSSLSSDGV142 (blue), and 156ERTIPITRE164 (purple).

Figure 1. Primary, secondary and tertiary structure for human α B crystallin.

The software Molecular Operating Environment (Chemical Computing Group, Montreal, Quebec, Canada) was used to construct the 3D homology model of human alphaB crystallin. MOE employs a number of algorithms including multiple sequence alignment, structure superposition, contact analyzer, and fold identification to develop homology models based on available high-resolution crystal and/or NMR structures of the template protein molecule (Levitt 1992). The program analyzed the stereochemical quality of the predicted models and took into account planarity, chirality, phi/psi preferences, chi angles, nonbonded contact distances, unsatisfied donors, and acceptors (Fechteler et al 1995). The predicted secondary structure of human alphaB crystallin (Figure 1) was aligned and found to be consistent with the secondary structure observed for wheat sHSP16.9. The alignment was used by MOE to create a series of 10 energy-minimized 3D models. Each model was evaluated using the ModelEval module of MOE. The best model was selected and superimposed on the monomeric subunits of Mj sHSP16.5 and wheat sHSP16.9 and the 3D coordinates of the three structures were fit with a final RMSD of 3.25 Å (Ghosh & Clark 2005). The three structures have the same basic topology. The hydrophobic N terminus has helical elements. The alpha crystallin core is an immunoglobulin-like beta sandwich that forms two anti-parallel beta sheets connected by a flexible loop. The C-terminal extension is charged and unstructured. The overall result was in good agreement with existing spectral and NMR data on the structure of alphaB crystallin (Jehle et al 2009; Koteiche & McHaourab 1999; Liang & Liu 2006). The homology model for human alphaB crystallin provided the molecular structure for mapping the interactive sequences identified using protein pin arrays onto the surface of human alphaB crystallin where the interactions with unfolding proteins occur.

Identification of Interactive Sequences and the Function of sHSPs

Following the discovery of a 19 amino acid peptide which corresponded to the $\beta 3/\beta 4$ strands in human alphaA crystallin and retained the protective activity against protein unfolding and aggregation (Sharma et al 2000; Bhattacharyya & Sharma 2001;), we investigated the interactive sites in human alphaB crystallin using protein pin array technology (Ghosh & Clark, 2005). It was recognized quickly that alphaB crystallin contained multiple interactive sequences that responded selectively, and rapidly to unfolding proteins with low affinity binding. The interactive sequences on alphaB crystallin were sensitive to the subtle, dysfunctional modifications which, if left unprotected, could disrupt the cellular functions of proteins. Structurally alphaB crystallin is a mosaic of interactive domains whose relative affinities must be coordinated to be effective, but not too effective, binding unfolding proteins. For a multifunctional stress response protein, specific, high affinity binding may not be as effective as selective binding which suggests the importance of weak non-covalent interactions including hydrogen bonds, hydrophobic and electrostatic interactions. The goal was to identify the interactive sequences in human alphaB crystallin and a protein pin array was designed to determine the interactive peptide sequences in alphaB crystallin (Ghosh & Clark 2005a; Ghosh et al 2005b; Ghosh et al 2007b; Ghosh et al 2007c). In the assay, peptides corresponding to residues 1–175 of human alphaB crystallin were

synthesized employing a simultaneous peptide synthesis strategy developed by Geysen, called Multipin Peptide Synthesis (Geysen 1990). The peptides were immobilized on derivatized polyethylene pins arranged in a microtiter plate format (Ghosh & Clark 2005a). Each peptide was eight amino acids in length, and consecutive peptides were offset by two amino acids. All peptides were covalently bound to the surface plastic pins. The first peptide on the first pin of the array was 1MDIAIHHP8, and the last peptide immobilized on the last pin of the array was 167PAVTAAPK174 of human alphaB crystallin. In total, eighty-four peptides corresponding to the 175-amino-acid primary sequence of human alphaB crystallin were synthesized and fixed to sequential pins in the array. To screen for binding to the peptides, fixed concentrations of each target protein were added to each well in the microtiter plate and incubated for reaction with the peptides immobilized on the pins. Repeated washes removed unbound target protein before a labeling procedure measured the amount of target protein complexed with each alphaB crystallin sequence. The pin arrays can be regenerated up to 90 times for repeated use.

The target proteins used in the pin array assays can be categorized as (a) crystallins, (b) cytoskeletal proteins, (c) unfolding proteins and (d) regulators of growth and differentiation that participate in pathways of protection/stabilization of normal cell structure. These interactive proteins are important for fundamental cell functions during normal differentiation and aging and have been reported to interact with alphaB crystallin. The pin array results determined that multiple interactive domains were important for the selectivity (not specificity) of the alphaB crystallin in multifunctional mechanisms. For example, the $\beta 3$ and $\beta 8$ strands in alphaB crystallin participate in self assembly of the alphaB crystallin complex, filament interactions, protection against protein unfolding, growth factors and beta catenin (Ghosh & Clark 2005a; Ghosh et al 2005b; Ghosh et al 2007b; Ghosh et al 2007c). In contrast, the $\beta 4$ and $\beta 5$ strands interact with signaling proteins and not in complex assembly or filament interactions. The $\beta 3$ - $\beta 8$ strands form an interactive domain that is a key binding region where the effects of mutations on association/dissociations constants measured using SPR are expected to correlate closely with altered activity of alphaB crystallin. Understanding the complex mechanism of the protective activity of sHSPs requires characterization of the specific functions of the individual sequences involved in interactions with unfolding proteins. The fact that sHSPs interact selectively with multiple proteins suggests that diversity in the multiple interactive domains rather than specificity is an important parameter in the protective mechanism(s) of sHSPs against protein unfolding and aggregation and accounts for the ability of alphaB crystallin to recognize and bind a variety of unfolding proteins and other self assembling target proteins with different affinities. Once the multiple interactive sequences were identified, they were mapped to the 3D computer homology model based on the X-ray diffraction structures of Mj sHSP16.5 and wheat sHSP16.9 where it was possible to visualize, for the first time, the exposed surface domains that were most likely to be responsible for the interactive functions of sHSPs in cells. It was hypothesized that, in the presence of external stress, sHSPs detect protein unfolding with an extraordinary sensitivity that could be difficult to duplicate outside biological cells and tissues.

Exposed Surface Domains and Protective Interactions

To establish the relationship between alphaB crystallin structure and biological activity, the multiple interactive sites on the surface of the alphaB crystallin molecule needed to be defined and visualized in 3D structural models. Space-filling models of alphaB crystallin were generated and the distributions of the exposed interaction domains were mapped to the surface, using the 3D molecular viewing software PyMol (Figure 2). All surface exposed side chains in the interactive regions are yellow and the surfaces not involved in protein – protein interactions are gray. While there are similarities in the surface exposed interactive domains, small variations are obvious. It is easily imagined that the surface variability is necessary to adapt to the differences in the surfaces of the diverse unfolding target proteins. Interactions occur on the exposed surface of the core domain of alphaB crystallin, on the N-terminus and on the C-terminus which is consistent with reports of the involvement of the C-, N- termini and core domain in the variety of selective activities of sHSPs (Derham et al 2001; Muchowski et al 1999; Pasta et al 2003; 2004; Saha & Das 2004; Sharma et al 1997; Shroff et al 2001). The results support the hypothesis that the molecular mechanisms for the multifunctional activity of sHSPs in disease involves diverse and multiple interactive domains. Visualization of the diverse domains permits the design of mutation experiments to confirm the precise residues involved in the recognition and binding of selected families of proteins, a major functions of human alphaB crystallin. Point mutations in the surface exposed amino acids confirmed the models and synthetic peptides based on the interactive domains were tested for functional activity in vitro (Ghosh & Clark, 2005a; Ghosh et al 2005a, 2006a, 2006b, 2007a, 2008). The results indicated that diversity in the interactive domains has functional importance and may account for the selectivity of alphaB crystallin for several families of proteins and for the polydisperse dimensions of the complexes of alphaB crystallin (Haley et al 1998; Haley et al 2000; Horwitz 2009). If there is functional significance to heterogeneity of the complex size, this is a significant mechanistic difference between the small and large HSPs (Ghosh et al 2007a, 2007b, 2007c) that can be clarified through continued research on the dynamics of the functions of alphaB crystallin.

The results are consistent with the hypothesis that small variations in the interactive surfaces of a stress response protein can account for diverse selectivity in protein targets, an indication of the importance of surface exposed sequences in the stress response of alphaB crystallin to unfolding proteins and self assembling systems. Detailed studies of the N-terminal interactive sequence LFPTSTSLSPFYLRPPSF determined that the exposed surface was 79% hydrophobic. The hydrophobic surface for the sequences FSVNLDVK, LTITSSLS, and GVLTVNGP that form the beta3, beta8, and beta9 strands was calculated to be 65%, 68%, and 71% hydrophobic respectively, an average of 68% for the interface formed by all three beta strands. The calculated hydrophobic surface for the C-terminal extension PERTIPTREEK was 58% hydrophobic (Ghosh & Clark 2005). The high proportion of hydrophobic surface in the interactive sequences is consistent with the potential importance of hydrophobic side chains in interactions between human alphaB crystallin and its binding targets, alpha crystallin subunits, cytoskeletal proteins, signaling proteins and regulators of normal growth (Shroff et al). Mapping of the interactive sequences to the 3D models of

human α B crystallin determined that the binding regions on the N-terminus, C-terminus, and α B crystallin core domain were solvent-exposed in the monomeric subunit. The importance of understanding the variability in the exposure of the interactive sequences in α B crystallin was the basis for the plan to synthesize interactive peptides for testing in amyloid formation assays in vitro.

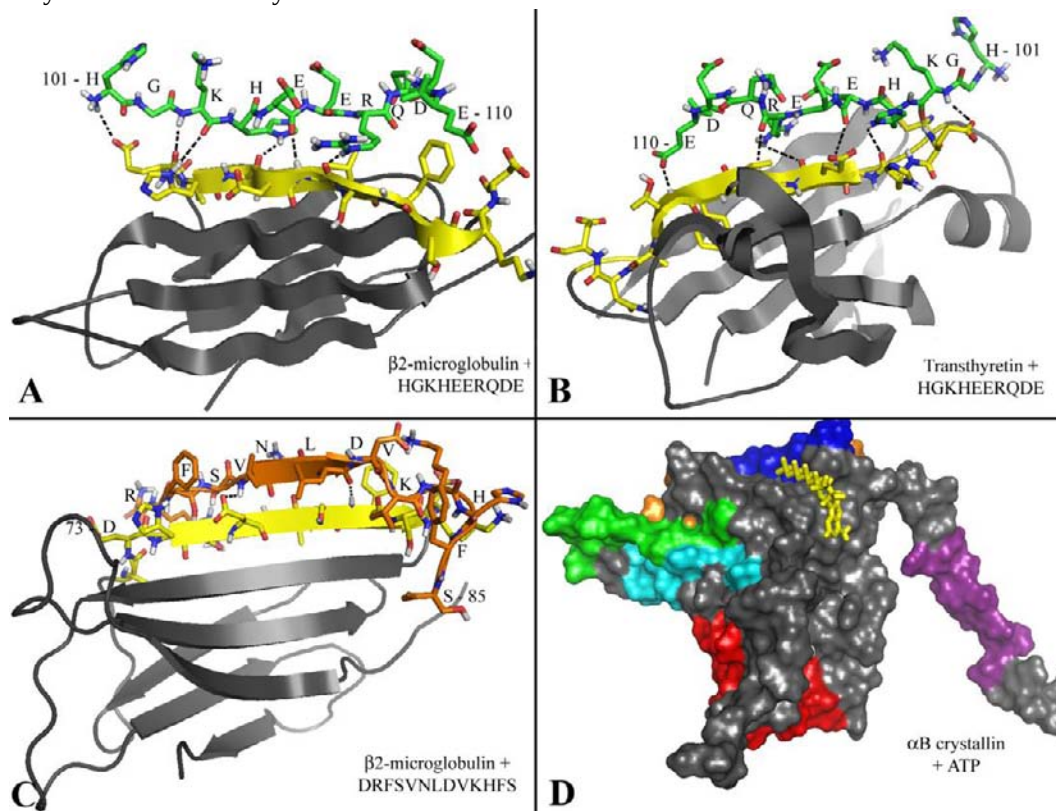


Figure 2. Diversity in the interactive surface domains of human α B crystallin.

Note: Synthetic peptides for potential use as therapeutics were based on the mapping of interactive domains to the surfaces of the 3D homology model of human α B crystallin and were colored (CENTER MODEL). The peptides are 41STSLSPFYLRPPSFLRAP58 (red), 73DRFSVNLDVKHFS85 (orange), 101HGKHEERQDE110 (light green), 113FISREFHR120 (dark green), 131LTITSSLSDGV142(blue), and 156ERTIPITRE164(purple). The surrounding models represent the surface domains (yellow) for the interactive sequences required for self-assembly of α B crystallin, binding to unfolding proteins, binding to intermediate and micro-filaments, binding to microtubules, binding to insulin, binding to β -catenin, binding to growth factors, and interactions with amyloid fibrils. All interactive sequences were identified using protein pin-arrays and functional assays with synthetic peptides. Surface domains for globular proteins were based on interactions with thermally unfolded alcohol dehydrogenase, citrate synthase, and β/γ crystallin. Surface domains for filaments were based on the interactions with actin, GFAP, vimentin, and desmin. Surface domains growth factors were defined by the interactions with NGF, VEGF, and FGF. Surface domains for amyloid proteins were based on interactions with β -amyloid 1-42, α -synuclein, β 2-microglobulin, and transthyretin. The spatial distribution of the surface domains is diverse which may be necessary to adapt to the surface of the individual target proteins.

Synthesis and Functional Evaluation of Bioactive Peptides

The alphaB crystallin interactive peptides, DRFSVNLDVKHFS (DR), HGKHEERQDE (HG), STSLSPFYLRPPSFLRAP (ST), LTITSSLSSDGV (LT), ERTIPITRE (ER) and FISREFHR (FI), were synthesized or purchased for experimental tests using a thioflavin T fluorescence assay for amyloid fibril formation of β -amyloid ($A\beta(1-42)$), α synuclein, transthyretin (TTR), and β 2-microglobulin (β 2m) (Ghosh et al 2008). Thioflavin T is a cationic benzothiazole that has high affinity for amyloid fibrils and enhanced fluorescence when bound to aggregating amyloid fibrils. The effectiveness of individual interactive sequences against amyloid fibril formation was measured using the Thioflavin T assay and was found to vary with the amyloid protein (Table 2). For example, the DR peptide inhibited the formation of α synuclein fibrils but increased the fibril formation of $A\beta(1-42)$. In contrast, ST inhibited fibril formation of Abeta but increased formation of alpha synuclein fibrils. Truncated bioactive sequences as small as four amino acids retained activity. Similar to the large synthetic bioactive peptides, the effectiveness of the four amino acid peptides varied with the target amyloid. For example, the KHFS peptide was a very effective inhibitor of fibril formation of alpha synuclein and a poor inhibitor of fibril formation of beta2 microglobulin (Table 2). The results were an impressive demonstration of the bioactivity of individual synthetic peptides on fibril formation of various amyloid proteins. The experimental results using synthetic bioactive peptides were important for several reasons and a number of conclusions can be made. First, the stress protein, human alphaB crystallin, may have a more significant and direct function in the pathophysiology of amyloid fibril formation than previously thought. Second, the results were a clear demonstration that individual bioactive peptides in alphaB crystallin have activity similar to that of the parent molecule. Third, individual bioactive peptides as small as four amino acids were effective inhibitors of fibril formation of amyloid proteins. Fourth, the multiple interactive domains on the surface of alphaB crystallin had multiple effects on the formation of self assembling fibrils. The variation in response of fibril assembly to individual interactive peptides accounted for the adaptive capability of alphaB crystallin in protection against multiple protein targets in aging diseases, stabilization of filaments and regulation of microtubule assembly during differentiation and development (Ghosh et al. 2007b, 2007c, 2008). The collective effects of the multiple interactive surface domains on human alphaB crystallin need to be clarified to understand the function of sHSPs in vivo. At this point in our understanding of the mechanism(s) of alphaB crystallin activity, we can hypothesize that the activity of a single stress protein resembles a component of a multiprotein complex in which a mosaic of interactive sites with variable affinities modulate diverse regulatory proteins and the dynamic organization of protein self assembly. While it is unlikely that these results will translate immediately into new therapies for protein unfolding diseases including cataract or neurodegeneration, knowledge of the structure-function relationships will be useful for identification of specific activities of each interactive domain with selected target proteins and a better understanding of the molecular mechanisms for the protective activity of sHSPs.

Table 2. Effects of α B crystallin peptides on amyloid fibril formation.

peptide	Residues	$A\beta(1-42)$		α synuclein		TTR		B2m	
		1x	5x	1x	5x	1x	5x	1x	5x
no peptide	-	100%	100%	100%	100%	100%	100%	100%	100%
STSLSPFYLRPPSF LRAP	41-58	0%	4%	446%	23%	92%	64%	62%	52%
DRFSVNLDVKHFS	73-85	49%	165%	0%	6%	55%	35%	38%	36%
DRFS	73-76	41%	26%	0%	9%	22%	38%	122%	217%
NLDV	78-81	0%	53%	0%	0%	25%	43%	50%	27%
KHFS	82-85	72%	71%	1%	0%	38%	45%	157%	196%
HGKHEERQDE	101-110	0%	0%	31%	34%	28%	29%	46%	42%
HGKH	101-104	100%	100%	2%	14%	13%	23%	13%	23%
HEER	104-107	151%	0%	43%	26%	8%	14%	8%	14%
RQDE	107-110	136%	114%	100%	161%	3%	22%	3%	22%
FISREFHR	113-120	96%	98%	100%	96%	93%	105%	229%	183%
LTITSSLSDGV	131-142	168%	103%	30%	60%	86%	85%	37%	49%
ERTIPITRE	156-164	154%	140%	49%	59%	40%	93%	46%	59%

Note: The effects of the α B crystallin peptides on amyloid fibril formation of β -amyloid ($A\beta(1-42)$), α synuclein, transthyretin (TTR), and β 2-microglobulin (β 2m) using Thioflavin-T fluorescence. The molar ratios of peptide:amyloid were 1:1 (1x) and 5:1 (5x). 100% is defined as the fibril formation of amyloid protein in the absence of peptide and 0% is defined as no fibril formation. The effectiveness of individual bioactive peptides derived from alphaB crystallin varies with the peptide sequence, the concentration and the protein target. The results suggest that different interactive sequences are specialized for different protein targets.

The AlphasB Crystallin Peptide – Amyloid Protein Complex

The protective effects of alphaB crystallin against fibril formation in protein unfolding diseases that include Alzheimer's and Parkinson's is well documented (Wyttenbach, 2004; Ecroyd & Carver, 2009). The experiments using the bioactive peptides confirmed the activity of the individual interactive sequences in the function of alphaB crystallin and provided a valuable model for determination of the importance of specific protein – protein interactions in sHSPs for recognition and binding to amyloid proteins. 3D molecular modeling was conducted to better characterize the structure activity relationships and the specific interactions responsible for the effects of the bioactive peptides on fibril formation of amyloid proteins. Three-dimensional co-ordinates for the interactive peptides were extracted from the computed homology model of human alphaB crystallin (Figure 1) (Ghosh & Clark 2005a; Ghosh et al 2005b; Ghosh et al 2007a). The three-dimensional co-ordinates for human transthyretin(1DVQ) and human β 2-microglobulin(1LDS) were obtained from their crystal structures in the protein databank (Klabunde et al 2000; Trinh et al 2002). Co-

ordinates for the peptides and the target proteins were used in the molecular docking program ClusPro (Comeau et al 2004a, b) which computed docking models for each protein–peptide combination (Figure 3) on the basis of free energy minimization, and filters for complex selection based on minimization of solvation and electrostatic energies to obtain the best structural fits between the bioactive peptides and the amyloid forming proteins. The 3D models characterized the interactions between transthyretin and beta2-microglobulin and the alphaB crystallin peptides DRFSVNLDVVKHFS which forms the beta3 strand and HGKHEERQDE which forms part of the loop connecting the beta5 and beta7 strands on the exposed surface of the alphaB crystallin core domain (Figure 3A-C). Residues His-101, Lys-103, His-104, Glu-105, and Arg-107 from the 101HGKHEERQDE110 peptide interacted with residues Glu-51, His-52, Ser-53, and Leu-55 in beta2-microglobulin (Figure 3A). Similarly, residues His-104, Glu-105, and Arg-107 from the 101HGKHEERQDE110 peptide interacted with residues His-79, His-81, Glu-83, and Val-85 in transthyretin (Figure.3B). Residues Phe-75, Val-77, and Leu-79 from the 73DRFSVNLDVVKHFS85 peptide interacted with residues Glu-51, Ser-53, Asp-54, Leu-55, and Phe-57 in beta2-microglobulin (Figure.3C). Both α B crystallin peptides bound to the D-strand of β 2-microglobulin and the F-strand of transthyretin. These β strands share sequence homology and are known to be involved in critical intra-fibril interactions (Ghosh et al 2008; McParland et al 2000; Nelson & Eisenberg 2006; Schormann et al 1998; Trinh et al 2002). While homology models cannot be definitive, the interactions at the simulated sites of peptide - amyloid interactions are expected to be weak and non-covalent, consistent with the hypothetical mechanism proposed for protective activity of alphaB crystallin against aggregation diseases. The models represent the direct interactions between residues of the alphaB crystallin peptides and residues of these amyloidogenic fibril forming beta strands of transthyretin and beta2-microglobulin that can be expected to prevent abnormal self-assembly of transthyretin and beta 2-microglobulin, beta strand rich amyloid-like fibrils, as was observed in the thioflavin T assays.

For comparison, the interactive surface for ATP was mapped onto the 3D model for alphaB crystallin (Figure 3D). Fluorescence resonance energy transfer (FRET) and mass spectrometry determined that the ATP interaction with alphaB crystallin occurs in a shallow groove between the beta 4 and beta 8 strands of the exposed surface of the alpha crystallin core domain. The ATP binding occurs at the surface exposed interactive sequence in the beta 4 strand in alphaB crystallin that is similar to the Walker B ATP binding motif (Ghosh et al 2006c) and is not at the same surface site as the interactive domain for amyloidogenic proteins. The 3D molecular models generate new and detailed structure activity relationships at the atomic level for the design of novel therapeutics based on the molecular structure and function of the small stress protein, human alphaB crystallin.

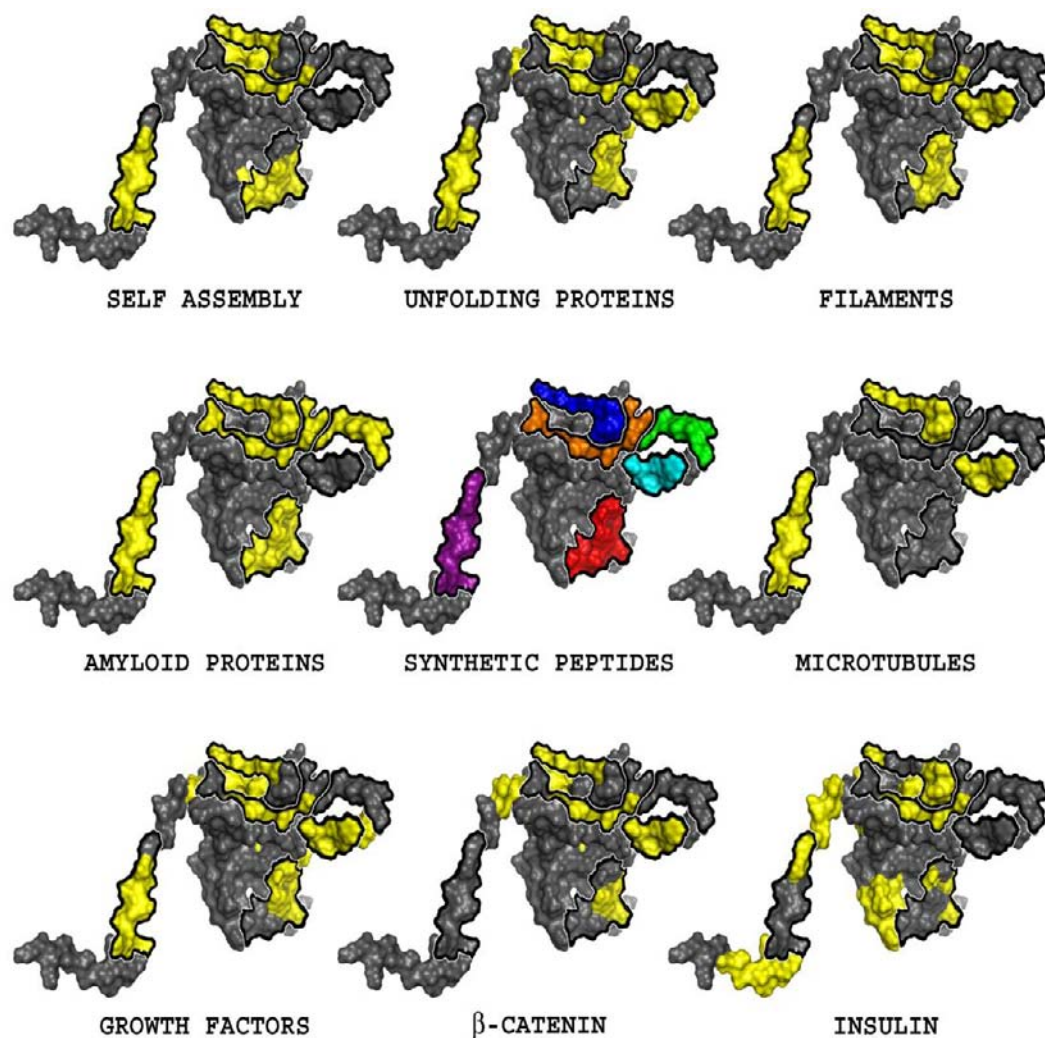


Figure 3. Models for molecular interactions between α B crystallin and amyloid proteins.

Note: The structures of α B crystallin peptides were docked with the amyloidogenic proteins and β 2-microglobulin(PDB: 1LDS) and transthyretin(PDB:1DVQ) using the ClusPro molecular docking program. (A) The peptide $_{101}\text{HGKHEERQDE}_{110}$ (green) binding β 2-microglobulin at the D strand(yellow). (B) The peptide $_{101}\text{HGKHEERQDE}_{110}$ (green) binding transthyretin at the critical amyloidogenic F strand(yellow). (C) The peptide $_{73}\text{DRFSVNLDVKHFS}_{85}$ (red) binding β 2-microglobulin at the critical amyloidogenic D strand (yellow). The peptide has the potential to form a β -strand apposed to the β -sheet of β 2-microglobulin, disrupting amyloid formation. (D) The surface domains of the interactive peptides in α B crystallin: $_{41}\text{STLSLPFYLRPPSFLRAP}_{58}$ (red), $_{73}\text{DRFSVNLDVKHFS}_{85}$ (orange), $_{101}\text{HGKHEERQDE}_{110}$ and $_{113}\text{FISREFHR}_{120}$ (green), $_{131}\text{LTITSSLSSDGV}_{142}$ (blue), and $_{156}\text{ERTIPITRE}_{164}$ (purple). Note the ATP (yellow) binding domain in a shallow groove at a Walker-B ATP binding motif between the β 8-strand ($_{131}\text{LTITSSLSSDGV}_{142}$) and the β 4 strand (Ghosh et al 2006c).

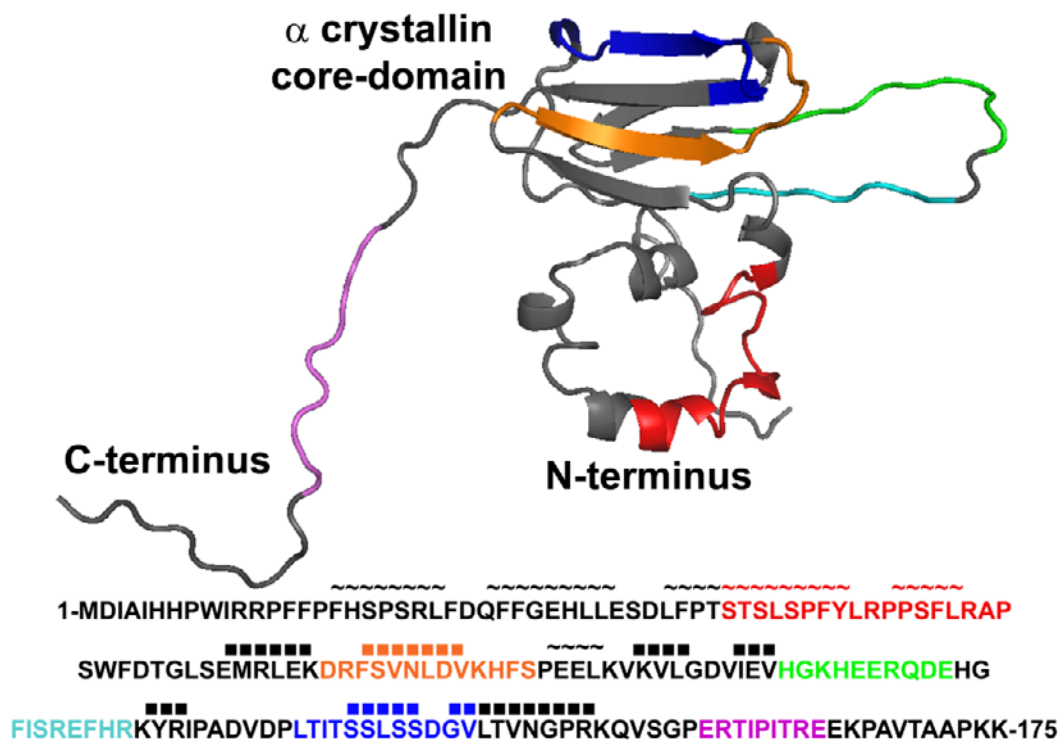


Figure 4. Model for the protection against protein unfolding and aggregation.

Note: In the first row, the colors label the exposed interactive sequences on the surface of an individual α B crystallin subunit (left image) which are partially buried in an assembled α B complex (right image). The surface domains for each interactive peptides are colored; $_{41}$ STLSSPFYLRPPSFLRAP $_{58}$ (red), $_{73}$ DRFSVNLDVKHFS $_{85}$ (orange), $_{101}$ HGKHEERQDE $_{110}$ (light green), $_{113}$ FISREFHR $_{120}$ (dark green), $_{131}$ LITSSSSDGV $_{142}$ (blue), and $_{156}$ ERTIPITRE $_{164}$ (purple). The second row models the dynamic equilibrium between the pool of α B subunits (grey ovals) and subunits assembled into complexes under normal conditions when interactions with native folded proteins (green) are minimal. Under conditions of cell stress, protein unfolding/misfolding increases the interactions between α B subunits and the unfolding/misfolding proteins (third row) and stronger subunit-unfolding protein interactions favor formation of α B crystallin-unfolded target protein complexes. The bottom row models the outcome of protein unfolding without (left) and with α B crystallin (right). The model suggests that the dynamics of complex assembly are integral to the function of the stress response protein, human α B crystallin.

Surface Accessible Interactive Domains and the Protective Mechanism of Human AlphaB Crystallin

One of the mysteries of stress proteins and sHSPs is the functional importance of the protein – protein interactions in the dynamic assembly of complexes in protection against protein unfolding (Figure 4). A well known characteristic of α B crystallin and most sHSPs is the assembly of polydisperse complexes that are in dynamic equilibrium with the subunits. The dynamic equilibrium is sensitive to physical conditions in the environment that

include temperature, pH, and ionic strength because subunit assembly is the results of weak non-covalent hydrogen bonds, electrostatic and hydrophobic interactions. The distribution of the multiple interactive domains on the surface of the space filled model of alphaB crystallin is consistent with multiple interactions between subunits (colored domains in Figure 4, top row left). The orange and blue areas are the exposed interfaces of the α crystallin core domain that contain the $\beta 3$ (orange) and $\beta 8$ (blue) strands for self-assembly of the αB crystallin complex (Ghosh & Clark, 2005a). In an assembled complex (top row right) the $\beta 3$ (orange) and $\beta 8$ strands (blue) are partially buried and inaccessible. In the absence of stress induced protein unfolding, αB crystallin subunits are in a dynamic equilibrium with the αB crystallin complex and interactions with native folded proteins are minimal (second row). In the presence of stress, unfolded proteins can shift the dynamic equilibrium to favor access to the $\beta 3$ and $\beta 8$ strands in the core domain of the dissociated αB crystallin subunits. Access to the interactive domains permits protective binding with unfolding proteins to inhibit the abnormal function and aggregation (middle row). The multiple interactive sites on the alphaB crystallin subunits interact with unfolding protein to form heterogeneous complexes that can grow to very large sizes (Arai & Atomi 1997; Friedrich et al 2004; Lee et al 1997; Horwitz 2009). On the basis of a dynamic model, it can be hypothesized that the strength of the interactions between subunits and unfolding proteins influences the protective activity of the stress protein. For example, strong interactions between subunits could be expected to favor formation of homomeric complexes decreasing accessibility to the interactive domains on alphaB crystallin subunits and reducing protective interactions with unfolding proteins. In contrast, weak interactions between subunits could be expected to favor dissociation of the complexes making the interactive domains on alphaB crystallin subunits accessible for protective interactions with unfolding proteins. The model predicts that (a) the protective activity of the stress proteins is regulated dynamically by access for unfolding proteins to interactive domains on the surface of the stress response proteins and (b) the relative affinities between stress protein subunits and unfolding proteins have regulatory functions. In this dynamic model, the function of the complex is to sequester the protective interactive surface domains until stress shifts the dynamic subunit – complex equilibrium to favor dissociation of subunits for interaction with unfolding proteins. The emphasis of this model is on the dynamic nature of the subunit - complex equilibrium which can regulate access to the multiple interactive surfaces on alphaB crystallin and other sHSPs. Detailed characterization of the relative strengths of the interactions between alphaB crystallin subunits and unfolding proteins will be needed to test this hypothesis on the functional relationship(s) between the interactive surfaces on the stress protein, human alphaB crystallin, and unfolding target proteins.

Conclusion

It is more important than ever before to understand the fundamental biological mechanisms responsible for the protective activity of stress response proteins and to maximize the development of therapeutics that can utilize these mechanisms. The multifunctional activity of human alphaB crystallin can be accounted for by the diversity and

variation in the interactive domains on the surface of the stress response protein. Similar to many sHSPs, alphaB crystallin interacts selectively, not specifically, both with unfolding proteins and with proteins important for cell differentiation and development including proteins in regulatory pathways, apoptosis and filaments. The mechanisms for these activities distinguish the small from the large families of heat shock proteins. Similar to several sHSPs, alphaB crystallin has special affinity for self assembling protein systems in the formation of complexes, filaments, microtubules and amyloid fibrils. Under the stress of normal differentiation, growth and aging, alphaB crystallin is a dynamic modulator of cell proliferation, migration and elongation which are sensitive to subtle changes in hydrophobicity, pH, ionic strength, charge, hydration, proteolysis and posttranslational modifications which are important factors in protein – protein interactions. As stress response proteins, the protective function of alphaB crystallin is beneficial against protein unfolding leading to amyloidogenesis observed in a variety of aging diseases. The alpha crystallin core domain and the N- and C- termini contain interactive sequences that are critical for the normal activity of sHSPs and individually retain protective functions against the deleterious effects of unfolding proteins and unstable of cytoskeletal assembly. Future experimental studies are expected to define the functional significance of the polydisperse complexes that can respond rapidly and dynamically to cellular stress. Our approach is to use the 3D molecular structures of the stress proteins and the interactions as the basis for identifying innovative therapies. Few proteins hold greater therapeutic promise than sHSPs for safe endogenous protection against protein unfolding and aggregation diseases (Table 1).

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Chapter XII

The Eukaryotic Chaperonin CCT (TRiC): Structure, Mechanisms of Action and Substrate Diversity

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Abstract

The chaperonin containing TCP-1 (CCT) is found in the cytosol of all eukaryotic cells. It is an oligomer formed from two back to back rings, each containing eight subunits that surround a central cavity. Each CCT subunit contains three domains: an equatorial domain containing an ATP binding site, a substrate binding domain which displays the least sequence similarity between other CCT subunits and a linker domain. The folding cycle of CCT is ATP driven and the subunits of each chaperonin ring hydrolyse ATP in a sequential manner. For actin and tubulin it has been demonstrated that these proteins initially bind to CCT in an open conformation. Following the nucleotide cycle of CCT they become compact whilst remaining bound to one or more chaperonin subunits. This is in contrast to the mechanism of the prokaryotic chaperonins for which it is thought that folding substrates are encapsulated within the chaperonin cavity without direct interactions with chaperonin subunits.

The sequence diversity of the CCT substrate binding domains provides a complex binding interface for potential substrates. The major folding substrates of CCT are the abundant cytoskeletal proteins actin and tubulin, whilst other less abundant proteins such as the cell cycle regulating proteins Cdc20 and Cdh1 are known to require interactions with CCT to reach their native state. The numbers of CCT substrate proteins and the way in which CCT binds to its substrates is a matter for debate. At present it is estimated that in yeast up to 300 proteins may bind to CCT but it is not yet known how many proteins

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are obligate substrates. There are two major theories regarding how CCT recognises its folding substrates. The first proposes that CCT is a relatively general chaperone, recognising hydrophobic binding determinants, whilst the second, at least in the case of actin, proposes that binding to CCT is sequence specific.

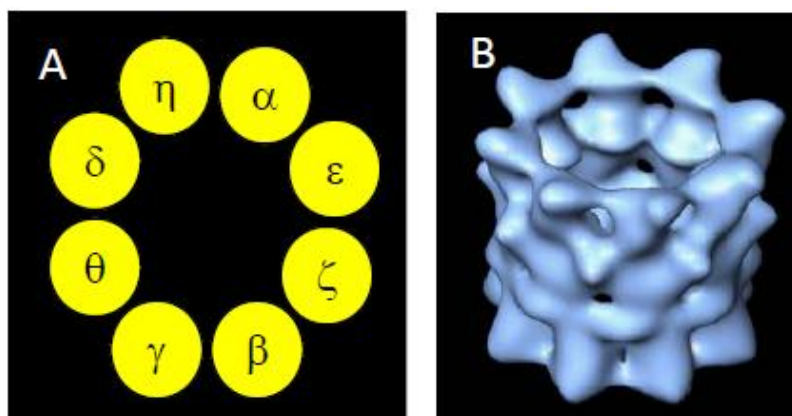
The mechanisms of CCT action and the diversity of potential CCT substrates will be discussed in relation to the activity of CCT having far reaching implications on the many cellular functions that depend on the activity of its folding substrates.

Introduction

Newly synthesised proteins must attain their correct native conformations in the crowded environment of the cell where the high protein concentration and effects of molecular crowding make aggregation a serious threat. In order to combat the dangers of aggregation, which are particularly relevant to newly synthesised or unfolded polypeptides with exposed hydrophobic regions, the group of proteins known as ‘molecular chaperones’ has evolved. These proteins function by a variety of mechanisms, some having a rather passive mode of action providing a stabilising interface for vulnerable proteins, whilst others are able to exert force on their substrate proteins thereby overcoming energetically unfavourable barriers. This chapter will focus on the eukaryotic member of the chaperonin group of molecular chaperones, Chaperonin Containing TCP-1 (CCT) which is also known as TCP-1 Ring Complex (TRiC). CCT was initially identified as a chaperone required for the formation of native actin and tubulin proteins (Sternlicht et al., 1993) although it is now established that CCT interacts with numerous protein substrates (reviewed by Brackley and Grantham, 2009).

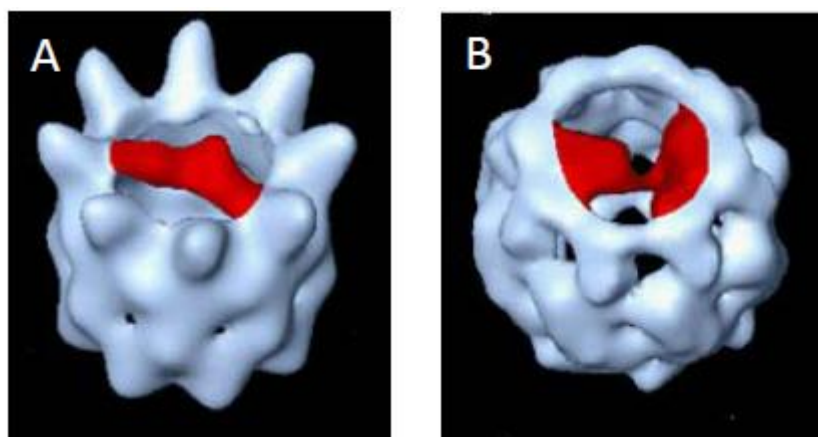
Structure of CCT

The chaperonins are a sub group of molecular chaperones that are characterised by their cylindrical structures formed from two back-to-back rings of subunits that surround a central cavity. All chaperonin subunits share the same general domain architecture: an apical substrate binding domain, an equatorial domain which contains an ATP binding site and is involved in interactions between neighbouring subunits and an intermediate linker domain. Changes in conformation upon nucleotide binding and hydrolysis are transduced from the equatorial domain via the intermediate linker domain to the substrate binding domain. This provides a basic mechanism by which all chaperonins can couple their ATPase activity to substrate folding. Despite this underlying common mechanistic feature, there are fundamental variations between the chaperonins which have been divided into two groups: group I (chaperonins from eubacteria and endosymbiotic organelles) and group II (chaperonins from archaea and the cytosol of eukaryotes). The major difference between these groups of chaperonins is the requirement of a co-chaperone. The group I chaperonins have a single ring co-chaperone that acts as a lid to close the chaperonin cavity during the folding reaction (eg GroEL and its co-chaperone GroES, (reviewed by Saibil, 1996)). In contrast, group II chaperonins have a helical protrusion that extends from the apical substrate binding domains and acts as an inbuilt lid during the folding cycle (Klumpp et al., 1997).



A) Cartoon to show the arrangement of CCT subunits within a single ring (Liou and Willison, 1997). CCT subunits are named α - θ in mammals and numbered 1-8 in yeast. B) 3-dimensional reconstruction of CCT from cryo-electron microscopy (Llorca et al., 2001).

Figure 1. Subunit arrangement of CCT



Cryo-electron microscopy and 3-dimensional reconstructions of actin (A) and tubulin (B) shown in red binding to CCT in open conformations (Llorca et al., 2001).

Figure 2. Actin and tubulin binding to CCT

The prokaryotic chaperonin, GroEL and the archaeobacterial chaperonin, the thermosome are formed from one or one/two/three subunit species respectively. However, CCT is unique amongst chaperonins in that it is formed from eight distinct subunit species (all products of individual genes). These subunits share sequence homology particularly in their equatorial regions whilst the apical, substrate binding domains of CCT subunits display the most divergence in sequence (Kim et al., 1994). Each CCT subunit appears to be highly conserved during evolution, for example yeast and mammalian orthologues share approximately 60% identity (Kim et al., 1994). The eight CCT subunits each occupy a fixed position within the chaperonin ring (Liou and Willison, 1997) (Figure 1). This provides a complex binding interface for potential substrates formed from the divergent sequences of the apical domains where geometry may also play a role in substrate:chaperonin interactions. Indeed, Archibald

et al., (2000) speculate that during evolution, the order of the CCT subunits within the rings must have been established prior to, or occur at the same time as, the evolution of subunit:substrate interactions. This is based upon the positioning of subunits within the ring being important for substrate binding (as is thought to be the case for actin and tubulin (Llorca et al., 2001; Llorca et al., 2000; Llorca et al., 1999) and would be particularly relevant for substrates that interact with CCT through more than one binding site.

Mechanisms of Action

The exact mechanisms of action of CCT during the folding cycle are not fully understood and it is likely that different substrates will have variations in their requirements from their interactions with CCT. However, insights into the folding mechanism of CCT have been gained from the use of cryo-electron microscopy followed by 3-dimensional reconstructions (to study actin and tubulin binding) and also from studies using CCT mutants in yeast to understand the cycle of ATP hydrolysis.

Upon dilution out of denaturant, the major substrates of CCT, actin and tubulin, bind to specific CCT subunits: actin binds to CCT β and δ or CCT ϵ and δ and tubulin binds either CCT α , β , γ , η and θ or CCT β , δ , ϵ , ζ and θ (Llorca et al., 2000; Llorca et al., 1999). Both actin and tubulin bind to CCT in open conformations (Figure 2) and appear to have already formed a degree of native structure, described as ‘quasi-native’ by Llorca et al., (2000). This is indicative of these substrates binding to CCT as late folding intermediates. The direct interaction between these substrates and specific CCT subunits is supportive of the theory that recognition of substrates by CCT is sequence-specific (see below). As is the case with other chaperonins, CCT utilises ATP to drive conformational changes that play a key role in substrate folding reactions. Upon AMP-PNP binding (which induces lid closure), both actin and tubulin adopt globular conformations and move to one side of CCT cavity as seen by cryo-electron microscopy (Llorca et al., 2001). Mutations in the ATP binding regions of CCT subunits in yeast have revealed a hierarchy in ATP binding/hydrolysis, suggesting that nucleotide-induced conformational changes occur sequentially around the chaperonin ring (Lin and Sherman, 1997). This was also shown directly using electron microscopy (Rivenzon-Segal et al., 2005). These observations are in good agreement with the model proposed by Llorca et al., (2001) where for both actin and tubulin, the N-terminal domain is released during the folding cycle, leaving the C-terminal domains bound to CCT. Furthermore, Neiryntck et al., (2006), utilised a rabbit reticulocyte lysate *in vitro* translation system to study the binding and release of actin mutants by CCT, and predicted that the final stage of actin folding involved the arrangement of the C-terminus. These major substrates of CCT are therefore not released into the central chaperonin cavity, in contrast to the proposed mechanisms for the GroEL chaperonin system (eg Roseman et al., 1996).

An example of the importance of the CCT ATPase allostery for the function of this folding machine is demonstrated by the *anc2-1* (G345D) mutation in yeast Cct4p (CCT δ). This point mutation is situated in a flexible loop on the outside face of the Cct4p subunit (Llorca et al., 1999) and was shown by Shimon et al., (2008) to affect both inter and intra-ring cooperativity and reduce the levels of native actin produced in *in vitro* folding assays. In

yeast, this mutation results in the disorganisation of the actin cytoskeleton and an increased sensitivity of microtubules to benomyl (Vinh and Drubin, 1994) indicating that *in vivo* the folding of these substrate proteins is compromised (although the impact upon the actin cytoskeleton appears greater than on microtubules).

It is not known exactly what information/assistance CCT confers to its major folding substrates during their folding reactions. CCT may stabilise these open conformations of actin and tubulin until nucleotide binds (the tubulin GTP binding site matures whilst tubulin is bound to CCT (reviewed by Lewis et al., (1997)). Alternatively, CCT may be required to overcome a particular kinetic barrier in the folding of these two proteins. Altschuler and Willison, (2008) suggest that this is the case for the CCT mediated folding of actin, whereby CCT is required by actin to overcome a particularly challenging kinetic barrier, forming a folding intermediate that can then proceed to the native state upon the binding of nucleotide and cation. The work of Stemp et al., (2005) and Pappenberger et al., (2006) both show that in an *E. coli* lysate, *in vitro* translation system, actin forms a stable folding intermediate that can only reach its native state in the presence of CCT, and clearly demonstrates that prokaryotic chaperones are unable to fold actin. This suggests that CCT is needed to accommodate specific folding requirements of substrate proteins, yet actin and tubulin do not share any obvious structural similarities. This question was addressed by Yam et al., (2008) who searched for common features amongst the proteins that were found to bind to CCT during small pool expression cloning and suggest that CCT may be involved in folding proteins where there is a need to protect β -sheet from aggregation, and also assisting proteins that form oligomers.

CCT as a Component of Chaperone Networks

It is thought that in order to guard against the dangers of aggregation in the complex environment of the cell, chaperone networks have evolved to ensure that the opportunity for off-pathway interactions are minimised. This seems to be the case especially for eukaryotic cells where there is an increase in the number of larger, multi-domain proteins in comparison to prokaryotes, resulting in a shift towards co-translational folding (Ellis and Hartl, 1999). Additionally, rates of translation are significantly slower in eukaryotes, providing both the time for co-translational folding to occur and the need for folding to begin in order to minimise the chances of aggregation occurring. The chaperones of eukaryotic cells have recently been divided into two groups, the first being chaperones whose expression is stress inducible, the second, chaperones that do not respond to stress but instead their expression levels are associated with translation (Albanese et al., 2006). CCT falls into the second category, indicating that it functions in *de novo* protein folding. This study also demonstrated that CCT co migrates with polysomes by sucrose gradient fractionation and immunoprecipitation experiments indicated an association with newly translated polypeptides (Albanese et al., 2006). This is consistent with the work of Etchells et al., (2005) where CCT was shown to bind to nascent polypeptides via photo-crosslinking.

However, in contrast to CCT functioning as a co-translational chaperone, and in keeping with the observations that actin and tubulin bind to CCT as late folding intermediates (Llorca

et al., 2001; Llorca et al., 1999), prefoldin was identified as a chaperone that could bind to unfolded actin polypeptides (in an ATP-independent manner) and deliver them efficiently to CCT but not to hsp60 (Vainberg et al., 1998). Actin and both α and β tubulin have been shown to bind co-translationally to prefoldin and in the case of actin, it is necessary for the first 145 amino acids to have been translated for binding to prefoldin to occur (Hanson et al., 1999). Prefoldin is an oligomer of six subunits formed from six distinct subunit species in eukaryotes and two subunit species in archaea. The structure of prefoldin from the archaeobacterium *M. thermoautotrophicum* reveals that each subunit possesses a coiled coil region forming a long protrusion giving the overall appearance of a jellyfish (Siegert et al., 2000). The extreme ends of these six coiled coil 'tentacles' are hydrophobic and when deleted, a reduction in the ability of prefoldin to suppress aggregation was observed (Siegert et al., 2000). This observation, taken together with cryo electron microscopy and 3 dimensional reconstructions confirming that actin is grasped by the tips of the prefoldin subunits (Martin-Benito et al., 2002), indicate that prefoldin binds to actin at an early stage of its folding. Images obtained by cryo-electron microscopy of prefoldin bound to CCT in the absence of actin, reveal that prefoldin binds across the top of the chaperonin cavity in an arch-like structure (Martin-Benito et al., 2002). These binding studies show prefoldin to span the CCT cavity via interactions with CCT subunits in a 1,4 position. This is in good agreement with the work of (Llorca et al., 1999) where actin is shown bound to either CCT β and δ or ϵ and δ (ie in a 1,4 arrangement). The prefoldin subunits are not essential in yeast: deletion of prefoldin5 is not lethal (Vainberg et al., 1998) and only a reduction in the folding of actin is observed upon deletion of single prefoldin subunits (Siegers et al., 1999). However, in the crowded environment of the cell, a chaperone system in which the substrate protein is delivered directly to the next chaperone would reduce the chances of off pathway events occurring.

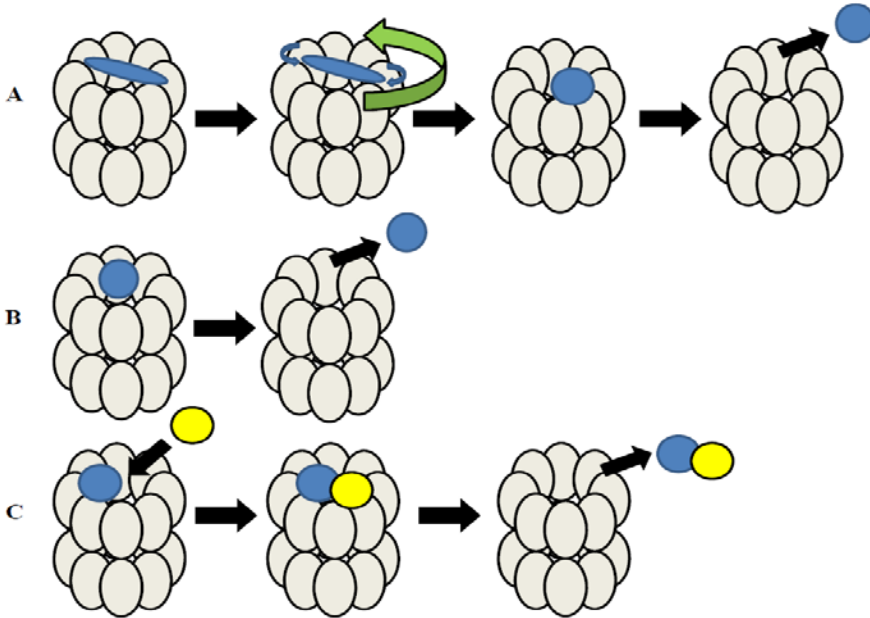
Another way in which CCT may take part in a chaperone network has been illustrated by the findings that Hsp70 can interact directly with CCT *in vitro* (Cuellar et al., 2008). It was shown biochemically that the nucleotide binding domain (but not the protein binding domain) of Hsp70 bound to CCT and analysis by electron microscopy revealed that this interaction occurred via the apical domain of the CCT β subunit. It is important to note that CCT β is implicated as one of the subunits involved in the initial binding of actin to CCT (Llorca et al., 1999) and it is possible that the binding of Hsp70 to this CCT subunit would orientate the substrate protein ready for binding to CCT. The docking of the atomic structure of Hsp70 onto the mass seen by cryoelectron microscopy suggests that the putative CCT binding site of HSP70 is a loop that is well conserved in eukaryotes but not in prokaryotes. Biochemically these authors saw no interaction between GroEL and DnaK, consistent with the theory that eukaryotes have developed coordinated chaperone networks.

The observations that prefoldin and Hsp70 can both interact directly with CCT raises an interesting question: are some substrates targeted to CCT specifically via prefoldin whilst others rely on Hsp70? Taken together with the observation that CCT may be able to bind substrates co-translationally (Etchells et al., 2005) it appears that different delivery systems may be used for the targeting of substrate proteins to CCT. Indeed the work of Yam et al., (2008) suggests that the way in which substrates are presented to CCT in the context of both folding intermediates and other chaperones may confer specificity of the chaperonin system.

However this is at odds with the theory that the interactions between CCT and its major folding substrates actin and tubulin are sequence-specific.

A Question of Specificity: Substrate Diversity and Substrate Recognition

The number of folding substrates of CCT has long been a matter of debate which is closely linked to speculation regarding how CCT recognises its substrate proteins. The first substrates of CCT to be identified were the major components of the cytoskeleton, actin and tubulin (Sternlicht et al., 1993). These proteins are very abundant and therefore not surprisingly, are the dominant proteins that co immunoprecipitate with CCT (eg Grantham et al., 2006). It is likely that actin and tubulin occupy a large share of the folding capacity of CCT, and in the case of actin, it is estimated that in yeast up to 50% of CCT oligomers may be occupied by actin at any one time (Brackley and Grantham, 2009). However it is clear that the number of CCT binding proteins extends beyond that of the actins and tubulins. For example, a group of proteins all involved in cell cycle regulation have been identified as CCT interacting proteins (Cdc20p, polo-like kinase I, Cdh1p and cyclin E), as have proteins that contain the WD40 repeat structural motif and also the Von Hippel Lindau tumor suppressor protein (reviewed by Brackley and Grantham, 2009; Horwich et al., 2007; Valpuesta et al., 2002). Two recent studies have aimed to evaluate the range of proteins that interact with CCT in yeast. The first uses a combination of proteomic and genomic approaches to establish an interaction network for CCT (Dekker et al., 2008). This study identified the proteins that co purify with CCT when implementing a tap-tag strategy of purification for the CCT oligomer and complemented this with a synthetic genetic array screen using the temperature sensitive *cct1-2*. This approach would therefore reveal proteins that have a physical interaction with CCT and also identify genes that are functionally linked to CCT. In the case of the latter group of interactions, the impact of the temperature sensitive CCT mutation could result in the miss-folding of a substrate protein and therefore reveal connections between CCT substrates and the deleted genes in the screen. Using these approaches, Dekker and co workers were able to identify an interaction network consisting of 136 genes/proteins including the major substrates actin and tubulin and CCT regulating proteins phosducin-like proteins 1 and 2. Of particular note is the discovery that the septin proteins (which are involved in bud formation) interact with CCT. Whilst these proteins do not require interactions with CCT to reach their native conformations, Dekker et al, suggest that CCT may have an organisational role in septin assembly as some mutant CCT alleles result in abnormal septum formation. This is an intriguing observation: septins form filaments and also bind to GTP, and is reminiscent of the major properties of actin and tubulin, in that they bind nucleotide and assemble into filamentous structures. Their utilisation of an ATP wash step has allowed for some functional insights into the nature of the observed interactions in terms of understanding which binding proteins are released from CCT upon ATP hydrolysis and therefore are likely to be folding substrates. This type of approach will be of great value for dissecting the nature of components of the CCT interactome.



A) Obligate substrates of CCT may need to bind via two interaction sites to enable mechanical input from CCT (blue arrows). B) non-obligate substrates may only require stabilisation from CCT which could occur via a single binding site. C) CCT as a platform for oligomer assembly, as for the non-obligate substrates, binding to CCT could occur via a single binding site per component of the assembling oligomer. The green arrow depicting the wave of nucleotide hydrolysis around the chaperonin ring to which substrate is bound is shown only in (A) but would also be expected to be required during reaction cycles with during other modes of binding.

Figure 3. Different classes of CCT binding proteins

The second recent study aimed to address the range of CCT subunits used small pool expression cloning to screen for the binding of newly synthesised proteins in a mammalian cell free expression system and suggested that CCT interacts with 6-7% of cytosolic proteins (Yam et al., 2008). This study was able to observe differences in the way in which proteins bound to CCT: some bound rapidly and transiently, whilst the binding of some proteins to CCT occurred later and the authors speculate that this delay in binding to CCT could be due to interactions with other chaperones occurring first (Yam et al., 2008).

It is important to note that in many cases, although a protein may have been shown to bind to CCT, the nature of this interaction is not always clear and it is likely that different classes of CCT binding protein exist. When understanding the extent of CCT function in the context of the cell it is important to consider different groups of CCT interacting proteins:

1). Obligate CCT Substrates (Figure 3A)

These are substrates that are dependent upon CCT to enable them to reach their native conformations and include the major CCT substrates actin and tubulin. These two proteins do not share any obvious similarities in their structures or sequences that reveal a common mode

of binding. Instead, cryo-electron microscopy has demonstrated that these two proteins bind to CCT using different but overlapping groups of CCT subunits. What is common is the utilisation of two binding sites which result in these substrate proteins being held in open conformations whilst bound to CCT. When a substrate is grasped by CCT in such a way then it should be possible to pull, push or twist the substrate protein via the conformational changes of CCT apical domains that occur during the ATP hydrolysis cycle of CCT. In this way CCT can directly manipulate the conformation of the substrate protein. It is therefore possible that a common feature of the obligate CCT substrates is the need for mechanical input during their folding. This certainly appears to be the case for actin, where the work of Altschuler et al., (2005) identifies an intermediate of actin, (that requires a high activation energy to reach the native conformation) which can be folded by CCT. Therefore, the free-energy landscape proposed by Altschuler and Willison, (2008) predicts that nascent actin forms intermediate I_3 which then requires input from CCT to overcome a high energy barrier in order to reach the I_2 state which is then able to bind nucleotide and proceed to the native conformation.

2). Non-Obligate CCT Substrates (Figure 3B)

In addition to providing a complex substrate binding interface where geometry and sequence appear to be important, the CCT apical domains may also be able to offer a non-specific binding surface. This may enable some proteins to utilise CCT when an off pathway event occurs during folding. Binding in this situation may occur via hydrophobic interactions between unfolded substrates and the hydrophobic amino acids located in the CCT helical protrusions.

Such interactions may act to stabilise a vulnerable folding intermediate which could then either spontaneously proceed to its native state or be assisted by the hsp70 chaperone system. It is probable that these interactions do not require the substrate to bind to CCT via more than one binding site as it would be predicted that the role of CCT would be passive.

3). Components of Oligomers (Figure 3C)

Proteins that are part of oligomeric assemblies may need to be held in an 'assembly competent' conformation until interactions with their binding partners have occurred. An example of CCT acting as a platform for oligomer assembly is demonstrated by the requirement of CCT in the formation of the Von Hippel Lindau Tumor suppressor protein/Elongin BC complex *in vivo* (Melville et al., 2003). It is important to note that the work of Yam et al., (2008) indicated that within the group of identified CCT binding proteins, there was a high number of proteins that were components of oligomeric assemblies, indicating that CCT may well play a role in the formation of oligomers. As with the non-obligate substrates, it can be predicted that proteins that utilise CCT as an assembly platform do not require more than one CCT binding site.

4). Regulatory Proteins

In addition to substrates of CCT some binding proteins may act as regulators of CCT activity. (see next section).

There is some debate regarding the exact location and nature of substrate binding sites on CCT. One theory argues for a rather general recognition of substrates with binding to CCT occurring via hydrophobic amino acids, whilst the second argues that binding to CCT occurs in a sequence-specific manner. In favour of the first theory, Yam et al., (2008) estimate that 6-7 % of proteins in yeast bind to CCT and that whilst CCT itself has the potential to bind to a wide range of proteins, some degree of specificity occurs when folding is occurring in the presence of other chaperones during translation. The binding of a substrate protein to CCT via a hydrophobic binding determinant is also demonstrated by Spiess et al., (2006) where the Von Hippel Lindau tumor suppressor protein was shown to bind to two CCT subunits within the oligomer via interactions with a hydrophobic helix in the apical domain situated at the base of the helical protrusion.

However, there is a great deal of evidence that argues strongly for the major CCT substrates actin and tubulin utilising binding sites that are sequence specific rather than a hydrophobic (ie, more general) interaction. This includes the observations already described here that the binding of actin and tubulin (as late folding intermediates) occurs via specific CCT subunits (Llorca et al., 2001; Llorca et al., 2000; Llorca et al., 1999) together with the mapping of the CCT binding sites to surface loops on actin (Hynes and Willison, 2000) and the inability of GroEL to fold actin (Pappenberger et al., 2006).

The divergence in the sequence between the CCT subunit apical domains together with the observation that the lining of the CCT central cavity contains an abundance of polar and charged residues (Pappenberger et al., 2002) suggests that CCT provides a complex binding interface where geometry and sequence specificity would certainly have the potential to have a major impact on interactions with substrates.

It has recently been discovered that a single amino acid (I297) in muscle α -actin is responsible for this isoform of actin being unfoldable by yeast CCT (Altschuler et al., 2009). These authors found that although muscle α -actin is able to bind to yeast CCT (consistent with I297 not being situated in any known CCT binding site), it was not folded, suggesting that the yeast CCT cannot proceed with the folding of α -actin at later stages of folding. These observations of species specificity illustrate the close coupling of the CCT:actin systems and strongly support the theory of Altschuler and Willison, (2008) where CCT is required by actin to overcome a specific folding barrier.

As previously suggested (Brackley and Grantham, 2009) CCT may well have a dual role in protein folding, acting as both a specific chaperone folding machine and as a general chaperone by providing a binding interface to stabilise vulnerable protein conformations. This would reconcile much of the debate in the literature regarding substrate specificity and the way in which folding substrates are recognised by CCT.

Regulation of CCT Activity

Little is known about how CCT activity may be regulated *in vivo*. It is probable that amongst the proteins now identified as CCT binding proteins some are regulators of CCT function. Some of these may be general enhancers of CCT activity and an example of this is the chaperone cofactor Hop (Hsp70/Hsp90-organising protein) which has been shown to bind directly to CCT and is able to enhance nucleotide exchange (Gebauer et al., 1998). As the number of potential substrates of CCT rises, questions regarding whether CCT activity is regulated in order to focus the activity of CCT towards obligate substrates become of interest. This issue is raised by McCormack et al., (2009) who suggest that one possible role of the phosducin-like proteins is to channel CCT folding towards particular substrates. The family of phosducin-like proteins have recently been shown to have a role as regulators of CCT activity (reviewed by Willardson and Howlett, 2007). In humans there are 3 forms: PhLP1, PhLP2 (Plp2p in yeast) and PhLP3 (Plp1p in yeast). PhLP1, 2 and 3 bind to CCT in their native forms (McLaughlin et al., 2002; Stirling et al., 2006; Stirling et al., 2007) and PhLP1 has been shown to bind to CCT forming an arch across the top of the central cavity (Martin-Benito et al., 2004). The mechanisms by which this family of proteins acts upon CCT is not understood. In some cases there appears to be an inhibitory action: the folding of newly synthesised actin can be inhibited by PhLP1 *in vivo* (McLaughlin et al., 2002) and PhLP3, when excess *in vitro*, inhibited the folding of actin and tubulin (Stirling et al., 2006). However, positive effects upon CCT mediated folding have also been observed: in a purified protein refolding system, yeast Plp2p (but not Plp1p) can increase the yield of CCT-folded actin molecules substantially (McCormack et al., 2009). Furthermore, when the yeast homologue of PhLP3 (PLP1) is deleted, cells are rescued from the lethal over expression of β -tubulin, possibly due to a reduced ability to correctly fold tubulin, which may result in the excess tubulin forming aggregates (Lacefield and Soloman, 2003).

In addition to protein co-factors/regulators, CCT activity may also be influenced at the level of post-translational modification. CCT β can be phosphorylated by p90 ribosomal S6 kinase and p70 ribosomal S6 kinase at S260 which is situated in the apical domain at the tip of the helical protrusion (Abe et al., 2009). These authors demonstrated that phosphorylation at this site is required for cell proliferation and speculate that due to its location, may influence either the chaperonin lid or the folding of a specific group of protein substrates.

CCT Function: Implications for a Wide Range of Cellular Events

The dependency of actin and tubulin upon CCT for their correct folding intrinsically links CCT function and activity to large array of cellular processes that require a functional cytoskeleton. Other known obligate substrates of CCT, Cdc20 and Cdh1, link CCT function to cell cycle progression (Camasses et al., 2003; Passmore et al., 2003). When CCT levels are reduced by siRNA in cultured mammalian cells there is a halt in cell cycle progression and a disorganisation of the actin based cytoskeleton with changes in cell motility (Grantham et al., 2006). As noted by Dekker et al., (2008)

it may be difficult to dissect the involvement of CCT during cell cycle progression as reduced levels of CCT would have effects both on cell cycle-specific substrates and the actin and tubulin cytoskeletal systems and this argument is of course true for many other cellular events.

Sphingosine kinase 1 (SK1) was recently identified as a folding substrate of CCT, with the CCT η subunit in particular being implicated in binding (Zebol et al., 2008). If SK1 is an obligate folding substrate of CCT, this would link CCT to a further array of cellular events via the role of SK1 in the production of the sphingosine 1-phosphate. CCT has also been shown to associate with some regulatory and catalytic subunits of the human protein phosphatase 2A system, with these interactions being conserved between yeast and humans, again providing further links between CCT activity and many cellular processes (Glatter et al., 2009).

In addition to the folding of newly synthesised proteins, CCT may have an additional role in the biogenesis of the actin and tubulin based cytoskeletal systems. *In vitro* CCT has been shown to influence initial rates of actin polymerisation and behave as a potential barbed (fast-growing) end actin capping protein (Grantham et al., 2002), while CCT α , γ , ζ and θ bind to microtubules polymerised *in vitro* (Roobol et al., 1999). This latter observation is in keeping with the growing evidence that CCT subunits when monomeric may have activity (reviewed by Brackley and Grantham, 2009).

Conclusion

The CCT chaperone is a unique protein folding machine that offers a complex binding interface for its folding substrates. Although we are now gaining insights into its mechanisms of action via cryo-electron microscopy to view its interactions with actin and tubulin (Llorca et al., 2001; Llorca et al., 2000; Llorca et al., 1999) and a greater understanding of the role of the allostery of the CCT ATPase (Shimon et al., 2008) the exact mechanisms of CCT action are not known. How CCT functions as a nano machine coupling nucleotide fuelled conformational changes to protein folding, especially with regard to actin, where it is known that CCT is required to overcome a particular energy barrier (Altschuler et al., 2005) remains an intriguing question.

Clearly the range of folding substrates and the way in which CCT may cooperate with other chaperones are areas that still require clarification in order to understand fully how CCT participates in the folding of proteins in the complex environment of the cell. CCT is often referred to as a general chaperone despite a great deal of evidence supporting the view that its interactions with actin and tubulin are sequence specific. This issue may well be resolved by the categorisation of CCT binding proteins as either obligate or non-obligate folding substrates together with consideration of the extent/abundance of these interactions in the cell.

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Chapter XIII

The Role of Molecular Chaperones in Plant Stress Response

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Abstract

Plant growth is greatly affected by abiotic stresses, such as drought, high salinity and cold. Therefore, plants need to have defending systems against many stresses for survival. Molecular chaperone interacts with unfolded or partially folded protein subunits, e.g. nascent chains emerging from the ribosome, leading to stabilizing native proteins, preventing aggregation of denatured proteins and degradation of defective or improperly folded proteins. Many molecular chaperones are stress proteins and most of them, but not all, have been identified as heat shock proteins (HSPs). HSPs/molecular chaperones and the heat stress elements in their promoters are conserved in all the eukaryotes, suggesting that they essentially have same function in response to high temperature stress. Participation of HSPs/molecular chaperones in other stresses has been also reported in plants. Furthermore, other types of proteins with chaperone functions, such as protein disulfide isomerase (PDI) and calnexin/calreticulin are up-regulated by the stress. They play a role for preventing aggregation by assisting refolding of nonnative proteins. It has been shown that expression of HSPs/molecular chaperones increases for assisting the deposition/assembly of high abundant secretory storage proteins in endoplasmic reticulum (ER) lumen during seed development. HSPs/molecular chaperones in plants are not well understood, however, they are elucidated little by little.

In this review, we first describe the characteristics of HSPs/molecular chaperones in plants. Then we describe the roles of HSPs/molecular chaperones in abiotic stress (heat and cold etc.) and ER stress during seed development, including enhanced

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chaperones accompanying by deposition of recombinant products in transgenic plants.

Introduction

In plant growing processes, HSPs/molecular chaperones are key components responsible for cellular homeostasis. Quality control systems are involved in protein folding and assembly, contributing on stabilizing native proteins, preventing aggregation of denatured proteins and dispatching nonnative and improperly folded proteins for destruction (1-3). HSPs/molecular chaperones act as key components leading to stress tolerance of plants. They are expressed as stress proteins and many of them were originally identified as heat shock proteins (HSPs). This heat shock response and heat shock factors are highly conserved from archaeobacteria to eubacteria and from plants to animals. Several of the proteins induced by heat shock are also induced by a variety of other stresses in order to protect cells from the toxic effects as an emergency response (4, 5).

1. Plant Chaperone

HSPs/molecular chaperones are localized in a distinct cellular compartment, including the cytosol, chloroplast, ER, and mitochondrion (6). Five major families of HSPs/molecular chaperones are conservatively recognized: the Hsp100/Clp family; the Hsp90 family; the Hsp70 family; the Hsp60 family; and the small Hsp (smHsp) family. Different classes of molecular chaperones appear to bind to specific non-native substrates and states. To date there are a few reports about them in plants.

Hsp100/Clp Family

The Hsp100/Clp, widely distributed and conserved in both prokaryotes and eukaryotes, are characterized by two conserved boxes (7) each of about 200 amino acid residues harboring an adenosine triphosphate (ATP) binding site. The proteins varies in size from 78 to 100 kDa dependent on difference in size of nonconserved spacers between the two conserve boxes and additional N- and C-terminal regions. Hsp100/Clp proteins are also synthesized in a variety of expression patterns and, are localized at different subcellular compartments in eukaryotes (7-11). They bind to denatured proteins and deliver proteins to the catalytic core of proteolytic complexes. They have ability to undo protein aggregates resulting from heat shock or other stresses and to disassemble protein complexes. They form homo-hexameric or -heptameric complexes in the presence of ATP and utilize the energy released from ATP hydrolysis to catalyze protein unfolding (11, 12). The Hsp100/Clp have a great diversity of functions, such as enhanced tolerance to high temperatures, promotion of proteolysis of specific cellular substrates and regulation of transcription (8, 13). They consist of two different polypeptides with different functions: a proteolytic subunit (mainly ClpP,

encoded on the chloroplast and nuclear genome); and regulatory subunits (mainly ClpA, C, D and X, encoded by the nuclear genome) in plants. Computer and proteomic analyses of the *Arabidopsis* genome revealed 26 orthologues (14-17).

Hsp90 Family

In eukaryotic cells, Hsp90 is the most abundant protein (~2% of cytosolic proteins) and is essential for viability (18). Hsp90 families bind to ATP and have autophosphorylating activity (19). Hsp90 acts as part of a multichaperone machine together with Hsp70 and co-operates with co-chaperones in order to protect aggregates of denatured proteins. Avian progesterone receptor (PR) has been used as an model system regarding the study on the Hsp90 chaperoning pathway (20-22). Although PR is inactive in the absence of the hormone, it can bind and respond to progesterone. This inactive receptor is in the protein complex. The study on Hsp90 complexes has revealed that five crucial chaperone components participated in the conformational regulation of Hsp90 client proteins, including Hip (Hsp70-interacting protein), Hop (Hsp70/Hsp90 organizing protein: p60/Sti1), p23, the immunophilins FKBP51/54(FK506 binding protein 51/54) and FKBP52, and Cdc37(cell division cycle protein 37)/p50 (23, 24).

The role of Hip, a tetratricopeptide repeat protein, is known to regulate the eukaryotic 70 kDa heat shock cognate Hsc70. Hip stabilizes the ADP state of Hsc70 that has a high affinity for substrate protein. Through its own chaperone activity, Hip may contribute to the interaction of Hsc70 with various target proteins (24-26).

Hop can bind either Hsp70 or Hsp90 independently and in an ATP-independent manner. Mutagenesis analysis of Hop indicates that the N terminus is required for Hsp70 binding, and a central region containing tetratricopeptide repeat motifs is necessary for binding Hsp90 and Hsp70. The role of Hop at this early stage of assembly appears significant in that it targets Hsp90 to Hsp70-client protein complexes. After loading of Hsp90 onto the client protein, Hop and Hsp70 dissociate and the Hsp90-client protein complex progresses to the mature form (27, 28). GmHop-1, a co-chaperone homologous to the mammalian Hop protein, was isolated from soybean and its transcripts were detected even under normal growth conditions but their levels increased under heat and cold stress (24).

p23 interacts with the ATPase domain of Hsp90. Although p23 can itself act as a chaperone in binding unfolded polypeptides (29, 30), p23 and its yeast homologue Sba1 specifically recognize the ATP-bound state of Hsp90 (31, 32), implying a more specialized function of p23 in regulating the Hsp90 ATPase cycle. The binding site for p23 is located within the NH₂-terminal half of Hsp90 and is supported by induced dimerization of Hsp90 (33). p23 appeared to stabilize the interaction between the Hsp90 heterocomplex and its substrate (34). CS domain of *Arabidopsis* SGT1 interacting with the N-Hsp90 is structurally similar to p23. It suggests that SGT1 has a similar binding property and activity as p23; however, the CS domain does not affect the ATPase activity of Hsp90(35).

FKBP51 and FKBP52 possess peptidyl-prolyl *cis/trans* isomerase (PPIase) domains that bind immunosuppressant drugs such as FK506 that are used for prevention of allograft rejection and tetratricopeptide repeat (TPR) domains, which provide binding sites for Hsp90.

The interactions of FKBP51 and FKBP52 with Hsp90 were demonstrated by coimmunoprecipitation and Western blot analyses (36-38). *Arabidopsis* ROF1 (AtFKBP62) is a peptidyl-prolyl cis/ trans isomerase and a member of the FKBP family. ROF1 expression is induced by heat stress and developmentally regulated. It binds Hsp90.1 via its TPR domain (39).

Cdc37/p50, having the potential to exhibit chaperone activity, is characterized as a protein kinase-specific cofactor for Hsp90. The C-terminal domain of Cdc37/p50 interacts with the N-terminal ATP-binding domain of Hsp90. In the crystal structure, Cdc37/p50 binds to the open face of the Hsp90 N-terminal domain, interfering with conformational changes of Hsp90 crucial for its ATPase activity; this accords well with the finding that Cdc37/p50 inhibits Hsp90 ATPase activity (40-42). There are many reports about Hsp90 multichaperone machine together with Hsp70 and co-operates with a cohort of co-chaperones except plant.

Recent report showed that Hsp90 may provide mechanistic insight into the cooperative interactions between molecular chaperones and proteolysis systems. Most cellular proteins in eukaryotic cells are targeted for degradation by the 26S proteasome, usually after they have been covalently attached to ubiquitin in the form of a poly-ubiquitin chain functioning as a degradation signal. *In vivo* inactivation of Hsp90 using the temperature-sensitive (*ts*) *hsp82-4Δhsc82* mutant cells (43) caused almost complete dissociation of the 26S proteasome into its constituents. Hsp90 interacts with the 26S proteasome and plays a principal role in its assembly and maintenance (44, 45).

Hsp70 Family

Proteins constituting the Hsp70 family exist in many cellular compartments. Hsp70 binds and releases unfolded/non-native proteins with its C-terminal domain. Hsp70 participate in a wide variety of processes, such as prevention against aggregation of denatured proteins (46), refolding of stress-denatured proteins (47), translation (48), translocation processes (49), steroid receptor function (20-22) and negative repressors of HSF1-mediated transcription by directed interaction to the transactivation domain (50, 51).

Plant Hsp70s are encoded by a multiple gene family. 12 full-length *Arabidopsis* Hsp70 sequences are available in the database, five genes encoding cytosolic proteins, three encoding endoplasmic reticulum (ER) luminal members, and remaining two each for plastid or mitochondrion-localized proteins, respectively. *Arabidopsis* Hsp70s exhibit organ- and developmental-specific expression, and their expression are in response to temperature extremes(52).

Developmental regulation of the heat-inducible Hsp70 in maturing seed and roots independent of temperature stress suggests physiological roles in seed maturation and root growth for this member of the Hsp70 family. The analysis of reverse transcriptase-polymerase chain reaction exhibited the complex differential expression pattern for the Hsp70s in *Arabidopsis* having specialized functions even among members localized at the same subcellular compartment (52). At least 12 members of this family have been detected, and their sequences and DNA blot analyses provided the diversity of genomic structure organization for this family despite evolutionarily conserved amino acid sequences in spinach

(53). Three similar cytosolic Hsp70s (*PsHSP71.2*, *PsHSP71.0*, and *PsHSP70b*) in pea have been reported to be regulated differentially (54).

One of Hsp70 homologues, BiP in the endoplasmic reticulum (ER) are implicated in the correct folding and assembly of secretory proteins in ER lumen after posttranslational transport across the ER membrane. BiP was termed as immunoglobulin heavy-chain binding protein which was later identified as GRP78 (Glucose-regulated protein 78). Expression of BiP is regulated at the transcriptional level and is induced under various stress conditions such as glucose starvation and treatment with calcium ionophores, calcium-chelating agents such as EGTA and compounds that block cellular glycosylation, such as tunicamycin and glucosamine. Expression of BiP is one of index for ER stress degree. BiP has been shown to regulate the unfold protein response (UPR) by controlling the activation status of the three transducers, IRE1, PERK and ATF6. They generally bind to extended polypeptide chains in ER lumen, independent of exposed hydrophobic features (55-57). Translational initiation of the human BiP mRNA is directed by an internal ribosomal entry site (IRES) located in the 5'-untranslated region (5'-UTR)(58, 59). However it is unclear how to induce *BiP* transcription in plants.

Hsp60/Hsp10

Hsp60/Hsp10 consists of two distinct family members, chaperonin 60 (Cpn60) and chaperonin 10 (Cpn10) as co-chaperone, which function together in an ATP-dependent manner. Hsp60/Hsp10 is molecular chaperone that is evolutionarily homologous to GroEL of *E. coli*. The intracellular localization is in the mitochondria and plastids of eukaryotes(2, 60-62).The products of the *groEL* and *groES* genes of *E. coli*, constituting the *groE* operon, are known to be required for growth at high temperature (42°C) and are members of the heat shock regulon (63). In plant, chloroplast chaperonins consist of two distinct polypeptides, Cpn60 α and Cpn60 β , which share only 50% amino acid identity (64, 65).

Small Hsp Family(Smhsp)

The smHsps range in size from 17 to 30 kDa and share a conserved C-terminal domain common to all eukaryotic smHsps and to the crys-tallin proteins of the vertebrate eye lens (2). Hsp20, smHsp form high molecular weight oligomeric complexes which serve as matrix for stabilization of unfolded proteins; Hsp70/40 and/or Hsp101 are inevitable for release. The complexity is variable, with a single representative in yeast (Hsp26) and 3-4 in mammals. In plants, 20-30 different smHsps are divided into six families based on DNA sequence analysis, immunological cross-reactivity and intracellular localization (66).

Aside from these major Hsp families, there are other groups with chaperone functions, such as protein disulfide isomerase (PDI) and calnexin/calreticulin, which assist in protein folding in the endoplasmic reticulum (ER). They act as key factors participating in quality-control of secretory proteins, ensuring that correctly folded proteins are transported to ER (3, 67, 68). Protein disulfide isomerase and peptidyl prolyl isomerase are known to catalyze the

formation and isomerization of disulfide bonds (rearrangement of disulfide bonds) or isomerization of peptide bonds around Pro residues, respectively (69). 22 PDILs (Protein disulfide isomerases-like), 19 PDILs and 22 PDIL proteins were identified in *Arabidopsis*, *rice* and *maize*, respectively (70).

Calnexin is type 1 transmembrane lectin protein of 64 kDa, whereas calreticulin is a soluble lectin polypeptide with M.W. of 46 kDa. They are highly homology to each other, and play an important role in folding of proteins carrying mono-glucosylated oligosaccharide. They are involved in addition of N-linked glycans in ER (71, 72).

Heat Shock Factor

The heat shock factors (HSFs) are involved in regulation of genes associated with the heat stress responses including heat shock proteins (Hsps), to protect against stress damage and many other important biological processes. Expression data using micro-array analysis and estimate of gene number from the complete genome sequences from *rice* and *Arabidopsis* indicates that HSF proteins are widely expressed in these plants (73). In *Arabidopsis*, there are 21 candidate genes divided into 3 classes and 14 groups, including a new class of *Hsfs* (*AtHsfCI*) closely related to *Hsfl* from *rice* and to *Hsfs* identified from frequently found expressed sequence tags of tomato, potato, barley, and soybean. They showed 19 tomato *Hsfs*, including 15 new genes (74). 25 HSF-like protein sequences were described in rice (73). There are 34 *Hsfs* in soybean (75).

Heat Stress Cis-Element

HSEs are reported as an inverted repeat region, which contains a varying number of the DNA sequence (5'-nGAAnnTTCnnGAAn-3') (76, 77), or as patterns of palindromic binding motifs (5'-AGAAnnTTCT-3') upstream of the TATA box of hsinducible genes (78, 79).

2. Chaperone Role in Stress in Plant

Abiotic Stress

Plant can't move to avoid from abiotic stress and it is greatly affected by environmental abiotic stresses, such as temperature, drought and high salinity. The relation between climate variation and crop production were investigated by synthesizing data on temperature, precipitation, solar radiation, and county corn and soybean yields throughout the United States for the period 1982–1998. Yield trends of crops were significantly correlated with observed temperature trends (80). Therefore, plants have systems of seed responses against defenses for survive. When plants are suffering from abiotic stress, two kinds of protein groups are induced. One group includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism. The others function in protecting cells from abiotic

stress, such as the enzymes required for biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes. Osmoprotectants (glycine, betaine, glycerol, proline and trehalose) can also act as 'chemical chaperones' by increasing the stability of native proteins and assisting in the refolding of unfolded polypeptides. Hsp/chaperone system plays pivotal roles in cells, both under normal growth conditions and when stressed (2, 81-84). It was analyzed by transcriptional response profiles of *Arabidopsis Hsfs* and *Hsps* to a range of abiotic and biotic stress treatments (heat, cold, osmotic stress, salt, drought, genotoxic stress, ultraviolet light, oxidative stress, wounding, and pathogen infection) and several genes belonging to the Hsp20, Hsp70 and Hsp100 families were specifically up-regulated in some condition (85). AtDREB2A, well-known transcription factor interacting with a *cis*-acting dehydration-responsive element (DRE) sequence, activates expression of many downstream genes associated with not only drought- and salt-stress response but also Heat stress (86). HSF-mediated cross talk was observed between HS and other abiotic stress signaling cascades. Among 21 members of the *Arabidopsis* HSF family, *HsfA3* is the only one that is transcriptionally induced during heat shock by DREB2A, and regulates the expression of genes encoding Hsps (87). Moreover, *AtHsfA9* has the unique role during seed developmental, which is not expressed during environmental stresses. It is notable that individual HSFs have the specific functions under different conditions. Actually, mutants analysis showed that expression of *AtHsfA9* depended on ABI3 (88).

Hsp100/Clp is essential for the survival of plants under high-temperature stress. ERD1 (early-responsive to dehydration 1) encoded by *ClpD* was up-regulated in response to dehydration and reached a maximum level after 10 h in *Arabidopsis* (89). The *ClpB* gene shows strong heat-inducible expression in bacteria and encodes a protein of approximately 100 kDa, called ClpB/Hsp100. ClpB proteins in *Arabidopsis*(90) and soybean (91), which was functionally complement to yeast ClpB/Hsp104 deletion mutants and restore thermotolerance, confer thermoprotection. Hsp101 has been shown to confer thermotolerance directly by the analysis of Hsp101 mutants of *Arabidopsis*, which are sensitive to high temperature (92), and *Hsp101* overexpressing plants have increased protection against high-temperature stress (93). APG6 (albino or pale-green 6), ClpB3 confer thermotolerance to chloroplasts during heat stress (94).

Plant *Hsp90* genes have been identified from tomato (95), periwinkle (96), maize (97), oil seed (98) and rice (99), and they are strongly induced by various abiotic stresses. Seven Hsp90 family members have been isolated (100), and their expression in *Arabidopsis* is developmentally regulated and induced by treatment of heat, cold, high salinity stress, heavy metals (copper, cadmium, lead and arsenite), phytohormones and light and dark transitions(100, 101). Overexpression of cytosolic and organelle *AtHsp90s* reduced tolerance to salt and drought stresses and expression of *RD29A*, *RD22* and *KIN2* was delayed under salt and drought stresses(102).

The expression of 10 spinach Hsp70 genes induced by heat stress was investigated in cold treatment. Some of them were transiently up-regulated, whereas others retained to be up-regulated. In tomato, 11 of 15 molecular chaperones tested exhibited elevated expression (1). *At-Hsc70-1/Hsp70-1* was shown to be expressed in leaves at normal temperature and further

induced by heat shock (52). The protective chaperone activities of Hsp70 serve to confer tolerance to heat, glucose deprivation, and drought in *Arabidopsis* (103).

Accumulation of Hsp22, mitochondrial smHsp in tomato cells (104) and Hsp26, the chloroplast-localized smHsp in rice (105) are induced by oxidative stress and play an important role in adaptive response. *AtHsp17.6A*, the smHsps encoding the cytosolic class II smHsp in *Arabidopsis* was induced by heat and osmotic stress, as well as during seed development. Overproduction of *AtHsp17.6A* enhanced salt and drought tolerance. Accumulation of *AtHsp17.6A* proteins could be detected by treatment with heat, but not with osmotic stress, suggesting stress-induced post-transcriptional regulation of *AtHsp17.6A* expression (106).

There are many reports on relationship between plant Hsps and stress. However, the function of Hsps in stress tolerance is not fully explored, especially Hsp60.

ER Stress in Rice

BiPs are induced at the maturation stage (1-2 weeks after pollination) during seed development where cells rapidly expand and their weight reach a maximum level toward the desiccation stage in pumpkin, rice and wheat (107-109). PDI and the calnexin are highly expressed at the early stage of seed development. They play critical roles in depositing storage proteins within the ER lumen in rice seed (110, 111). It has also been shown that ER stress in seed is induced by rapid and abundant accumulation of storage proteins.

We have recently generated transgenic rice plants accumulating several recombinant products such as the soybean ferritin (112), the T cell epitope peptide derived from cedar pollen allergens (7Crp) (113, 114), the modified ovokinin(2-7), (nobokinin) (115), the glucagon like peptide (GLP-1) (116, 117) and the house dust mite (HDM) allergen (Der p 1) (118) in seeds by expressing them under the control of the endosperm-specific glutelin *GluB-1* promoter (119). Pleiotropic phenotype with opaque or floury in their seed suggests different kinds of ER stress were induced, which was confirmed by electron microscopic observation of endosperm cells. Expression of ER stress associated proteins such as BiPs and PDIs has been shown to increase in maturing seeds in order to alleviate ER stress. UPR motifs are detected in promoter of several BiP and PDI genes, suggesting that high expression of heterogeneous products causes ER stress (Oono et al., in preparation).

The 44K Rice Oligo Microarray analysis was carried out using transgenic rice maturing seeds in which strong ER stress was induced by high accumulation of recombinant peptides. We found that some HSF and many heat shock protein genes were highly up-regulated. During seed development, Hsp/chaperones were generally induced (88). However, most of their genes exhibited quite different expressions pattern from those during seed development (Oono et al., in preparation). *BiP* transformants (*BiP OX*: Yasuda et al., in press, *BiP RNAi*: Wakasa in preparation) have similar phenotypes to those accumulating high concentrations of heterogeneous peptides, indicating that overexpression or suppression of BiP as one of ER stress sensors caused severe ER stress. Many heat shock proteins were induced, suggesting abnormal protein metabolism was easy to induce ER stress.

ER stress signal pathway and high temperature stress pathway may exhibit cross-talk of gene expression in rice. Actually, in *Arabidopsis*, *AtbZIP28* (120) was up-regulated in response to heat and that a *AtbZIP28 null mutant* has a striking heat-sensitive phenotype. The heat-inducible expression of genes that encode *BiP2*, ER resident chaperone, and *Hsp26.5-P*, a small heat shock protein, is attenuated in the *AtbZIP28 null mutant* (121). Furthermore, Signaling pathway of salt stress response is mechanistically related to ER stress responses (121, 122) in *Arabidopsis*, suggesting ER stress signal pathway is cross-talk with other stress signal pathway.

Conclusion

In plant growth, Hsps/molecular chaperones have important roles for adaptation to stresses and seed development. Furthermore, during germination stage before start of photosynthesis, immediate and efficient protein biogenesis are required (52). In Pumpkin, BiP was induced for assisting the folding and assembly of newly synthesized hydrolytic enzymes, which are required to degrade seed storage proteins used as nutrients for germinating seedlings (107).

However, many parts remain unclear for Hsps/molecular chaperones in plant. Further studies will be required to understand whole picture. Transformants containing heterogeneous recombinant products in our studies will be one of powerful tools to find novel Hsps/molecular chaperones to keep homeostasis cell, because kind of chaperons induced by ER stress depends on property of accumulated recombinant product.

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Chapter XIV

Chaperone Activity of Intrinsically Disordered Proteins

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Abstract

Intrinsically disordered proteins (IDP) are unfolded/unstructured under native conditions. They play important roles in living organisms, mostly in signal transduction and regulation of biochemical pathways. Biochemical studies showed that some IDPs also have protective effect on partner molecules, or enhance folding of a specific protein, i.e. they have chaperone activity. In our studies we have analyzed the chaperone activity of six IDPs and found that all of them are active in a variety of assays employed. The studies were carried out with two plant stress proteins from the dehydrin family (ERD10 and ERD14, *A. thaliana*), and four *E. coli* ribosomal proteins (L15, L16, L18, L19). We have tested these proteins in both the prevention of substrate aggregation and deactivation, and in the refolding/reactivation of a denatured partner molecule. We found that ERD10 and ERD14 hinder aggregation and also deactivation, however they do not assist refolding. The ribosomal proteins significantly enhance the refolding and reactivation of a denatured protein and also prevent the deactivation of substrate molecules under denaturing conditions. A combination of their chaperone activities and molecular properties (tolerance against mutation, resistance against aggregation, etc.) elevate these IDPs to a unique level of functionality and stability, due to which they play an essential role in the survival of strong environmental stress conditions and probably also in sudden changes in protein stability caused by a mutation for example.

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Introduction

Biophysical and Structural Properties of Intrinsically Disordered Proteins

Intrinsically disordered proteins (IDPs) are widely distributed in prokaryotic and eukaryotic proteomes (Tompa et al. 2006), where they play fundamental functions in regulation of downstream signaling in the cell, biochemical pathways or gene expression (Tompa 2002; Dyson and Wright 2005). These functions are usually closely related to the structural disorder of these proteins, such as high flexibility, structural adaptability and extended conformational states. This characteristics may appear either along the entire length of the protein, which is then called fully unstructured/disordered, or only in a part of the protein which is called partially unstructured/disordered. The preliminary condition of the formation of an IDP region is the special amino acid composition (Radivojac et al. 2007). IDPs are highly charged, mostly with hydrophilic characteristics. They are depleted of hydrophobic amino acids, such as Trp, Tyr, Ile, Phe, Val, Leu and the structure-promoting amino acid Cys, while charged and hydrophilic amino acids, such as Asp, Met, Lys, Arg, Ser, Gln, Pro and Glu are overrepresented within these proteins, compared to globular proteins (Figure 1.). According to this shift in the aminoacid composition Dunker and colleagues have suggested the term “order promoting” amino acids for those which are under-, and “disorder promoting” amino acids for those which overrepresented in IDPs (Dunker et al. 2001).

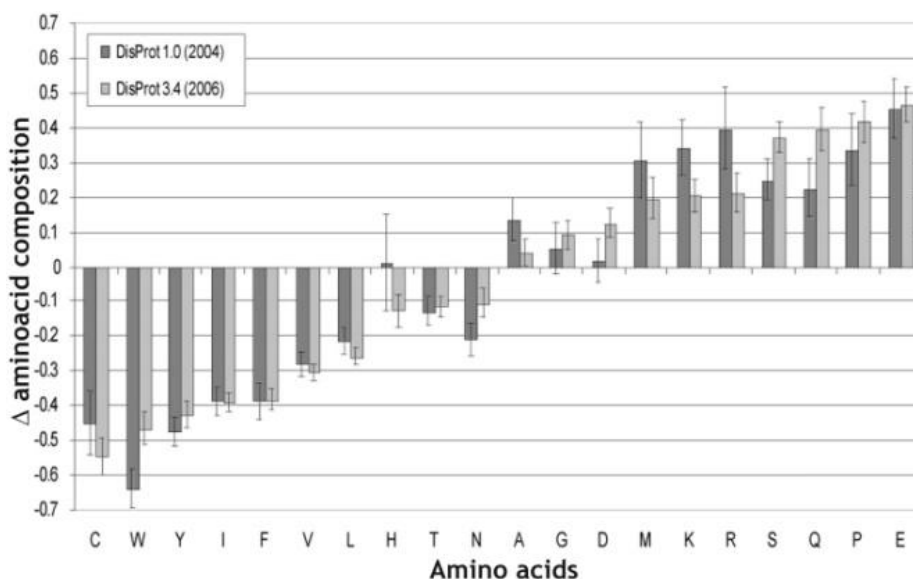


Figure 1. The difference in amino acid composition between IDPs and globular proteins. Amino acid composition datasets were established using Disprot (Sickmeier et al. 2007) and Globular-3D.dataset, the latter created from PDB entries. Columns indicate the difference in amino acid composition of IDPs and globular proteins, i.e. positive value indicates higher frequency, while negative value indicates lower frequency of the corresponding amino acid in IDPs. Dark gray indicates DisProt 1.0 (152 proteins), whereas light gray indicates DisProt 3.4 (460 proteins). Confidence intervals were estimated using per-protein bootstrapping with 10,000 iterations (Radivojac et al. 2007)

The hydrophilic character makes the formation of a hydrophobic core – known critical in the formation of most structured proteins – impossible. Accordingly, the soluble structure of IDPs is usually highly extended with similar characteristics observed for denatured proteins. In their typical structural state not all conformations are allowed, because some are sterically excluded by the amino acid side-chains. In solution the allowed conformers rapidly convert one into the other, without strong preferences to any particular conformation. Thus, in solution they constitute a structural ensemble of conformations, which is their biologically active state (Linding et al. 2003). This phenomenon observed by IDPs is against the classical concept of structural biochemistry, which claims that a specific function of a protein is determined by its unique and rigid three-dimensional (3D) structure. This idea was formulated more than 100 years ago as a lock-and-key model for explaining the amazing specificity of the enzymatic hydrolysis of glycosides (Fischer 1894). To contain these observations Wright and Dyson have called for the re-assessment of this paradigm (Wright and Dyson 1999).

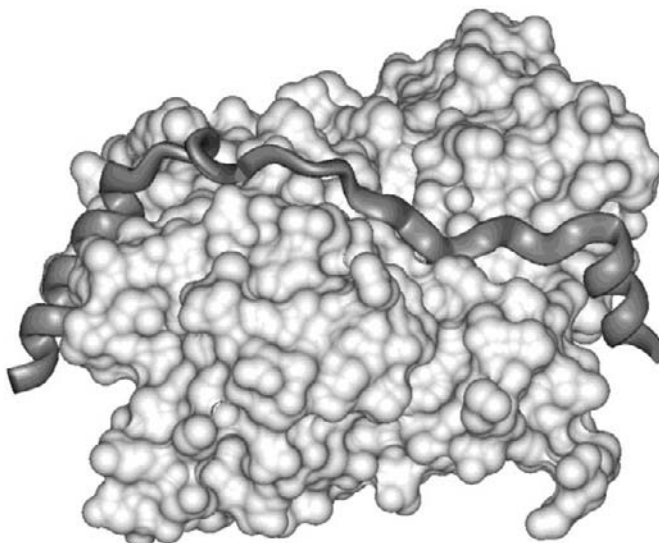
The biophysical and structural properties of IDPs are different from those of globular proteins. Their polypeptide chain is extended and wanders a bigger space, thus their apparent molecule size is much bigger than expected from the calculated molecular mass (Tompa 2002). This property entails several consequences such as lower mobility in size exclusion chromatography or that their polypeptide chain is easily accessible for other molecules. Latter makes them ideal for post-translational modifications, such as glycosylation, phosphorylation, proteolytic digestion etc. Also on the account of extended structure, they possess extended interaction site, which makes them suitable for rapid molecular recognition and binding (Shoemaker et al. 2000; Tompa 2002).

Biological Function of IDPs

The biological function of IDPs is strongly correlated with their structural nature. The extended polypeptide chain makes them suitable for the formation of extended interaction sites, as they often present more than one interaction site to one particular substrate or they even can bind distinct partners at the same time (Tompa 2002). The interaction formed at multiple sites make them very specific, like p21, the specific inhibitor of cyclin dependent kinases, which binds to the CDK with an extended interaction site or thymosine β 4 which binds to the G-actin in a similar manner (Figure 2). IDPs usually bind to the partner molecule via a structured motif formed during the interaction. This process is called induced folding (Turjanski et al. 2008), which usually occurs at a specific site of the protein called “molecular recognition feature”, “linear motif” or “preformed structural element” (Fuxreiter et al. 2004; Uversky et al. 2005).

IDP regions are unable to form a catalytic center, accordingly no enzymes can be found among IDPs (Tompa 2002). Nevertheless, they carry out essential functions in regulation of biochemical pathways, either by modifying enzyme activity, organizing cell compartments, maintaining the assembly of protein complexes, neutralizing small molecules or by providing sites for post-translational modification (Tompa 2002). However the most unexpected function is their chaperone activity. Chaperones play an essential role in folding of protein or

RNA substrates toward obtain their biologically active structure (Wandinger et al. 2008). Also, they are the molecular “body guard” under cellular stress, by protecting the active conformation of biomolecules against denaturation (Yahara et al. 1998; Wandinger et al. 2008). This function of IDPs is surprising because one would expect that a 3D structure is required for such an activity. However, IDPs carry out this function in their native structural state, strikingly with similar efficiency as globular chaperones. This biological function of IDPs was firstly reviewed by Tompa and Csermely (Tompa and Csermely 2004), who claimed that beside a few known fully unstructured chaperones, unstructured regions occur more often in globular protein-, and RNA chaperones than in other functional classes of globular proteins. In addition, the authors have suggested a possible mechanism for this activity, i.e. the entropy transfer model. The model claims that the highly flexible protein or protein region binds to the misfolded partner molecule and due to its high flexibility (entropy content) it breaks up the misfolded, locally stable structure to provide another chance for the substrate to explore the conformational space and fold into the native, biologically active conformation (also see in detail in the conclusion).



The structure of T β 4 (dark grey) bound to G-actin (light grey) has been assembled by combining the structures of two fusion proteins containing either half of T β 4, in complex with G-actin, cf. (Irobi et al. 2004).

Figure 2. Thymosine β 4 bound to G-actin

Protein Chaperone Activity

The relevance of IDP regions is nicely shown in the case of GroEL-GroES system, which is barrel-like chaperone complex in prokaryotic organisms (Lin and Rye 2006). The C-, and N terminal regions of GroEL are highly unstructured, and oriented into the central cavity of the barrel-complex, which is the actual site of chaperone action (Clare et al. 2009). Biochemical studies showed that the deletion of the C-terminal unstructured region reduces

the chaperone efficiency of the GroEL dramatically (Machida et al. 2008). This and similar studies have shown that IDP regions are indeed essential parts of globular chaperones. Detailed analysis of fully unstructured proteins, such as shown in this chapter, revealed that they may carry similar chaperone activity within their native, completely unfolded conformation as well.

Fully unstructured protein have chaperone function either in protecting the native structure of substrate(s) and preventing aggregation, or even during the folding or refolding processes. For example, α -synuclein, which is involved in Parkinson's disease aggregates into amyloid fibers in dopaminergic neurons and causes cell death, which is the causative event in the disease (Maguire-Zeiss et al. 2008). β -synuclein, the fully unstructured homologue of α -synuclein hinders the aggregation process, with an unknown mechanism (Bertoncini et al. 2007). Intriguingly, α -synuclein itself is an IDP that shows chaperone-like activity by protecting the enzyme activity of microbial esterases against heat, low pH, and organic solvents (Park et al. 2002; Ahn et al. 2006). Fully disordered α -casein was also described to prevent a variety of unrelated proteins/enzymes from thermally-, or chemically induced aggregation (Bhattacharyya and Das 1999). A similar relation is also apparent between caseins themselves, as α - and β -casein are potent inhibitors of fibril formation by κ -casein (Thorn et al. 2005). Microtubule-associated protein 2 (MAP2) can prevent the DTE-induced aggregation of insulin and the thermal aggregation of alcohol dehydrogenase, whereas it can also reactivate enzymes, such as lactate dehydrogenase, malate dehydrogenase, and α -glycosidase (Sarkar et al. 2004).

Upon cellular stress the general biological significance of chaperone function is unambiguous. Under environmental stress, such as cold, freezing, elevated temperature, drought or osmotic stress cell components (proteins) and cell compartments (cell organelles surrounded by membrane) are subject to degradation and deactivation, as they are extremely sensitive to changes in the environment (Vierling and Kimpel 1992). Without protective factors, these changes lead to cell death. Stress proteins are present in the cells under native conditions and carry out essential function in the physiological assembly of the cell, however upon stress their expression level is increased several fold and they promote viability of the cell, thus they represent a survival factor upon stress. Such proteins were analyzed in detail in immovable organisms, such as plants (Nesatyy and Suter 2008), which are extremely affected by the changes in environment, due to which strong molecular protection is essential. Plant stress proteins are members of late embryogenesis abundant (LEA) protein family (Tunnacliffe and Wise 2007), termed so due to their occurrence during the late stage of seed development. LEA proteins are highly disordered, several members are suggested to be fully disordered without showing any secondary structural motif under native condition. The expression level of LEA proteins is significantly increased in matured plant upon abiotic stress, such as cold or high salinity (Tunnacliffe and Wise 2007), their absence cause decreased tolerance against stress, which suggests that they possess essential role in the protection of the plant. The *in vitro* study carried out with AavLEA1 protein revealed that it protects citrate synthase (CS) against heat-induced aggregation and lactate dehydrogenase (LDH) against cold-induced aggregation (Goyal et al. 2005). These protective effects, however, manifest only in a trehalose-dependent manner, with low protective efficiency. Thence the authors suggested that this effect is based on the "molecular shield" mechanism,

rather than chaperone activity, which claims that the chaperone inhibit the interaction between denatured protein molecules, thus prevent the formation of aggregates. Nevertheless, further examination of AavLEA1 revealed that it inhibits the aggregation of polyQ sequences inside living cells, derived from the pathological factor of Huntington's disease, huntingtin (Qin and Gu 2004; Chakrabortee et al. 2007), furthermore it has a broad anti-aggregation effect on human cell extracts (Chakrabortee et al. 2007). Desiccation of globular proteins often induces denaturation and aggregation, which was effectively hindered with the addition of AavLEA1 protein at a 1/5 molar ratio. Furthermore, it was also shown that if AavLEA1 is added to the protein sample within 5 minutes after resolubilization, it still rescues protein from aggregation, but it does not have any effect afterwards. This suggests that AavLEA1 protects protein substrates against aggregation in a non-specific manner, but it can not resolubilize aggregates already formed. Cryoprotective activity on LDH has been demonstrated for two dehydrin-type proteins (Momma et al. 2003), and a similar effect was also shown for PCA60, a protein from winter bark tissues of peach (Wisniewskia et al. 1999).

RNA Chaperones

It is generally accepted that in the early stage of evolution RNA was both involved in information storage and catalysis (Gilbert 1986). Because RNA is prone to misfold (Herschlag 1995; Treiber and Williamson 2001; Cristofari and Darlix 2002), simple proteins with RNA chaperone activity, might have conferred a significant selective advantage in a world so highly dependent on RNA function. Several such proteins with RNA chaperone activity were described in the literature, which either partially or fully disordered. The classical example is the A1 protein of heteronuclear ribonucleoprotein (hnRNP), which promotes the renaturation of complementary nucleic acid strands. hnRNP has a Gly-rich unstructured carboxy-terminal domain that is involved in the renaturation activity (Pontius and Berg 1990). An other convincing example is the nucleocapsid protein (NCp7) which can be found in HIV. The NCp7 has a structured central domain with two zinc-finger motifs, while the amino-, and carboxy terminal regions are unstructured. The unstructured regions participate in strand transfer reactions during reverse transcription (Morellet et al. 1992). Ribosomal proteins (RPs) are known for their essential function in the assembly and stabilization of the ribosome (Herschlag 1995; Ramakrishnan and White 1998; Rajkowitsch et al. 2005), which they carry out with the partial or complete absence of structural motifs (Tompa and Csermely 2004; Timsit et al. 2006; Ivanyi-Nagy et al. 2008). Several RPs carry out functions outside of the ribosome (Wool 1996) denoted as extraribosomal function, some of which may critically contribute under ribosome misassembly and stress-response. These extra functions are often realized through interactions with RNA or DNA, such as the RNA chaperone activity in a trans-splicing assay of several large-subunit RPs (Semrad et al. 2004).

Research Studies

In this chapter we will review the chaperone activity of six IDP proteins, four ribosomal protein from *E. coli* (Kovacs et al. 2009); L15, L16, L18, and L19, which show significant chaperone activity in RNA trans-splicing assay (Semrad et al. 2004) and two LEA proteins (Kovacs et al. 2008) ERD10 and ERD14 (members of LEA group 2, also termed as dehydrins) from *Arabidopsis thaliana*. The chaperone assays were carried out with selected model substrates in a test tube under denaturing conditions, like elevated temperature or denaturing chemicals. The model substrates were chicken egg lysozyme, alcohol dehydrogenase (*Saccharomyces cerevisiae*) and firefly luciferase. We have both performed denaturing and refolding assays to probe both type of chaperone activity these proteins may have.

Chemically Induced Deactivation of Lysozyme

Lysozyme is an enzyme which catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in bacterial cell wall (Holtje 1996). Dithio-threitol (DTT) reduces the disulphide bonds in proteins and results the disruption of the active conformation. In the chaperone assay we treated lysozyme with DTT in the presence and in the absence of either LEA protein (Figure 3A and Table 1) or RPs (Figure 3B and Table 2) The denaturation process can be followed via enzyme activity assay, by following the decrease in enzyme activity, measured by the hydrolysis of bacterial cell-wall suspension.

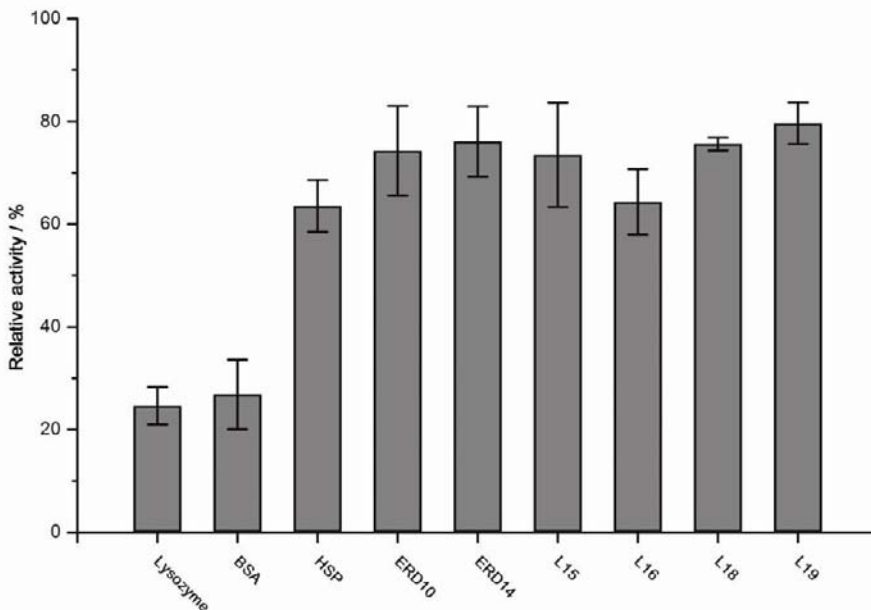


Figure 3. Chemically induced deactivation of chicken-egg lysozyme.

Lysozyme was treated with DTT, alone or in the presence of BSA, HSP90, ERD10, ERD14, L15, L16, L18 or L19, indicated on the horizontal axis. Columns represents relative enzyme activity observed after 30 min of treatment, relative to the activity of native lysozyme, SD were calculated from 3 consecutical experiments.

All six IDP has a similar, or even stronger protective effect than the well-known eukaryotic chaperone heat shock protein 90kDa (HSP90) (Figure 3., Tables 1. and 2.). The experiments were carried out in similar manner, whereas the lysozyme was preincubated alone or with one of either of IDPs or controls, and DTT was only added prior to the experiment. Enzyme activity was measured at every 5 minutes, and transformed into a relative term. After 30 minutes the enzyme activity has dropped to 25% of the initial activity, whereas in the presence of HSP90 the deactivation was significantly diminished, leading to a final activity of 65%. ERD10 and ERD14 protected lysozyme against deactivation to a slightly higher degree than HSP90 did; the final activity of lysozyme was 75%, while in the presence of RPs the remaining activity was between 65% and 70%. BSA — in the same concentration as the chaperones — did not affect the rate of deactivation.

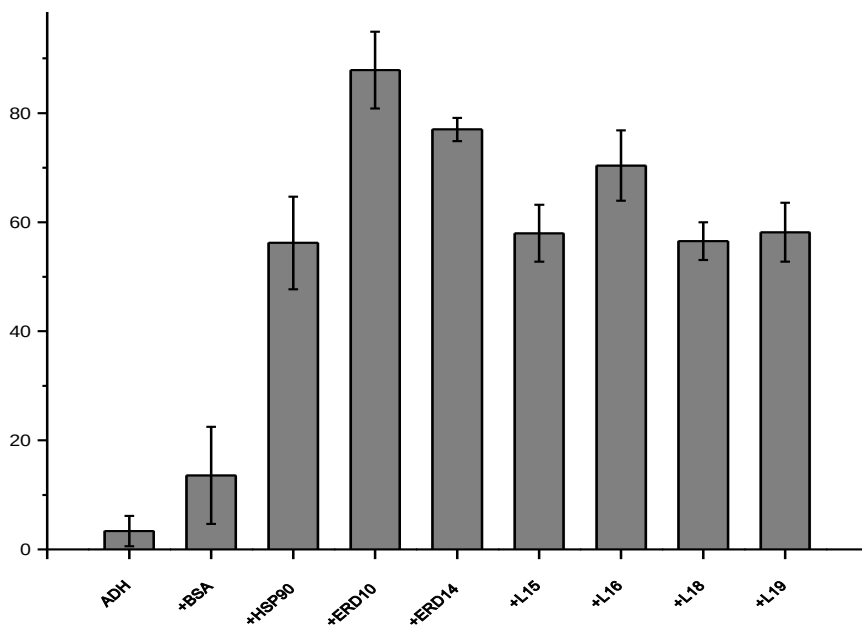


Figure 4. Heat-induced deactivation of alcohol dehydrogenase.

Heat-Induced Deactivation of Alcohol Dehydrogenase

Alcohol dehydrogenase (ADH) is a 160 kDa tetrameric enzyme, which catalyses the conversion of ethanol into acetaldehyde in the presence of NAD^+ coenzyme. ADH loses the active conformation required for enzyme activity at elevated temperatures. This characteristic of ADH has been used in a chaperone assay, by following the course of the activity loss in the presence and in the absence of a candidate chaperone. We analyzed all six IDPs for

chaperone activity in this assay, and found that all of them have a significant protective effect (Figure 4., Tables 1. and 2.).

ADH was treated at 43°C for 60 min alone and in the presence of either of BSA, HSP90, ERD10, ERD14, L15, L16, L18 and L19; as indicated on the horizontal axis. The enzyme activity of ADH were converted into relative term, related to the enzyme activity of native ADH. The diagramm shown the activity oserved after 60 min of heat-treatment; SD were calculated from 3 consecutical experiments.

In one hour the activity of ADH decreased to about 5% of the initial activity, similarly with the negative control BSA. HSP90 limited the deactivation to about 60% of the initial activity. ERD10 and ERD14 showed stronger protection at 5 times higher molar ratio, when the enzyme activity only decreased to 90% and 75%, respectively. They also protected ADH at lower molar ratios; however with lower efficiency, which can probably be accounted for by the 5 fold difference in molecular size. L15, L18 and L19 showed chaperone activity comparable to HSP90, while L16 protected ADH more efficiently than the other three RPs.

Heat-Induced Aggregation of Firefly Luciferase

The chaperone effect of ERD10 and ERD14 was also tested on firefly luciferase. Luciferase is a heat sensitive protein which denatures and aggregates upon heat treatment. Both ERD10 and ERD14 prevented the aggregation of luciferase completely, as did the positive control HSP90 (Figure 5., Table 1.). BSA again did not have significant effect in the assay. RPs have not yet been tested in this assay.

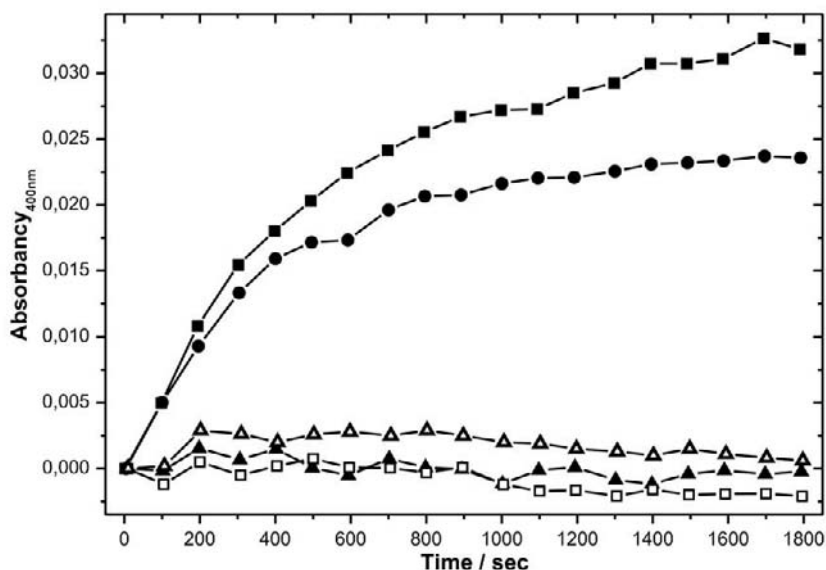


Figure 5. Heat-induced aggregation of firefly luciferase. The figure shows a typical experiment of thermal aggregation of luciferase. The denaturation of luciferase was induced at 45 °C alone (■) or in the presence of BSA (●), HSP90 (▲), ERD10 (□), or ERD14 (△). Aggregation was followed by measuring absorbance at 400 nm.

Reactivation of Lysozyme

Chaperone proteins usually also support complex non-trivial folding mechanisms. For testing such an activity of IDPs, we have performed refolding assays with entirely reduced and denatured chicken egg lysozyme. The refolding was induced with equilibrated red-ox system, composed of reduced and oxidized glutathione, and followed in time. The formation of structures was followed by determining of the enzyme activity at every 10 minutes. Chaperone effect was indicated by the increased speed of refolding and/or the higher activity regained in the same time. This study showed that neither ERD10 nor ERD14 affected the process of refolding (data not shown), while all four RP have significantly increased the activity of lysozyme regained (Figure 6. and Table 2.).

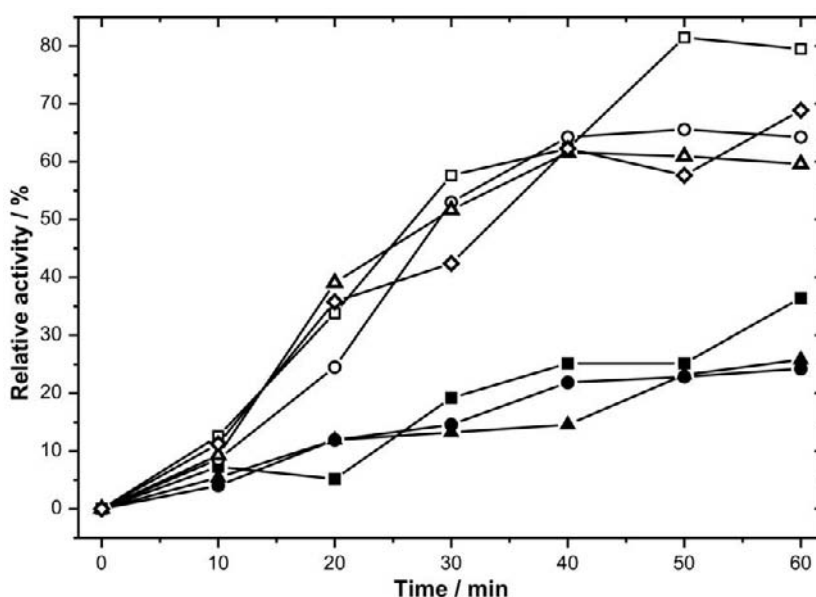


Figure 6. Reactivation of denatured lysozyme. Reactivation of lysozyme was initiated with rapid dilution into reactivation buffer. The recovery of enzyme activity was followed alone (■), or in the presence of BSA (●), HSP90 (▲), L15 (□), L16 (○), L18 (△) or L19 (◇) for 60 minutes; enzyme activity was measured by the specific reaction of lysozyme, the decomposition of *Micrococcus lysodeicticus* cell wall suspension. The activities are given relative to that of native lysozyme.

Conclusions

The experimental evidence reviewed in this chapter pertains to the “classical” chaperone activity of six intrinsically disordered proteins (IDPs) (Kovacs et al. 2008; Kovacs et al. 2009), two plant stress proteins belonging to the LEA group2 family (dehydrins), and four *E.coli* RPs.

Chaperone Activity of ERD10 and ERD14

The two LEA proteins show significant protective and/or anti-aggregation effect on a wide variety of model substrates, however they do not have significant effect in refolding reactions. This suggest that the physiological function of ERD10 and ERD14 might only be the protection of already available and functional proteins and not the folding or refolding of denatured or freshly synthesized proteins. These findings are in agreement with the expected function of LEA proteins expressed in the late stage of seed development and abiotic stress, when the main goal is to sustain the viability of the cell by protecting the cell components and compartments and generally not in the synthesis of new components.

Table 1. Chaperone efficiency of ERD10 and ERD14

	ADH loss of activity	Citrate synthase aggregation	Luciferase aggregation	Lysozyme loss of activity
BSA	20.14 ± 9.6	26.14 ± 3.79	23.93 ± 4.85	4.116 ± 6.3
HSP90	100 ± 10.4	100 ± 3.80	100 ± 2.64	100 ± 9.19
ERD10	158.6 ± 17.7	45.93 ± 3.71	112.1 ± 1.10	127.0 ± 15.8
ERD14	138.2 ± 6.5	50.28 ± 5.49	105.8 ± 2.20	131.7 ± 12.4

Table 2. Chaperone efficiency of ribosomal proteins

	ADH loss of activity	Lysozyme loss of activity	Lysozyme reactivation*
BSA	12,44% ± 6,92	67,58% ± 2,66	70,63% ± 1,67
HSP90	100% ± 8,35	100% ± 3,36	72,96% ± 1,94
L15	114,49% ± 7,53	183,01% ± 3,01	224,68% ± 2,35
L16	135,76% ± 7,62	120,37% ± 3,19	178,48% ± 2,86
L18	134,32% ± 8,19	188,74% ± 2,77	201,13% ± 1,54
L19	125,62% ± 8,08	231,3% ± 4,49	198,73% ± 3,51

Chaperone efficiencies of ERDs and RPs are expressed relative to the chaperone activity of HSP90. Activities within a particular measurement were background-corrected by subtraction of activity observed without additions and divided by the background-corrected activity of the HSP90 experiment. *In the case of the lysozyme refolding experiment in Table2, activities were only corrected to background (*cf. Results*). The table shows standard deviations of three parallel experiments (*cf. Figures 3-6*).

Janus Chaperone Activity of Ribosomal Proteins

The four RPs studied in this work have a well defined 3D structure when bound to the ribosome (Evers et al. 1994; Ban et al. 2000), but in solution they show the characteristics of intrinsically disordered proteins (Nishimura et al. 2004; Ivanyi-Nagy et al. 2005; Timsit et al.

2006). It was shown previously that functional promiscuity, i.e moonlighting shows a strong correlation with disorder of proteins, due to their inherent structural adaptability and extended interaction sites (Tompa et al. 2005). According to the moonlighting nature of IDPs, an interaction with distinct partners is only surprising in the light of radically different chemical nature of RNA and protein. It was shown recently that some of *E. coli* RPs have RNA chaperone activity in trans-splicing assay (Semrad et al. 2004), here we have provided evidence that L15, L16, L18 and L19, which have the highest RNA chaperone activity, also have protein chaperone activity. Due to this promiscuity in chaperone action we termed them “Janus chaperones”. The name Janus, a mythological Roman God, applies as example of two “similar” faces. The chaperone activity of ribosomal proteins shown in this study, nevertheless, is not significant if free ribosomal proteins *de facto* do not exist in the cytosol, as suggested previously (Ramagopal and Subramanian 1974; Warner 1999). However, it has been noted that during fast cell cycle, when protein synthesis is very rapid, or during environmental stress, when decomposition of the ribosome is one consequence of the stress, concentration of free RPs increase. The cellular concentration of RPs is under tight control; however the short-term presence of such chaperones under cellular stress may provide a crucial activity by supporting the chaperone machinery directly.

Specificity and Characteristics of IDP Chaperones

IDP chaperones shown in this chapter have broad substrate specificity. The chaperone activity of ERD10 and ERD14 was analyzed with five substrates, whereas it showed chaperone activity with four of them, RPs showed chaperone activity with three out of four substrates, in four assays including the refolding assay. Chaperones are usually specific to one protein or a protein family (Rudiger et al. 1997; Knoblauch et al. 1999; Park et al. 2008), while IDPs have chaperone activity with a variety of substrates. Similarly, AavLEA1 shows anti-aggregating activity in desiccation experiments on total protein extracts of human cell (Chakrabortee et al. 2007). Osmolytes (also termed as chemical chaperones) in general known for their broad anti-aggregation effect by *in vitro* comparative experiments. This effect is generally based on the physical (charged) properties of these molecules and not on direct molecular interactions (osmolytes are small charged molecules, amino acids and derivatives, polyols and sugars, methylamines, methylsulfonium compounds) (Kinjo and Takada 2002; Yancey 2005; Ignatova and Gierasch 2006). IDPs are highly charged which raises the possibility that they may also act as osmolytes and have chaperone activity only due to their charged nature. Osmolytes are only effective at high concentration, typically at molar concentration; whereas IDPs, shown in this chapter and at other places have chaperone activity at equimolar ratio, typically at micro molar concentration. This suggests that the chaperone action of IDPs is based on a direct, presumably on a non-specific interaction.

Molecular Mechanism of Chaperone Activity

Chaperone proteins in general may either i) prevent inactivation of a partner, ii) prevent the aggregation of a partner, iii) disperse its aggregate, or iv) help actively refold it. This raises the question of the molecular mechanism, of their chaperone activity. Tompa and Csermely (Tompa and Csermely 2004) have suggested that the chaperone activity of IDPs is based on their highly flexible (entropic) nature, whereas they bind to the trapped folding intermediate and move it out of a local energy minima by “transferring” part of their entropy to the substrate, thus giving another chance for the molecule to explore the conformational space. With this mechanism IDPs would only be able to support folding or refolding reactions, as the axiom which this mechanism is based on is that the substrate is stuck in a local energy minimum corresponding to a non-native structure. However, IDP chaperones can also protect the native conformation of the substrate and prevent its aggregation. For the anti-aggregation activity the “molecular shield” mechanism was suggested (Goyal et al. 2005), which assumes that IDPs inhibit the collision and sticking of denatured protein molecules together, by either filling in the space between them, or by binding to the denatured protein and increasing its solubility due to their highly charged nature. In addition, upon binding to the denatured substrate they may also cover interaction sites which initiate the formation of aggregates. However, until now no molecular mechanism has yet been suggested for the protection of IDPs of the native structure of substrates. We suggest that this function again can be accounted for by non-specific e.g. ionic interaction formed between the substrate and chaperone molecule, at multiple interaction sites, prior the denaturation of the substrate. Our model claims that the IDP chaperone binds to the peripheral charged residues of the substrate at several points and hinder the conformational change of the substrate induced by the stress condition. In this case hydrophobic interaction can be discounted as hydrophobic regions are usually completely covered and packed inside the protein. Thus if hydrophobic regions gets exposed the aggregation takes place very rapidly, so preventing of such an event would be an ideal way of protection. Also a constant refolding reaction can hardly be the case, as under the conditions (heat, freezing, drought, etc) these proteins are functional such an activity would be less favorable/affordable and very time consuming, than protecting the native structure. This suggestion is partially confirmed by recent studies carried out with AavLEA1 protein, which has a broad anti-aggregation activity on whole human proteome (Chakrabortee et al. 2007). Authors have shown that at 1/5 molar ratio AavLEA1 inhibited the aggregation of almost the whole proteome upon desiccation and resolubilization. Aggregation rate was also hindered if AavLEA1 was added 5 min post resolubilization. However it was not able to recover neither protect against aggregation of the substrates afterwards, when denaturation of the substrates can be taken into account. This suggests that AavLEA1 binds to a native protein and protects it against denaturation, and refolding is either too slow or it cannot support the refolding of such a highly variable mixture of proteins. In either case, it is clear that AavLEA1 have protective effect only on the native, and none in the case of denatured, or aggregated protein. For such an activity, as underscored by the two LEA proteins and RPs in this chapter, we suggest that the IDP binds in an extended structure to the surface area of the substrate via ionic interaction, and prevents the disruption of the native conformation. This interaction however does not affect the

biological activity of e.g. an enzyme, which is nicely shown in our experiments, in which the progress of the reaction was followed by measuring the decrease of enzyme activity of the partner molecule.

Relevance of IDP Chaperones

As shown in the introduction and throughout the chapter, IDPs possess unique molecular characteristics (Tomba 2002). These proteins or protein regions do not have stable folds, they are highly charged, and accordingly they are highly soluble. Their solution structure is usually open and extended, and they do not denature or aggregate (Wright et al. 2003; Tomba et al. 2006). This property might be essential during stress conditions, because neither the structure nor the function of the IDP protein is compromised. It is arguable whether IDPs or globular proteins have appeared earlier in evolution. Nevertheless, it has been proposed at several places that IDP chaperones might represent the early type of molecular chaperones, which are able to carry out chaperone action in the absence of ATP (Tomba and Csermely 2004). It is also notable that the anti-aggregation and/or protective function of IDPs might represent an essential function in stabilizing mutations, which might lower the solubility of proteins. This effect might be crucial by functional diversification and appearance of novel protein function by mutations, as also in the fine tuning of the original function (Tokuriki and Tawfik 2009). All of these diversification are considered to be essential in evolutionary leaps.

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Chapter XV

Fluorescence Study on Aggregation of α -Crystallin and Insulin

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Abstract

Fluorescence spectroscopy of tryptophan residues and the 8-anilino-1-naphthalenesulfonate probe and light scattering were used to study some properties of bovine α -crystallin, in particular, its thermo- and photo-induced aggregation. The effective diameter of the native α -crystallin globule, calculated from the polarization and life-time of 8-anilino-1-naphthalenesulfonate (ANS) using the Levshin–Perrin equation, amounts to 90 Å and increases during aggregation to at least 140 Å. The decrease in the tryptophan fluorescence intensity in the course of α -crystallin thermo- and photo-denaturation and aggregation is caused by local conformational alterations in the environment of the tryptophan residues and by light scattering. Tryptophan residues in the aggregates are hidden in the interior. Thermal aggregation of the protein takes place not only at high temperatures. Extrapolation of the experimental time dependence of slow spontaneous aggregation to the long time range allows one to find the “denaturation time” t_e . The t_e value for α -crystallin (at a concentration of 0.8 mg/ml in phosphate buffer at pH 8.4) is about 100 h. Using steady-state, polarized, and phase-modulation fluorometry, the DTT-induced denaturation of insulin and formation of its complex with α -crystallin in solution were studied. Prevention of the aggregation of insulin by α -crystallin is due to formation of a chaperone complex, i.e. tight interaction of chains of the denatured insulin with α -crystallin. The conformational changes in α -crystallin that occur during complex formation are rather small. It is unlikely that N-termini are directly involved in the complex formation. It has been shown that ANS is not sensitive to the complex formation. ANS emits mainly from α -crystallin monomers, dimers, and

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tetramers, but not from oligomers or aggregates. The possibility of highly sensitive detection of aggregates by light scattering using a spectrofluorometer with crossed monochromators is demonstrated.

Keywords: α -crystallin, insulin, chaperone complex, denaturation, aggregation, tryptophan fluorescence, 8-anilino-1-naphthalenesulfonate (ANS), dithiothreitol (DTT), UV irradiation, light scattering.

Introduction

It is known that the age-related cataract in human and animals is caused by physico-chemical alterations in α -crystallin, the main protein component of the lens [1]. Beside metabolic disorders leading to cataract, there are two more causes, which may often be the main ones: heating and solar ultraviolet [2].

As the temperature in the eyeball usually does not exceed 36° C, it may seem that thermal aggregation of α -crystallin should be excluded as a cause of cataract. There is a widespread opinion that only strong heating can cause protein denaturation and aggregation. This may be true if only short-term (minute-scale) heating is considered. A number of works have been devoted to α -crystallin aggregation in solutions entailed by pronounced heating and raising the protein concentration [3 – 5]. In reality, as will be shown here by the example of bovine α -crystallin, thermal denaturation (and ensuing aggregation) may proceed at usual temperatures, though very slowly, and this process cannot be ignored if we consider the time-scale of weeks, months, and years.

Intense UV irradiation is known to cause damage to aromatic amino acid residues and to impair the native protein structure [6]. Since α -crystallin contains tryptophan and tyrosine residues, UV light of a wavelength shorter than 300 nm is absorbed by the lens. Such absorption can lead both to destruction of these residues and to denaturation rearrangements in the α -crystallin molecule. This may be attended by protein aggregation.

Bovine α -crystallin comprises two similar subunits α A and α B. The molecular mass of either subunit is about 20 kDa [3]. Either subunit has Trp9; α B also has Trp60. Both Trp residues are in the N-proximal domain [7]. The protein has no disulphur bonds. The structural features, domain organization, dissociation, and oligomerization of α -crystallin subunits are described in [3, 8, 9]. The subunits are associated in spherical complexes. In aqueous solutions, α -crystallin is preferably in the form of dimers, tetramers, and oligomers. The molecular mass of oligomers reaches 540 kD (about 26 subunits) [9].

At high temperatures, α -crystallin in an aqueous solution denatures and aggregates [6], which is accompanied by a change in the tryptophan fluorescence intensity. The average size of crystallin aggregates reaches 800 kDa [3]. The structural properties and the domain organization of α -crystallin and its aggregates have been described [9].

The temperature dependence of α A and α B-crystallin denaturation and aggregation has been studied in [4] by circular dichroism, light scattering, fluorescence of tryptophan residues and 8-anilino-1-naphthalenesulfonate (ANS). Dynamic and stationary light scattering as well

as ANS fluorescence were used in [3] to assess the aggregation properties of various α -crystallin forms as dependent on temperature and UV irradiation (254 nm).

α -Crystallin is known to exhibit “chaperon” properties. Oligomerization of α -crystallin is not necessary for chaperone functions [3, 9]. The chaperon properties can be observed in model experiments, for instance, during dithiothreitol-induced protein denaturation [10]. A study was made in [11] on the chaperon activity of α -crystallin in the refolding of citrate synthetase.

Experimental

Tryptophan residues of bovine α -crystallin and the 8-anilino-1-naphthalenesulfonate (ANS) probe (Sigma, USA) were used as fluorescent indicators of denaturation and aggregation. Most experiments were conducted in 50 mM phosphate buffer at pH 8.4. Measurements were made with an SLM-4800 phase modulation spectrofluorimeter (SLM Inc., USA) in mirror micro-cuvettes enhancing the signal several-fold [6]. In all experiments the background (phosphate buffer signal) was subtracted. Emission spectra were registered with a 2-nm step at 4-nm monochromator slits. The extent of polarization was measured using Glan–Thomson prisms. Polarization of the tryptophan emission was assayed at 338 nm (excitation wavelength was 300 nm) and of ANS one - at 460 nm (excitation wavelength was 380 nm). The lifetime of the excited state was determined at a modulation frequency of 30 MHz. Signal accumulation time was 30 s per point. The data on polarization and lifetime were averaged over four independent measurements. The accuracy for the lifetime was 0.1 ns; for polarization extent, 0.002.

The volume and the diameter of protein molecules were calculated using the Levshin–Perrin equation [6] from the data on polarization and lifetime of the ANS fluorescence. The equation appears as

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{RT\tau}{\eta V} \right)$$

where P_0 is the limit polarization (in frozen solvents, $P_0 = 0.5$), P is the measured polarization, τ is the life time, η is the solution viscosity (1 cP for water), T is the absolute temperature, R is the universal gas constant, and V is the effective volume of a rotating particle.

Absorption spectra were recorded with a Specord M-40 instrument (Germany). Protein concentration was determined from the absorbance at 280 nm using the tryptophan extinction coefficient of $6 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ [6] with account for the number of residues. Light scattering was assessed in two ways: from the optical density in light transmission at 310–320 nm (where α -crystallin does not absorb) using a standard spectrophotometer compartment, and from the intensity of scattered light detected by the photomultiplier of the spectrofluorimeter at right angles (in this case the monochromators before and after the cuvette were both set to 310 nm, with 1-nm slits).

Part 1. α -Crystallin and Its Aggregation

Tryptophan Fluorescence

Preparations of α -crystallin from the bovine lens contain more α A subunits than α B; their ratio is about 3:1 [7]. Therefore, the main contribution (at least 80%) to the fluorescence of such preparations comes from Trp9. The α -crystallin emission spectrum has a maximum (λ_{max}) at 339 nm (Figure 1), which is 16 nm shorter than the λ_{max} of aqueous tryptophan (355 nm under the same conditions). This band shift indicates that the tryptophan residues are inside the protein globule rather than in the water phase. The α -crystallin emission band has been decomposed [7] into two components, one assigned to Trp9 and the other to Trp60; however, it has been incorrectly asserted that Trp9 in the protein has a hydrophobic environment. In reality, there certainly are polar groups in the vicinity of both residues, because tryptophan in a totally non-polar (hydrophobic) medium has a λ_{max} of 310–320 nm [6].

Using phase modulation, it was found that the mean lifetime of the excited state (τ) of α -crystallin Trp residues in phosphate buffer at pH 8.4 and 10°C is 3.9 ns. This value is close to the mean $\tau = 3.6$ ns, determined by the pulse method in [7] for this protein in a Tris-buffer at 20° C.

The Trp residues of α -crystallin show pronounced fluorescence polarization. The value $P = 0.284$ obtained here (excitation was at 300 nm, recording was at 338 nm) does not differ much from $P = 0.292$ obtained in another buffer and at different protein concentration by other authors [7]. Unfortunately, the data on Trp fluorescence polarization and lifetime cannot be used to determine the α -crystallin diameter (see below), because the rotational mobility of the residues themselves is superimposed on the rotational mobility of the protein globule. With the Levshin–Perrin equation [6] we found that the overall angle of the turn of tryptophans in aqueous α -crystallin is about 28° (this angle is composed of the rotational mobility of the protein globule and the rotational mobility of the residues themselves within the globule).

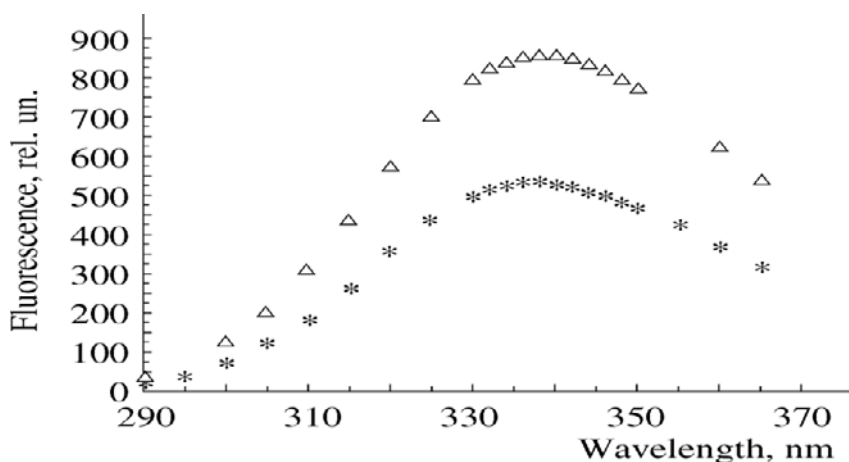


Figure 1. Emission spectra of α -crystallin before (upper curve) and after (lower curve) 15-min heating at 80°C. Protein concentration was 0.8 mg/ml in 50 mM phosphate buffer pH 8.4. Measurements were done at 10°C. Excitation was at 295 nm; slit width 4 nm.

ANS fluorescence and α -crystallin dimensions

The ANS probe binds with α -crystallin not instantaneously but rather slowly, over more than ten minutes [12], i.e., their interaction involves gradual penetration of the probe into the globule. In our control experiments with bovine serum albumin, the binding was instantaneous (surface binding, data not shown). The ANS molecule is tightly bound to α -crystallin owing to hydrophobic and polar interactions. A tightly bound large probe (length over 10 Å) is hardly able to appreciably rotate itself. In the first approximation, we can assume that the probe rotates with the globule as a unit whole [6]. Hence, the depolarization of ANS fluorescence is mainly due to the rotation of the globule, and the Levshin–Perrin equation can be applied to calculate the α -crystallin dimensions. Substituting the measured P and τ for ANS into the equation, we obtained the effective volume and diameter of α -crystallin and its aggregates (Table 1).

It is of interest to collate our data with those obtained earlier by different methods. From small-angle X-ray scattering, the α -crystallin diameter is 150 ± 50 Å [9]. The same authors obtained larger values, about 200 Å, by photon correlation spectroscopy [13]. Dynamic light scattering estimated the mean protein diameter at no less than 113 ± 20 Å [3]. We can conclude that our estimation is realistic enough.

Thermal aggregation

Protein denaturation and aggregation in solutions is known to depend on many factors: temperature, pH, ionic strength, protein concentration, storage time, etc. There are two points of view on denaturation: a cooperation transition [14] and a stepwise process involving accumulation of an intermediate state such as the molten globule [15]. Rather than comparing these models, here we shall focus on the phenomenology of thermally induced aggregation of α -crystallin.

Figure 1 displays the Trp fluorescence spectra of α -crystallin and its aggregates. The protein was dissolved at 0.8 mg/ml in 50 mM phosphate buffer at pH 8.4. Under such conditions it exists largely in a non-aggregated form. Aggregates were obtained by 15-min heating of this solution at 80°C, and then the spectrum was recorded at 10°C. Note that this aggregation is irreversible. The decrease in the Trp fluorescence intensity for the aggregates has two causes: local conformational alterations near the N domain, and aggregation-dependent light scattering. If aggregation is caused by large-scale conformational changes (complete denaturation), a markedly affected the λ_{max} and τ will be detected. However, no blue shift, expected for a fully denatured protein, is observed in Figure 1. The τ value for the aggregates was about 3.5 ns, the same as for the native protein.

Table 1. Polarization and lifetime of crystallin-bound ANS, and the effective volume and diameter of α -crystallin

	P	τ , ns	V, Å ³	d, Å
α -crystallin	0.36	15.1	379 000	90
its aggregates	>0.4	~17	>1 420 000	>140

During protein aggregation, the polarization degree (for both the Trp and the ANS emissions) was increased so drastically and the contribution of light scattering became such great as to preclude exact calculation of the aggregate size. A rough estimate was no less than 140 Å (Table 1).

Figure 2 collates the absorption spectra of α -crystallin and its aggregates obtained by 1-h heating at 70°C. One can see that the spectrum of aggregates is markedly “raised” because of light scattering. The fluorescence intensity, measured in a solution of aggregates, always differs from that for separate molecules [6]. This circumstance has not been taken into account earlier by other researchers. On the other hand, just this circumstance enabled us to follow α -crystallin aggregation by changes in the Trp fluorescence.

Table 2 gives examples of the influence of varying the concentration, temperature, and heating time on α -crystallin aggregation, assayed by the Trp fluorescence intensity. With increasing protein concentration, the probability of aggregation substantially rises. Thus, heating of α -crystallin at 0.1 mg/ml for 20 min at 80°C causes slight aggregation, whereas the aggregation at 0.7 mg/ml is quite pronounced. These data are consistent with another work [5] where the temperature and concentration dependences of α -crystallin aggregation were compared using steady-state and time-resolved Trp fluorescence.

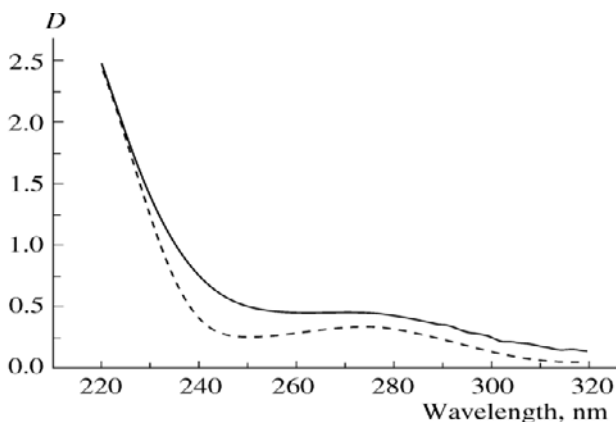


Figure 2. Absorption spectra of α -crystallin (lower curve) and its aggregates, obtained by 1-h heating at 70°C (upper curve). Measurements were done at 20°C. Protein concentration was 0.8 mg/ml.

Table 2. Influence of α -crystalline concentration, heating temperature and incubation time on the decrease in Trp fluorescence (50 mM phosphate pH 8.4, 10°C; excitation was at 295 nm, registration was at 340 nm, monochromator slits were 4 nm)

[Protein], mg/ml	t, °C	Time, min	Fluorescence decrease, %
0.2	10	1	0
0.2	10	70	1
0.2	50	70	14
0.2	70	60	28
0.2	70	120	40
0.1	80	20	11
0.7	80	20	33

As evident from Table 2, the aggregation at 10° and 50°C is much slower than at higher temperatures, and increases with the heating time. This result appears to be trivial. However, three points are noteworthy: (i) aggregation is not instantaneous, (ii) aggregation is not a cooperative “*all or none*” transition, and (iii) aggregation takes place not only at elevated temperature. Protein denaturation and aggregation are commonly described as instantaneous or at least very fast events. This is true only at high temperatures close to 100°C, when all protein molecules practically at once pass from the native state into a denatured (and aggregated) one. At lower temperatures, the transition is first experienced by only a fraction of molecules, then another portion, et seq., time-exponentially (see below).

Figure 3 shows the time curve for α -crystallin aggregation at 70°C, and Figure 4 gives the same data on a logarithmic time scale. The linear shape in the later case signifies that aggregation is caused by gradual spontaneous denaturation of the protein molecules. Such process can be described in the framework of the known theory of defect accumulation [16]. The process of spontaneous defect formation in any object (including proteins) can be described by the equation

$$t = v^{-1} \exp (E_a / kT)$$

Here t is the defect formation time, E_a is the corresponding activation energy (it is mainly the energy of H-bond rupture in protein), k is Boltzmann’s constant, T is absolute temperature, and v is the atom vibration frequency, $\sim 10^{13} \text{ s}^{-1}$. In the first approximation, we can take that appearance of a single defect (one rupture) in a protein molecule is sufficient for its denaturation and aggregation. Extrapolation of the experimental dependence to long times (Figure 4) allows one to find the “denaturation time” t_e wherein the number of native molecules decreases by the factor of 2.7. This value is a quantitative measure of “molecular aging” or instability of the given native protein under the given conditions. We found that for α -crystallin at 0.8 mg/ml and 70°C the t_e value is $\sim 100 \text{ h}$. At 36°C, this value is no less than one year, as derived by finding E_a at 70°C and substituting it into the equation for 36°C. This approach can, in principle, be extended to any times, temperatures, and proteins.

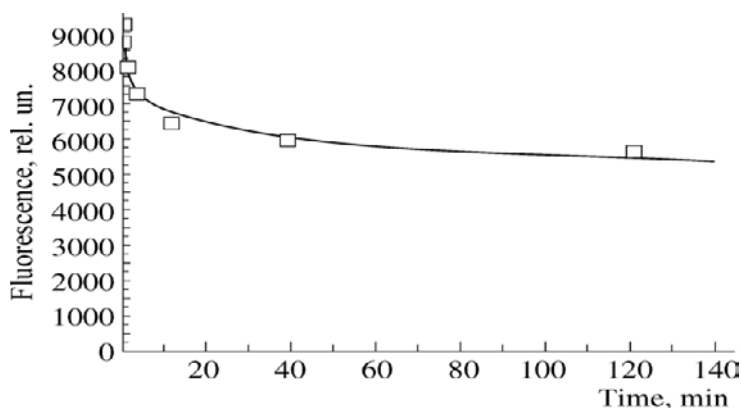


Figure 3. Time dependence of tryptophan fluorescence intensity during α -crystallin aggregation at 70°C. Fluorescence was measured at 10°C. Excitation was at 295 nm, recording at 340 nm. Protein concentration was 0.8 mg/ml.

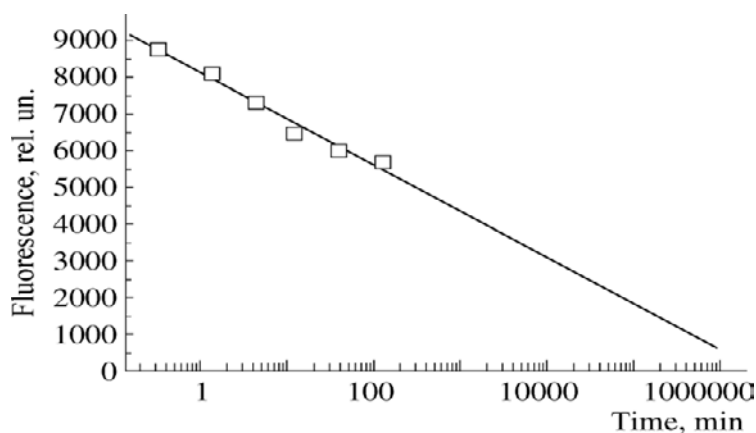


Figure 4. Semilogarithmic representation of the time dependence of tryptophan fluorescence intensity during α -crystallin aggregation at 70°C. Points are experimental data from Figure 3. The straight line is the averaging and extrapolation.

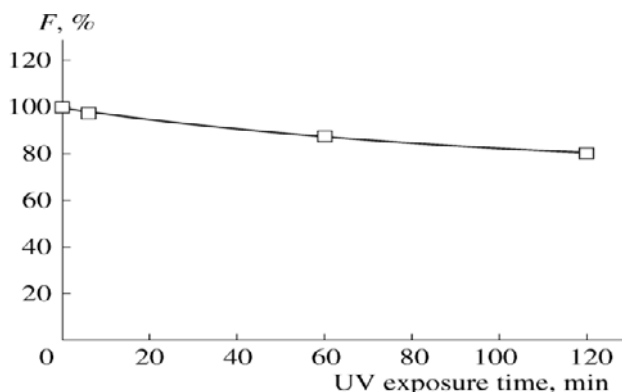


Figure 5. Influence of UV irradiation (~ 280 nm) on α -crystallin tryptophan fluorescence intensity. Exposure was 2 h in a mirror micro-cuvette at 10°C. Protein concentration was 0.8 mg/ml.

Photo-aggregation

Taking that the fluorescence quantum yield for tryptophan in water is 14% and the life time is 3 ns [6], the quantum yield for Trp in α -crystallin does not exceed 18%, i.e. 82% of the absorbed energy is spent non-radiatively. The destruction of tryptophan chromophores under UV irradiation is known to be quite slow, anyway, much slower than the UV-induced denaturation [6]. The photo-excited Trp in protein is efficiently deactivated by the neighboring groups, meaning that the energy of the UV quantum (~ 100 kcal/mol) is mostly spent on vibrations, i.e., on abrupt “molecular heating”. This may cause denaturation and aggregation.

Figure 5 shows the influence of UV irradiation on the α -crystallin Trp fluorescence intensity. The protein solution, hermetically sealed at 10°C (whereby one can neglect thermal aggregation) was continuously irradiated for 2h with UV light of ~ 280 nm, using a 450-W xenon lamp and an aperture holographic monochromator with 16-nm slits. Fluorescence intensity (excitation is at 295 nm, registration is at 340 nm, slit width is 4 nm) was rapidly re-

corded several times during irradiation. The observed decrease in fluorescence intensity, caused by α -crystallin photo-denaturation is accompanied by an increase in light scattering.

The emission spectrum of the photo-aggregated protein practically did not differ from that of the thermo-aggregated one. There was no appreciable blue shift of the λ_{max} (as could be expected for a completely denatured protein) in the course of photo-aggregation. Hence, UV irradiation does not cause complete denaturation of the entire protein globule. The lack of a blue shift also means that Trp residues are not exposed to water in the aggregates thus formed, but are hidden in the interior (just as in thermal aggregation).

It should be mentioned that the development of aggregation-related light scattering during UV irradiation of α -crystallin at 254 nm has been demonstrated earlier [3]. There is also a report [17] on α -crystallin aggregation photo-induced with hypericin, a natural pigment that generated reactive oxygen species upon light absorption.

Part 2. How α -Crystallin Prevents Aggregation of Insulin

Chaperon complex of α -crystallin with insulin

α -Crystallin is a chaperon protein. Chaperones are known to bind to denatured proteins providing their proper folding [18], delivery into corresponding cell compartments [19], and preventing aggregation [20].

An interesting feature of α -crystallin is its ability to interrupt thermal aggregation of various proteins. This chaperon ability is manifested not only upon heating, but also upon dithiothreitol (DTT)-induced denaturation at room temperature. In particular, the prevention of DTT-induced aggregation of insulin by α -crystallin was studied in [10]. Of the two insulin chains, *a* and *b*, formed upon reduction of S–S bonds, the *b*-chain is the best in binding with α -crystallin [10], and the complex formed is near equimolar [21].

Insulin is a small protein, composed of only 51 amino acid residues and formed by two chains connected by three S–S bridges, stabilizing its structure. It easily denatures and loses its physiological properties upon heating or reduction by dithiol compounds. Native insulin in aqueous solution is present mainly as a monomer. Denatured insulin forms aggregates.

Protein aggregation, occurring upon denaturation, can be detected by light scattering (commonly using a spectrophotometry). This light scattering is eliminated, when chaperone protein–protein complexes are formed [5, 7, 8, 10, 21]. The disaggregating effect of α -crystallin on various proteins was also studied using tryptophan and 8-anilino-1-naphthalenesulfonate (ANS) emissions [5, 7, 8]. In particular, ANS interacting with α -crystallin was thought to be sensitive to complex formation with denatured insulin. Researchers supposed that changes in the ANS fluorescence *intensity* resulted from conformational changes in the complex formed. However, other parameters of fluorescence were not measured.

To determine the mechanism of the prevention of insulin aggregation by α -crystallin, more exhaustive investigation of fluorescence is necessary, including determination of not only fluorescence intensity, but also polarization degree (*P*) and lifetime of excited state (τ).

The goal of this part of the work was to study the DTT-induced denaturation of insulin and formation of its complexes with α -crystallin in solution, using the steady-state, polarized,

and phase-modulating fluorometry of tryptophan and tyrosine protein residues and ANS. Moreover, unlike previously reported studies, in which the light scattering was determined with spectrophotometers by optical density, the light scattering in our study was determined in a more sensitive way: by intensity of scattered exciting light, entering the registration channel at crossed monochromators of a spectrofluorimeter.

Bovine α -crystallin and insulin (Serva, Fluka,) were used in the experiments. The proteins were dissolved in 50 mM sodium phosphate (pH 8.4). Unlike study [7], in which both proteins were taken at concentration of 2 mg/ml, the initial concentration of α -crystallin in our experiments was 4 mg/ml, corresponding to the subunit concentration of 2×10^{-4} M (molecular weight of each α -crystallin subunit is about 20 kD), and the initial concentration of insulin was 2 mg/ml, corresponding to 3.3×10^{-4} M (molecular weight of insulin is about 6 kD). In most experiments, 150 μ l of the initial α -crystallin solution were mixed with 100 μ l of the initial insulin solution, i.e. at nearly equimolar ratio (1.2×10^{-4} M α -crystallin and 1.3×10^{-4} M insulin). Insulin denaturation was initialized by addition of DTT (10 μ l of the initial 0.5 M solution). All control samples (150 μ l of α -crystallin in 100 μ l of the buffer; 100 μ l of insulin in 150 μ l of the buffer; and the same solutions with 10 μ l of DTT) were prepared in the same way. The tested solutions were placed at 22°C into mirror micro-cuvettes allowing several times increase of signal on a spectrofluorometer and use of small solution volumes (to save protein preparations) [22]. The cells were closed by lids for prevention of evaporation. Measurements were carried out on an SLM-4800 spectrofluorometer (SLM Inc., USA). All measurements were corrected for the light scattering of the buffer solution. Polarization degree (P) of the emission of α -crystallin tryptophan residues and ANS (Sigma) was measured in accordance with [22], using Glan–Thompson prisms in four positions with correction for the light scattering of the buffer to eliminate both the contribution of artifact polarization of the light by the monochromators and the contribution of light scattering. The final concentrations of α -crystallin and insulin, used in most experiments, were 1.2×10^{-4} and 1.3×10^{-4} M, respectively. Concentration of ANS in experiments was generally 10^{-5} M, one order lower than the protein concentration, to exclude self-association of the probe, ensure its complete binding with the proteins, and substantially reduce effects of light screening and reabsorption. Fluorescence lifetime (τ) was determined by the phase modulation method at modulation frequencies of 30 MHz (for tryptophan residues) and 18 MHz (for ANS). Signal accumulation was 30 sec for one point. The τ value was calculated as the arithmetic mean of values measured by phase and by modulation from four independent measurements.

Effective volumes (V) of proteins were determined using the modified Levshin–Perrin equation [22], proceeding from τ and P of the ANS probe:

$$V = 8498 \tau / (1/P - 2.381)$$

This equation is reasonably accurate for room temperatures and aqueous solutions. Herein τ is expressed in nanoseconds and V in cubic angstroms.

Intense light scattering was determined by the absorption value in transmitted light at 310 nm (neither insulin nor α -crystallin absorb at this wavelength) in a standard compartment of an M-40 spectrophotometer in 0.5-cm quartz cells.

Weak light scattering was determined by intensity of the scattered light registered by an SLM-4800 photomultiplier tube at right angle to the exciting light; in this case, both monochromators (before and after the micro-cuvette) were set to the same wavelength (310 nm; slit width was 1 nm).

Light Scattering

Light scattering in the UV region is known to be significantly higher than in the visible. Tyrosines and tryptophans absorb virtually no light at 310 nm. So, the light scattering by protein aggregates can be conveniently observed at this wavelength. Highly sensitive detection of the aggregates can be carried out on a spectrofluorimeter (with narrow slits of both monochromators crossed at 310 nm). Exciting light being scattered from the aggregates gets through into the detection channel. This gives large signal (Figure 6).

Solutions of native insulin are absolutely transparent even at high concentration (more than 10^{-4} M), and their optical density is very low at 310 nm. Nonetheless, a direct highly sensitive measurement of light scattering on a spectrofluorimeter indicates that a small portion of molecules is in an aggregated state, because the light scattering level is not zero (Figure 6), and this level depends on the insulin concentration. When DTT is added to the insulin solution, highly intense scattering develops (Figure 6), which becomes apparent to the naked eye. DTT reduces disulfide bonds resulting in dissociation of the molecule into two separate chains, *a* and *b*. As this takes place, insulin degrades into a denatured state, in which some of the hydrophobic protein groups are on the surface. As a result, the solubility in water drastically decreases, and individual chains stick together to form large aggregates, which leads [10] to high scattering.

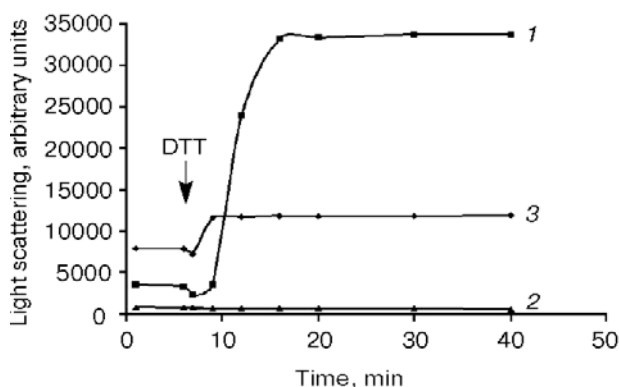


Figure 6. Changes in the light scattering of insulin (1), α -crystallin (2), and their mixture (3) upon addition of DTT. The light scattering was detected on an SLM-4800 spectrofluorimeter with crossed monochromators at the same wavelength of 310 nm. Concentrations: insulin, 1.3×10^{-4} M; α -crystallin, 1.2×10^{-4} M.

DTT added to α -crystallin has no effect on its light scattering (Figure 6). This means that DTT under the conditions of the control experiment does not cause denaturation and aggregation of α -crystallin. Low level of light scattering in the case of α -crystallin signifies that this protein does not form large aggregates at concentrations about 10^{-4} M and lower.

Addition of α -crystallin to insulin undergoing DTT-induced denaturation leads to suppression of the light scattering of insulin (Figure 6). This means that α -crystallin taken at near equimolar (per subunit) ratio to insulin effectively prevents the aggregation of insulin. However, the light scattering is still slightly increases after DTT addition. This can be due to both incomplete prevention of insulin aggregation and formation of very large crystalline–insulin complexes. Indeed, in excess of α -crystallin in relation to insulin, the binding achieves almost 100%, but light scattering does not vanish (not shown in fig).

Table 3. Intensity, degree of polarization, and lifetime of fluorescence of tryptophan residues of α -crystallin and ANS in the presence and absence of insulin as well as before and after 40-min incubation with DTT

In solution	F_{trp} (%)	P_{trp}	τ_{trp} (nsec)	F_{ANS} (%)	P_{ANS}	τ_{ANS} (nsec)
α Crystallin	100	0.284	3.9	100	0.36	15.1
α Crystallin + DTT	92	0.290	4.4	122	0.35	16.9
α Crystallin + insulin	97	0.283	3.9	86	0.35	16.9
α Crystallin + insulin + DTT	88	0.281	4.3	80	0.36	17.0
The same after 40 min	85	0.279	4.6	95	0.34	17.0
Insulin	–	–	–	3	0.32	12.7
Insulin + DTT	–	–	–	2	0.34	12.6
The same after 40 min	–	–	–	18	0.34	12.5

Note: Excitation of tryptophans was at 300 nm, emission was registered at 338 nm. Excitation of ANS was at 370 nm; emission was at 465 nm. Accuracy of measurement of phase τ of α -crystallin tryptophans was ± 0.1 nsec and that of phase modulation τ of ANS was ± 0.4 nsec. Accuracy in measurement of P of α -crystallin tryptophans was ± 0.002 and that of ANS was ± 0.01 .

It is worth noting that the light scattering level of the mixture of the two proteins without DTT, when measured in the highly sensitive way on the spectrofluorimeter with crossed monochromators (customary spectrophotometry fails in detection), was somewhat higher than the sum of light scattering levels of these proteins taken separately (Figure 6). This means that mixing of two native proteins can lead to formation of large, even if small in number, aggregation complexes. They seem to be formed due to nonspecific hydrophobic interactions between some molecules.

Tryptophan fluorescence of α -crystallin

The α -crystallin molecule contains three tryptophans (one in the first subunit and two in the second one). Although all three tryptophans are localized at the N-termini, they are faced to the inner space of the protein globule rather than to water. Their emission spectrum has a

maximum near 338 nm [23]. The tryptophan fluorescence of α -crystallin is very little affected by addition of insulin or DTT. This concerns its intensity, degree of polarization, P , and lifetime, τ (Table 3). When insulin and DTT were added together followed by incubation for 40 min, the intensity decreased by only 12%, in comparison to that of the mixture of proteins without DTT. Both P and τ also varied only slightly. This suggests that the α -crystallin conformation is little affected upon crystalline–insulin complex formation, and the N-termini of its subunits are not likely to be directly involved in the complex formation. Moreover, the polarization degree of tryptophan fluorescence did not increase. According to the last equation, this means that the binding of insulin with α -crystallin does not lead to marked increase of the particle volume, that is, the situation does not appear when one molecule of α -crystallin binds many molecules of insulin. Minor decrease of P after 40-min incubation is apparently caused by increase of τ . Since tryptophans of α -crystallin are localized at the flexible N-end, they possess some rotary mobility [22, 23], which partially depolarizes their radiation, thus impeding application of the equation for exact calculation of particle sizes.

Tyrosine fluorescence of insulin

Insulin does not contain tryptophans, but it has four tyrosines. Tyrosine fluorescence of insulin substantially decreases with its denaturation and aggregation (Figure 7). This is due to two causes: strong conformational changes and aggregation-caused light scattering. As one can see in Figure 7, the decrease of tyrosine fluorescence corresponds to drastic increase of the scattering. Observation of complex formation between α -crystallin and insulin by tyrosine fluorescence of the latter is hampered because of the obstructive effect of α -crystallin tyrosines (each subunit of α -crystallin contains two tyrosines).

Fluorescence of ANS in insulin aggregates

The ANS probe binds very little with native insulin. So, the probe fluorescence is very weak. Incubation of insulin for 40 min in the presence of DTT led to the protein denaturation and six-fold increase in intensity of the ANS fluorescence (Table 3). However, the absolute value of the intensity remained low. The observed six-fold increase of the intensity results from high increase of the probe binding by insulin aggregates; some contribution from elongation of the optical path of exciting light in a scattering medium may also occur (similar increase in intensity was reported elsewhere [10]). As this takes place, both parameters - lifetime and polarization degree of ANS - remain virtually unchanged: $\tau = 12.5 - 12.7$ nsec and $P = 0.32 - 0.34$. Note that free ANS molecules in water have $\tau = 3.8$ nsec and $P < 0.06$ [22]. The calculation by the Levshin–Perrin equation has shows that the mean effective volume of insulin aggregate in presence of ANS is about $164\,000\text{ \AA}^3$, that is, its diameter is $69\text{ \AA}$. It should be stressed that we are dealing with precisely those insulin aggregates ANS binds with and from which depth it emits rather than with all aggregates (ANS hardly penetrates into large aggregates).

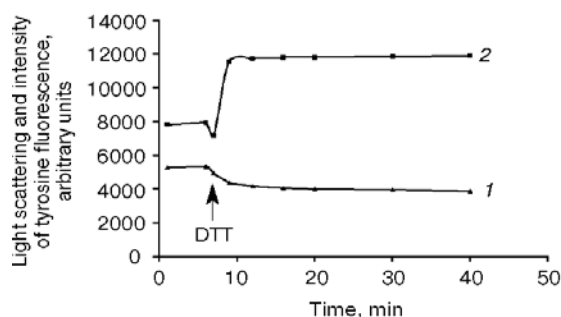


Figure 7. Changes in tyrosine fluorescence (1) and light scattering (2) of insulin during the process of its DTT-induced aggregation. Tyrosine fluorescence was excited at 275 nm and detected at 310 nm. Light scattering was measured on an SLM-4800 spectrofluorometer with crossed monochromators at 310 nm. Insulin concentration was 10^{-5} M.

Fluorescence of ANS in α -crystallin

ANS binds with α -crystallin not instantly, but rather slowly [23], for about 10 min under the conditions of the experiments. The probe gradually penetrates into the protein, between subunits (the quickest penetration occurs into dimers and tetramers, not into oligomers), which is accompanied by many-fold intensification of the probe fluorescence and shift of the emission spectrum to shorter wavelengths [22, 23]. The ANS lifetime on binding with α -crystallin is 15.1 nsec. This value is only slightly greater than that in the case of insulin, but the intensity change is much greater, i.e., it increases 33-fold (Table 3). This is due to complete transition of the probe from the polar aqueous phase into the hydrophobic areas of the complex. The polarization degree of ANS in α -crystallin ($P = 0.36$) is slightly higher than in insulin aggregates. The P value for the bound ANS mainly depends on rotation of the probe together with the aggregate; to a first approximation, self-rotation of the probe is negligible [22]. The mean volume of α -crystallin molecules calculated by the Levshin–Perrin equation with parameters of ANS emission is $379\,000\text{ \AA}^3$, diameter $90\text{ \AA}$ [23], that is, ANS emits preferentially from dimers and tetramers rather than from large oligomers.

Fluorescence of ANS in the presence of α -crystallin upon denaturation of insulin

Incubation of α -crystallin with insulin in the presence of DTT is accompanied by noticeable change in intensity of ANS fluorescence (Figure 8). Addition of DTT to α -crystallin alone (in the absence of insulin) results in first increase in intensity of the probe fluorescence followed by its decrease to a level below the initial one. This seems to reflect kinetics of conformational changes of protein subunits. Since both kinetics and magnitude of the increase in ANS fluorescence intensity in the mixture of two proteins in response to addition of DTT are close to those in the absence of α -crystallin (DTT added to insulin with ANS), one can suppose that the main cause of gradual increase in the intensity is enhancement of ANS binding in insulin aggregates. If this is the case, no appreciable response of the probe to the crystalline-insulin complexes occurs, that is, ANS does not “feel” the complex formation. This is also evident from the very small changes of P and τ values of ANS (Table 3) after incubation of the proteins mixture with DTT. Calculation by the Levshin–Perrin equation shows that the volumes of all protein particles (α -crystallin,

insulin, and their complex) determined from the ANS parameters did not increase after incubation with DTT.

The binding of ANS with α -crystallin was studied in detail in work [12]. The authors demonstrated that even in a 24-subunit oligomer only one binding site is present (with the dissociation constant of $9 \times 10^6 \text{ M}^{-1}$). The binding of the probe occurs in a hydrophobic area localized near the surface. It is not very sensitive to the number of subunits, that is, ANS responds little to the interactions between subunits. No difference was found in binding of the probe to *a* and *b* subunits of this protein. The increase in ANS fluorescence intensity in the mixture of α -crystallin and insulin denatured by DTT was found to be caused by the enhancement of binding of the probe to insulin (primarily to its *b*-chain), but not to α -crystallin [10]. These data are in agreement with the data reported here. The intensity of the probe fluorescence in the mixture of α -crystallin and native insulin was equal to the sum of the intensities from the two individual proteins [10], so the authors concluded that the proteins did not form a complex. It is of interest that the overall intensity in the mixture with denatured insulin was almost the same as in the case of the individual proteins, which is confirmed by the data of the present study.

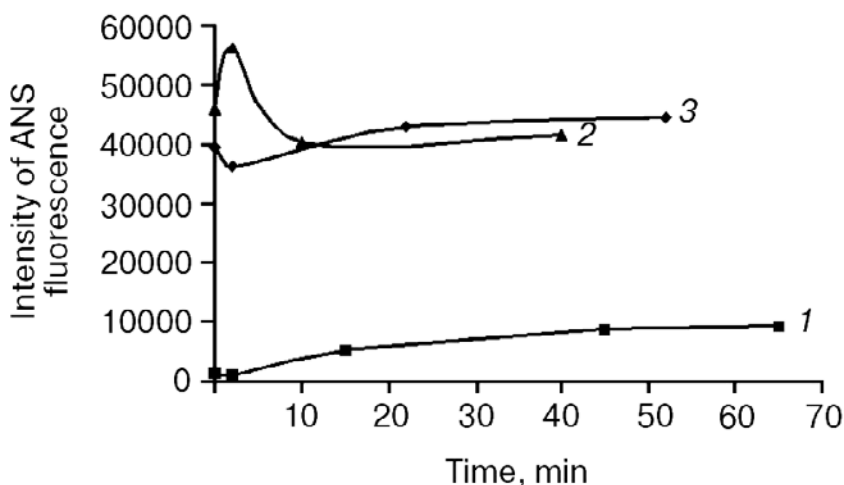


Figure 8. Change in intensity of ANS fluorescence in solutions of insulin (1), α -crystallin (2), and their mixture (3) immediately after addition of DTT. The fluorescence was excited at 370 nm and recorded at 465 nm. Concentrations: ANS, 10^{-5} M ; insulin, $1.3 \times 10^{-4} \text{ M}$; α -crystallin, $1.2 \times 10^{-4} \text{ M}$.

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Chapter XVI

The Induction of Hepatic Heat Shock Protein 27 and 70 by an Anti-Hepatitis Drug-Bicyclol and Its Role in Protection against Liver Injury

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Abstract

Bicyclol is a new potent anti-hepatitis drug and has been approved to treat viral hepatitis in China since 2004. Pharmacologically, bicyclol protects against drug and chemical-induced liver injury, and inhibits hepatitis virus replication in duck viral hepatitis and in a HepG2.2.15 cell line. Bicyclol exerts most of its effects by eliminating free radicals, maintenance of mitochondrial glutathione redox status, anti-inflammation and anti-apoptosis. However, further elucidation of the molecular mechanism of bicyclol in protection against liver damage is still needed.

Heat shock proteins (HSPs) are a family of constitutive and inducible expressed gene products that collectively function to maintain cellular protein conformation during stressful conditions. HSPs can be induced during acute or chronic stress as a result of protein misfolding, aggregation or disruption of regulatory complexes. Prior induction of HSPs protects cells from subsequent lethal insults. The up-regulation of HSPs expression constitutes an important cellular defense mechanism. Therefore, it would be of great therapeutic benefit to discover compounds that are clinically safe and able to enhance the expression of HSPs.

Since bicyclol has cytoprotective action against liver injury, a question arises whether the effect of bicyclol on liver injury is mediated through induction of hepatic

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HSPs. Our study demonstrated that bicyclol significantly induces the hepatic HSP27 and HSP70, which play a key role in protection against liver injury.

Oral administration of bicyclol alone significantly induced hepatic HSP27 and HSP70 expression both in protein and mRNA levels in a time- and dose-dependent manner through activation of heat shock factor-1 (HSF1) in mice, but no inducing effect on HSP27 and HSP70 in mouse spleen and kidney was observed. In *in vitro* study of HepG2 cells, bicyclol can enhance HSP27 and HSP70 promoter activities, indicating that bicyclol induces transcription of the HSPs genes.

The hepatoprotective effect of HSP27 and HSP70 induced by bicyclol was confirmed in acetaminophen and concanavalin A (ConA) induced liver injury in mice. HSP27 and HSP70 suppressed the elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), liver tissue necrosis, the release of cytochrome c and apoptosis inducing factor (AIF) from mitochondria as well as hepatic DNA fragmentation caused by acetaminophen and ConA. Moreover, overexpression of HSP27 and HSP70 by bicyclol attenuated NF- κ B activation induced by ConA either by inhibiting I κ B degradation or by directly suppressing the NF- κ B transcriptional activity, and also inhibited D-galactosamine-induced activation of JNK signal transduction pathway. These results suggest that the protective action of bicyclol against liver injury is mediated by its induction of HSP27 and HSP70, which provide new evidence for elucidating the mechanism of cytoprotective effect of bicyclol against liver injury both in animals and patients.

Keywords: Bicyclol; heat shock protein; apoptosis; NF- κ B; JNK.

1. Introduction

The liver is a very important organ in metabolism and actively participates in the synthesis of various proteins. It is an extremely powerful organ in detoxification and removal of toxic chemicals and other materials, and is also frequent target organ of toxins. Hepatitis virus infection is the most cause of liver disease. Long term of liver injury is often the onset of liver fibrosis, even liver sclerosis and hepatocarcinoma. Apoptosis and necrosis will nevertheless occur as a consequence of liver injury. It is still a worldwide problem to prevent and treat liver diseases until now. So, it is very important to investigate the mechanism of liver injury and to develop effective and safe drugs for liver diseases as well as elucidation of the molecular mechanism of the developed new drugs.

2. Bicyclol: A Novel Anti-Hepatitis Drug

The development of bicyclol was based on the study of traditional Chinese medicine. In the early 1970s, clinical observation found that *Fructus Schizandrae*, one of the Chinese medicinal herbs, was effective in improvement of the abnormal liver function of chronic hepatitis B (CHB). Following this clinical lead, seven dibenzo (a, c) cycloocten lignans were isolated from the kernels of *Fructus Schizandrae* and their protective action against liver injury was screened in carbon tetrachloride (CCl₄)-intoxicated mice. Of seven lignans,

schizandrin C was shown to be the most active compound in protection against liver injury in CCl₄-intoxicated mice. In order to develop novel drug for therapy of CHB, a number of analogues of natural schizandrin C were synthesized and screened in liver injury models. Fortunately, dimethyl-4, 4'-dimethoxy-5, 6, 5', 6'-dimethylene dioxybiphenyl-2, 2'-dicarboxylate (called DDB, Figure 1) was found to be effective in protection against liver injury in animals[1]. Clinical trial proved that DDB markedly improved the impaired liver function and ameliorated the main symptoms of hepatitis patients. However, the anti-viral activity and bioavailability of DDB were poor. In order to develop a novel drug better than DDB both in inhibition of HBV replication and bioavailability, a number of DDB derivatives were synthesized and screened in liver injury model. Finally, a novel substitute of DDB (named bicyclol) by a 6-hydroxymethyl (-CH₂OH) instead of 6-the carboxylate group (-COOCH₃) in the side chain (Figure1) was found to be more effective than the parent DDB in protection against liver injury, and was also shown to inhibit hepatitis virus replication in 2.2.15 cell line and duck hepatitis [2]. Through study for 15 years, China FDA issued license to manufacture bicyclol in 2004. Since then bicyclol has been widely used to treat chronic viral hepatitis in the mainland of China.

Clinical trials have shown that bicyclol 20~50 mg given orally twice daily for 3~6 months to patients markedly reduced the elevated serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to the normal limits by 50%, and also inhibited hepatitis virus B replication by approximately 20% (serum HBeAg conversion from positive to negative) [3]. No noticeable side effects were observed.

Pharmacologically, bicyclol inhibited replication of hepatitis virus in duck viral hepatitis and HepG2.2.15 cell line transfected with human hepatitis B virus [2]. Moreover, bicyclol protected against experimental liver injury in several animal model, for example, bicyclol reduced mouse liver injury induced by CCl₄ [4], acetaminophen [5], and concanavalin A (ConA) [6,7]. The mechanistic study revealed that bicyclol exerts most of its effects by multiple pathways such as eliminating free radical, maintenance of mitochondrial glutathione redox status, anti-inflammation and anti-apoptosis [8]. However, the primary molecular mechanism underlying these actions of bicyclol remains unclear.

3. Heat Shock Proteins

HSPs are highly diverse and conserved family of proteins containing both constitutive and inducible members. They are classified into distinct families based on molecular weights, including HSP100, HSP90, HSP70, HSP60, HSP40, small HSPs (sHSP) and HSP10. HSPs families are well known as molecular chaperones since they play critical roles in protein folding in the cell: 1) to prevent aggregation and misfolding during the folding of newly synthesized chains, 2) to prevent nonproductive interactions with other cell components, 3) to direct the assembly of larger proteins and multiprotein complexes, and 4) during exposure to stressors that cause previously folded proteins to unfold. Therefore, molecular chaperones are considered to be endogenous cytoprotective factors, lifeguards or guardians of proteome.

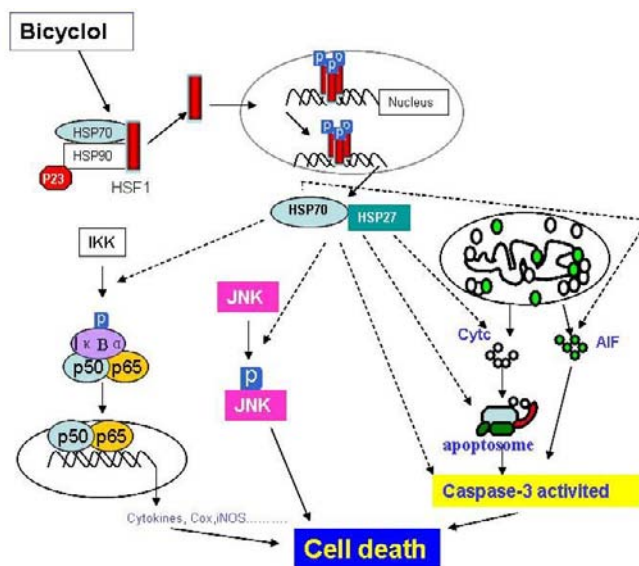


Figure 1. The picture of *Fructus. Schizandrae* and chemical structures of schizandrin C, DDB and bicyclol.

HSPs can be induced at the protein and mRNA levels to promote cell growth and protect tissues from injury [9]. Prior induction of HSPs by a mild stress can provide protection to cells and tissues against a subsequent severe stress that would normally be damaging or lethal. For example, HSPs can enhance the survival of mammalian cells exposed to oxidative injury [10] and upregulation of HSPs by thermal treatment can provide protection against lethal heat stress in mice [11]. Induction of HSPs in rats and mice were also found to diminish liver injury caused by hepatotoxins such as acetaminophen [12], bromobenzene [13] and from ischemia–reperfusion [14]. In general, the cytoprotective effects of HSPs are most often attributed to their ability to bind nonnative proteins and chaperone their refolding or elimination. In the cells undergoing chemical or physical protein denaturation or adduction (i.e., proteotoxicity), HSPs are believed to enhance the solubility of denatured proteins by preventing the aggregation of exposed hydrophobic areas.

4. The Relationship of Hepatic HSPs and Hepatoprotective Activity of Bicyclol

Since HSP is an endogenous defense mechanism in protecting against cell injury under various pathological conditions, we hypothesized that the hepatoprotective effect of bicyclol might result from the induction of hepatic HSPs. To test the hypothesis, we first investigated the effect of bicyclol on hepatic HSPs expressions in mice.

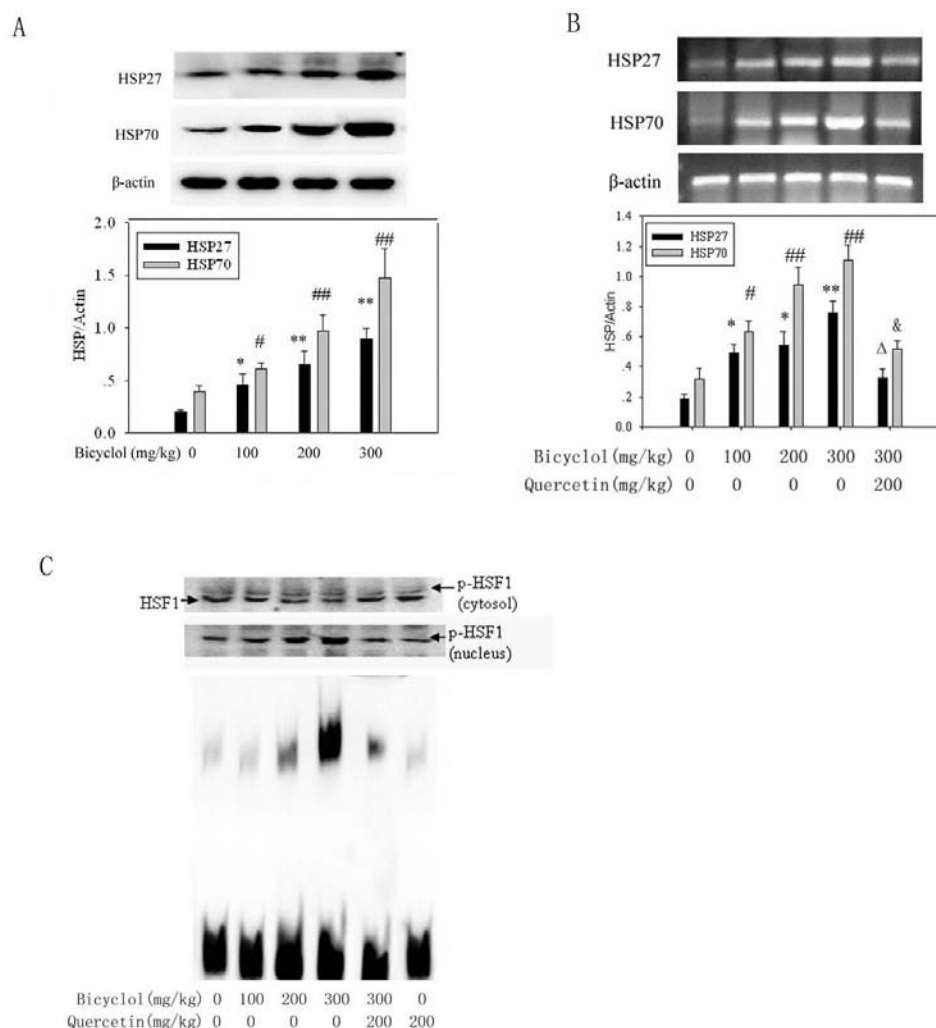


Figure 2. Effect of bicyclol on the expression of hepatic HSP27 and HSP70 and HSF1 in mice. (A) Western blot analysis for HSP27 and HSP70 induced by bicyclol 100 mg/kg, 200 mg/kg and 300 mg/kg administered three times in 24 hours. The inductive action of bicyclol 300mg/kg was more potent than those of 100 mg/kg and 200 mg/kg. Mean $\pm$ SD results of three to four separate experiments are graphed. *,# $P < 0.05$, **,## $P < 0.01$ versus control mice. (B) Whole liver mRNA was extracted for RT-PCR assay (2 μ g per lane). The inductive action of bicyclol was in a dose-dependent manner. Mean $\pm$ SD results of triplicate experiments are graphed. *,# $P < 0.05$, **,## $P < 0.01$ versus control mice, respectively. (C) Effect of bicyclol on the activation and nuclear localization of HSF1 in mouse liver. A representative from three results is shown.

4.1. Bicyclol Induces Hepatic HSP27 and HSP70 Expression in Protein and mRNA Levels in Mice

Male Kunming mice were orally administered three doses of bicyclol 200mg/kg (dissolved in polyethylene glycol 400) in 24 hours. After the last administration of bicyclol, both HSP27 and HSP70 levels in the livers increased significantly, but the increase of HSP70

was earlier than HSP27. The level of HSP27 increased about 2.6-fold within 6 hours, and remained higher than that of the control at 8 hours, then declined to a normal level at 24 hours. The expression of HSP70 began to increase within 2 hours, reached a maximum level (about 2.1-fold) at 4 hours, remained at the higher level for 2 hours, and then gradually decreased to the control level at 24 hours after the last administration of bicyclol. However, bicyclol did not induce HSP90 accumulation at the time point indicated above.

In the dose-effect relationship study, mice received bicyclol 100, 200 and 300 mg/kg three times in 24 hours, respectively. The accumulations of HSP27 and HSP70 protein and mRNA in mouse liver were assayed 6 hours after the last administration of bicyclol. The inducing effects of bicyclol on both protein and mRNA were in good dose-dependent manners. The effect of bicyclol 300 mg/kg on HSP27 and HSP70 accumulations was more potent than that of 100 mg/kg and 200 mg/kg. The accumulations of HSP27 and HSP70 were associated with the pronounced expressions of mRNA (Figure 2).

The synthesis of HSPs is regulated both at the transcriptional and posttranscriptional levels. In addition, one of the first cellular responses to stress may be an increase in HSPs mRNA “half-life”. As to bicyclol, its up-regulation of HSP27 and HSP70 synthesis occurred at the transcriptional level as indicated by increases of HSP27 and HSP70 mRNA expressions in mouse liver. Moreover, in the *in vitro* study of HepG2 cells, the effect of bicyclol on HSP27 and HSP70 promoter activities were further examined. The HSP27 and HSP70 promoter sequences were amplified and cloned into a luciferase reporter vector, and then transfected into HepG2 cells. After 12 hours, bicyclol 50 μ M was added to the cells. The luciferase activity was assayed 4 hours later. Bicyclol alone significantly enhanced luciferase activity of both HSP27 and HSP70 in HepG2 cells in dose-dependent manners, indicating that the induction of mouse liver HSP27 and HSP70 by bicyclol was at transcriptional level by up-regulating the transcription of HSP27 and HSP70 genes.

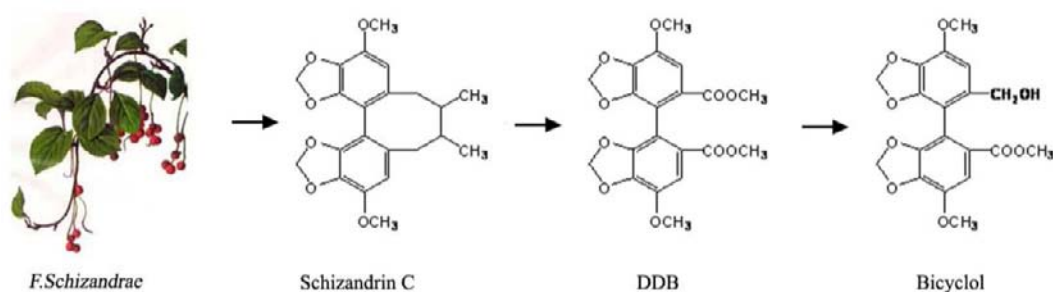


Figure 3. The hepatic HSP27 and HSP70 induced by bicyclol in protected against ConA-induced liver injury in mice. Mice were orally administered bicyclol 300 mg/kg for three times in 24 hours, and then they were injected with ConA 25mg/kg 1 hour after the last dosing of bicyclol. The liver tissues were obtained 6 hours after ConA injection for different determinations. (A) Liver lesions (hematoxylin-eosin stain, scale bar =50 μ m). (B) Western blot of cytochrome *c* and AIF in mitochondria and cytosol. A representative of three separate experiments is shown. **,### P <0.01 vs ConA, bicyclol+ConA+quercetin and ConA+quercetin. (C) Representative hepatic DNA fragmentation (DNA ladder). **,### P <0.01 vs ConA, bicyclol+ConA+quercetin and ConA+quercetin.

Quercetin, a flavanoid compound, is a known inhibitor of HSPs biosynthesis by inhibiting HSF1 activation. In our study, quercetin was used as a tool to determine whether it could reverse the inducing effect of bicyclol on HSP27 and HSP70 expression. Simultaneously oral administration of quercetin 200mg/kg significantly attenuated the expressions of HSP27 and HSP70 induced by bicyclol 300mg/kg, suggesting that bicyclol efficiently induced the synthesis of specific heat shock proteins.

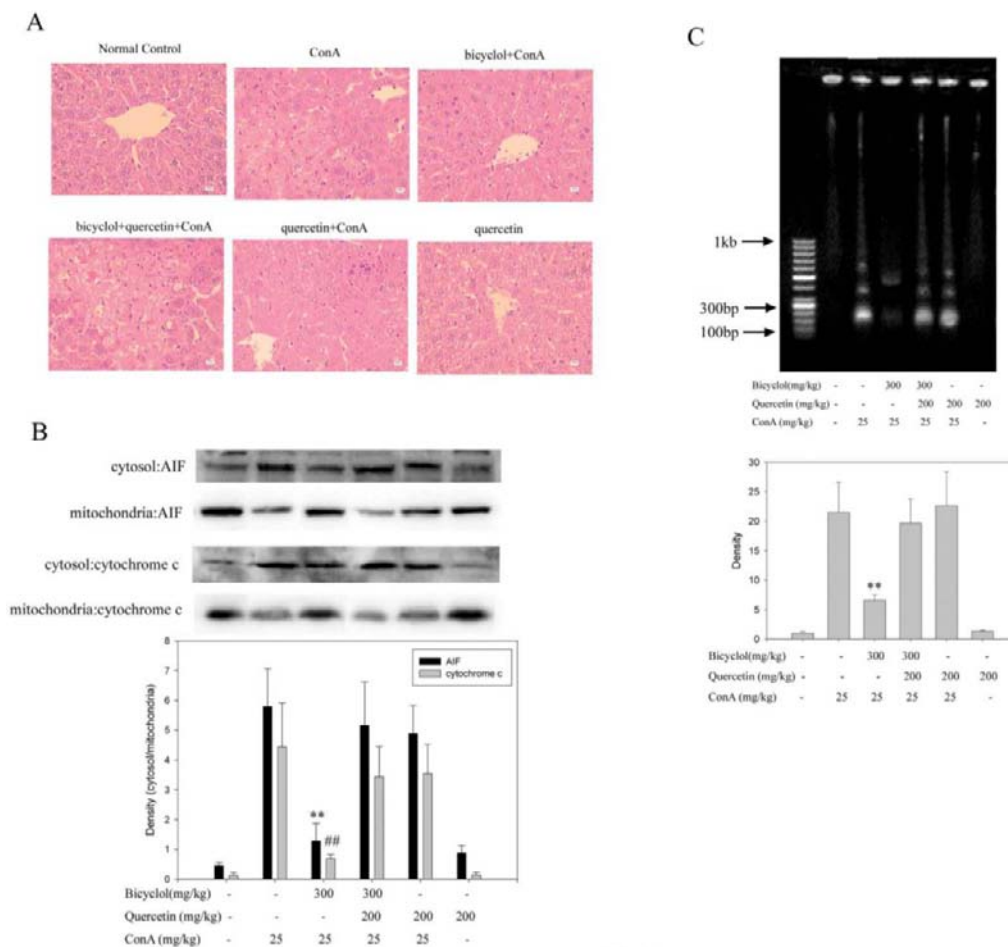


Figure 4. Inhibition of I κ B degradation and NF- κ B activation by bicyclol-induced HSP27 and HSP70 in ConA treated mice (A) Effect of bicyclol on the degradation of I κ B- α . Mice were administered bicyclol 200mg/kg for three times in 24 hours, and ConA 25mg/kg was given 1 hour after the last administration of bicyclol. I κ B- α was detected at the indicated time. (B) Dose-effect of HSP27 and HSP70 induced by bicyclol on the degradation of I κ B- α in ConA treated mice. Mice were treated described above. * P <0.05, ** P <0.01 vs ConA treated alone, $\Delta\Delta P$ <0.01 vs bicyclol 300mg/kg +ConA group. (C)Effect of MG132 on NF- κ B activation in ConA-treated mice. ** P <0.01 vs ConA treated alone. (D) Inhibitory effect of bicyclol-induced HSP27 and HSP70 on nuclear localization of NF- κ B in ConA treated mice. * P <0.05, ## $\Delta\Delta P$ <0.01 vs ConA treated alone, && P <0.01 vs bicyclol 300 mg/kg + ConA group. (E) Inhibitory effect of bicyclol-induced HSP27 and HSP70 on NF- κ B-DNA binding activity in ConA treated mice. **,## P <0.01 vs ConA treated alone, $\Delta\Delta P$ <0.01 vs bicyclol 300 mg/kg + ConA group. A representative for each group is shown. Similar results were obtained in three separate experiments.

The effect of one dose and three doses of bicyclol 300 mg/kg on HSP27 and HSP70 expression was further compared. Three doses of bicyclol significantly up-regulated HSP27 and HSP70 expression, while a single dose of bicyclol did not significantly induce expression of either HSP27 or HSP70, suggesting that the induction of HSPs needs accumulation of bicyclol in the liver.

It is worth noting that the inducing effect of bicyclol on HSPs is only occurred in the liver. In the same experiment system described above, no significant accumulations of HSP27 and HSP70 in spleen and kidney were found. These results suggest that there was organ specificity of bicyclol in the induction of HSPs.

4.2. Bicyclol Enhances Heat Shock Factor 1 (HSF1) Localization and Activation

The synthesis of HSPs is tightly regulated at the transcriptional level by HSF1. The activation of HSF1 is associated with its oligomerization, nuclear localization, and acquisition of binding activity to heat shock element (HSE) in the heat shock gene promoter to exert a transcriptional activation [15,16]. The HSE consists of a contiguous array of alternately oriented pentanucleotide 5'-nGAAn-3' units found in the promoter region of heat shock genes.

To explain the mechanism of inductive action of bicyclol on HSP27 and HSP70, the effect of bicyclol on the activation HSF1 was examined. HSF1 mainly presents in the cytosol of resting cells and its phosphorylation is a good indicator of cells under stressful conditions. The phosphorylation of HSF1 was detected as an upward band shift by Western blot. When mice were treated with a dose of bicyclol 100, 200 and 300 mg/kg three times in 24 hours, respectively, the liver cytosol HSF1 level gradually decreased, and concomitantly, the nucleus HSF1 (indicated as p-HSF1) increased 6 hours after the last administration of bicyclol. The effect of bicyclol on HSF1 showed a good dose-effect relationship. The specific DNA-protein complexes in the liver were further detected by gel mobility shift assay using biotin-labeled oligonucleotide. The treatment of bicyclol produced an HSE-binding activity in 6 hours. This HSF1-HSE binding activity was confirmed to be an HSF1-HSE complex. Simultaneous administration of quercetin 200 mg/kg attenuated the effects of bicyclol on nuclear HSF1 and its DNA binding activity.

5. The Role of HSP27 and HSP70 Induced by Bicyclol in Protection against Liver Injury

5.1. Role in Protection against Acetaminophen Induced Liver Injury in Mice

Acetaminophen is a safe and effective analgesic/anti-pyretic drug when used at therapeutic levels [17]. However, an acute or cumulative overdose can cause severe liver injury with the potential to progress to liver failure. In fact, acetaminophen overdose is the

most frequent cause of drug-induced liver failure [18]. It has been well accepted that the hepatotoxicity of acetaminophen is initiated through hepatic P450 metabolism of acetaminophen to *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI has high reactivity with sulphhydryl groups, which results in glutathione (GSH) depletion in hepatocytes. Once GSH is depleted, the remaining NAPQI covalently binds to intracellular proteins. Special binding of NAPQI to mitochondrial proteins could trigger mitochondrial dysfunction and, consequently, necrosis of hepatocytes [19].

To test whether there is a correlation between HSP27 and HSP70 induced by bicyclol and its protection against acetaminophen hepatotoxicity, the hepatic HSP27 and HSP70 in mice of acetaminophen alone and bicyclol treated acetaminophen groups were determined. The intraperitoneally injection of acetaminophen 200 mg/kg alone induced accumulations of HSP27 and HSP70 in mouse liver, while pretreatment of bicyclol 300 mg/kg before injection of acetaminophen caused overexpression of HSP27 and HSP70 at 6 hours. The levels of HSP27 and HSP70 induced by bicyclol plus acetaminophen group doubled compared with that of acetaminophen treated alone. Simultaneous administration of quercetin blocked acetaminophen and bicyclol plus acetaminophen-induced HSP27 and HSP70 accumulations.

In the experiment of HSP27 and HSP70 induced by bicyclol in protection against acetaminophen-induced hepatotoxicity, the HSPs inhibitor quercetin was used as a tool and administered in association with bicyclol. Acetaminophen 200 mg/kg was intraperitoneally injected 4 hours after the last administration of bicyclol 300 mg/kg and quercetin 200 mg/kg. 6 hours after acetaminophen injection, the mice were sacrificed for different determinations. The biomarkers of liver injury, such as serum transaminase (ALT/AST) increased markedly, and liver tissue toxicity and necrosis were serious. Pretreatment of bicyclol 300 mg/kg significantly decreased the elevated serum ALT/AST and hepatocyte necrosis induced by acetaminophen. Hepatocyte mitochondrion is the key target of acetaminophen intoxication. Bicyclol also inhibited the leakage of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria and inhibited nuclear DNA fragmentation induced by acetaminophen. Co-administration of quercetin 200 mg/kg with bicyclol 300 mg/kg before acetaminophen injection markedly attenuated all the protective effect of bicyclol on serum ALT/AST levels, liver necrosis, the release of cytochrome *c* and AIF from damaged mitochondria and hepatic DNA fragmentation in mice. While quercetin itself showed no effect on the above biomarkers both in normal and acetaminophen-poisoned mice.

5.2. Role in Protecting against ConA-Induced Liver Injury in Mice

Liver injury induced by ConA differs from that of acetaminophen. ConA can induce hepatitis with manifests of elevated serum ALT levels, T cell infiltration as detected by histological analysis, massive granulocyte accumulation, and hepatocyte apoptosis and necrosis. This type of liver injury is a T cell-mediated immunoinflammatory condition similar to human autoimmune hepatitis, while acetaminophen is through the production of NAPQ I by liver microsome mechanism.

The first experiment was to determine whether bicyclol also induces the expression of hepatic HSP27 and HSP70 in ConA-injected mice, the experiment of time-course effect of

bicyclol was first conducted. Mice livers were collected at 1, 2, 4, 6, 8, 24 hours after ConA injection. ConA alone increased the hepatic levels of both HSP70 and HSP27 significantly, while HSP70 was peaked earlier than HSP27. Interestingly, pre-administration of bicyclol (200mg/kg for three times within 24 hours) further enhanced the accumulation of both hepatic HSP27 and HSP70 proteins in ConA injected mice. The level of HSP27 was clearly increased by 2.0-fold in contrast to that at 0 hour. The same response was also observed in HSP70, as its level increased by 2.8-fold within 2 hours and remained high until 4 hours, and then gradually decreased to a level equivalent to that for the control at 24 hours. Thus, bicyclol given orally can markedly elevate the level of HSP27 and HSP70 in ConA-injected mouse livers.

As mentioned above, the increase of hepatic HSP27 and HSP70 expression varied with time after bicyclol (200mg/kg) administration in ConA-injected mice. To test whether this induction is dose-dependent, mice were administered bicyclol 100, 200 and 300 mg/kg thrice with 24 hours, the hepatic HSP27 and HSP70 accumulation was in a dose-dependent manner in ConA treated mice. The inducing effect of bicyclol 300 mg/kg on HSP27 and HSP70 expression was more potent than those of 100 and 200 mg/kg. Co-administration of quercetin, an inhibitor of HSPs biosynthesis attenuated the inductive effect of bicyclol on HSP27 by 50% and HSP70 by 67%.

We next investigated whether bicyclol pretreatment could also affect the expression of HSP27 and HSP70 mRNA. RT-PCR analysis showed that bicyclol 300 mg/kg up-regulated hepatic HSP27 and HSP70 mRNA expression in mice injected with ConA. The induction of HSP27 and HSP70 mRNA by bicyclol 300 mg/kg was highly significant, and there was a good correlation of the accumulation of HSP27 and HSP70 with the expression of HSP27 and HSP70 mRNA. Co-administration of quercetin decreased the inductive effect of bicyclol on mRNA of HSP27 by 55% and HSP70 by 76%. All these results suggest that the inductive effect of bicyclol on liver HSP27 and HSP70 accumulation is via up-regulation of transcription of HSPs genes.

To study the role of HSP27 and HSP70 induced by bicyclol in protection against liver injury, a dose of ConA 25 mg/kg was injected intravenously to mice 1 hour after the last administration of bicyclol. Pretreatment of 300 mg/kg bicyclol markedly alleviated ConA-caused liver injury in mice as indicated in reduction of serum aminotransferase, liver necrosis, the release of cytochrome *c* and AIF from mitochondria and hepatic DNA fragmentation. Co-administration of 200 mg/kg quercetin together with 300 mg/kg bicyclol significantly attenuated all the protective actions of bicyclol as mentioned above in ConA challenged mice. While quercetin itself showed no effect on the above biomarkers in both normal and ConA treated mice (Figure 3). It is apparent that the HSP27 and HSP70 induced by bicyclol may act as protector against ConA-induced liver injury in mice.

6. Bicyclol-Induced HSP27 and HSP70 Inhibits NF- κ B-Mediated Inflammation in ConA Treated Mice

NF- κ B is a ubiquitous transcription factor that regulates a number of genes involved in inflammation and immune response [20], which is normally sequestered in the cytoplasm where it associates with a family of inhibitory proteins known as I κ B. In response to external signals, I κ B is phosphorylated by the IKK complex, and subsequently degraded through a ubiquitin-dependent proteolysis [21]. The IKK complex is a potential target for inhibition of NF- κ B pathway by HSPs[22]. I κ B inhibits the transcriptional activity of NF- κ B by preventing the nuclear translocation of NF- κ B in cytoplasm. HSP70 has been found to associate with the p65 subunit of NF- κ B and inhibits the nuclear transport of the latter in T-cells [23], and HSP27 has been shown to be a ubiquitin-binding protein regulating the degradation of I κ B expression, thereby indirectly influencing NF- κ B activation [24].

As mentioned in ConA-induced liver injury, bicyclol exhibited significant protection against liver injury. To further clarify whether the mouse liver injury induced by ConA was mediated by NF- κ B signaling pathway, the proteasome inhibitor MG132, which is a well-known NF- κ B inhibitor by blocking degradation of I κ B- α , was employed. A dose of 2 mg/kg MG132 was injected intraperitoneally to mice 1 hour before injection of ConA 25mg/kg. The injection of MG132 reduced the elevated serum TNF- α and IL-2 levels 6 hours after ConA injection. Similarly, pretreatment of bicyclol 300mg/kg decreased the serum levels of TNF- α and IL-2 challenged with ConA. These data indicated that the productions of TNF- α and IL-2 induced by ConA were NF- κ B-mediated, and that bicyclol decreased TNF- α and IL-2 productions through the direct inhibition of NF- κ B activity.

I κ B is an inhibitor that binds to and sequesters NF- κ B in the cytoplasm. 1 hour after the injection of ConA 25 mg/kg, the degradation of I κ B- α was observed in mice. And bicyclol alone had no effect on the level of I κ B- α in normal mice. Pretreatment of bicyclol 200 and 300 mg/kg inhibited ConA-induced I κ B- α degradation (bicyclol 200mg/kg: 2.7-fold increase; bicyclol 300mg/kg: 3.9-fold increase). Co-administration of quercetin to inhibit HSPs biosynthesis counteracted the inhibitory effect of bicyclol on I κ B- α 's degradation by 69% decrease (Figure 4A,B). Thus, the inhibitory effect of bicyclol on the degradation of I κ B- α is through its induction of HSP27 and HSP70.

To further explore the interactions of bicyclol-induced hepatic HSP27 and HSP70 with IKK and I κ B, a set of co-immunoprecipitations with IKK- α or I κ B- α antibodies were performed using liver protein isolated from ConA treated mice. The interactions of HSP27 and HSP70 with IKK- α and I κ B- α were different, as HSP27 efficiently interacted with IKK- α , but not with I κ B- α , whereas HSP70 interacted with I κ B- α more markedly than with IKK- α . Blocking of HSP27 and HSP70 biosynthesis with quercetin reduced these interactions, respectively, suggesting that the inhibition of IKK activity and I κ B- α degradation by bicyclol was through its induction of hepatic HSP27 and HSP70.

Then the effect of hepatic HSP27 and HSP70 induced by bicyclol on NF- κ B activation in ConA treated mice was studied. The hepatic NF- κ B was activated after the injection of ConA 25mg/kg in mice, and this activation of NF- κ B was inhibited by MG132. Pretreatment of bicyclol 300mg/kg inhibited nuclear translocation of NF- κ B p65 subunit and NF- κ B-DNA-

binding activity induced by ConA. However, when the hepatic HSP27 and HSP70 synthesis was inhibited with quercetin, the suppression of nuclear translocation of NF- κ B p65 subunit and NF- κ B-DNA-binding activity by bicyclol was abrogated (Figure 4C,D,E), suggesting that the HSP27 and HSP70 induced by bicyclol is involved in the suppression of NF- κ B activation.

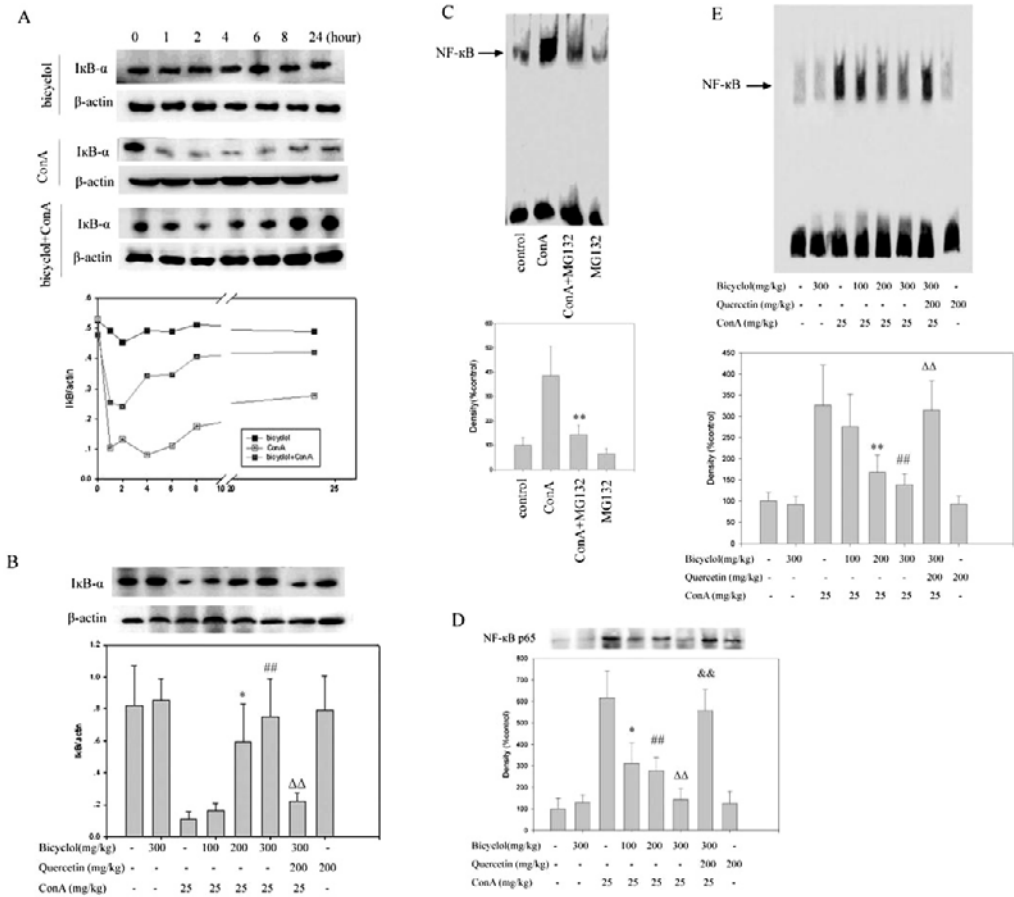


Fig4

Figure 5. Schematic of the induction of HSPs of bicyclol and its protection against liver injury. Bicyclol activates HSF1 to form trimers from its inactive monomers. Activated HSF-1 induces the expression of HSP27 and HSP70. The induced HSPs protects against liver injury caused by acetaminophen and ConA. The protective effects of HSPs attributed to the inhibition of apoptotic bodies, suppression of NF- κ B and JNK pathways. These results provide a complete and new explanation of the mechanism of bicyclol in protection against liver injury. indicates activation and indicates inhibition

To verify this conclusion, the in vitro experiment of HepG2 cells intoxicated with D-galactosamine (D-GaIN) 50 mM for 8 hours was performed. Pretreatment of the cells with bicyclol 100 μ M markedly suppressed D-GaIN-induced IKK phosphorylation, IκB degradation, NF- κ B p65 translocation and NF- κ B-DNA binding activity. Using RNA

interference (RNAi) to knock down HSP70 expression, the above inhibitory effects of bicyclol were all attenuated. This indicated that bicyclol suppressed NF- κ B activation through its induction of HSPs.

7. The Role of Bicyclol-Induced HSP27 and HSP70 in the Regulation of Hepatocyte Apoptosis

It was reported that apoptosis is a key pathologic change in liver disease, including viral hepatitis [25], liver ischemia [26], chemical [27] and drug-induced [28] liver injury. Recent paper pointed out that mitochondria play an important regulatory role of in the apoptotic process [29]. Mitochondrial dysfunction is the commitment step in hepatocyte apoptosis [30]. Cell death signals induce the release of cytochrome *c* from the mitochondria, which then binds to Apaf-1, facilitating the formation of the apoptosome. Apoptosome formation results in the processing and activation of caspase-9, which triggers the caspase pathway by activating the downstream caspase-3[31]. AIF is another mitochondrial intermembrane protein released upon apoptotic stimulus. AIF translocates to the nucleus and triggers caspase-independent nuclear changes upon activation of the intrinsic pathway [32].

To study the role of HSP27 and HSP70 induced by bicyclol in the apoptosis of hepatocytes, HepG2 cells intoxicated with D-galactosamine (D-GaIN) 50 mM for 8 hours. Pretreatment the cells with of bicyclol 100 μ M markedly suppressed D-GaIN-induced apoptosis in HepG2 cells. Using RNAi knock down HSP70 expression markedly attenuated the anti-apoptosis by bicyclol. Co-immunoprecipitation assay indicated that there was a quite apparent interaction of HSP27 with cytochrome *c*, but very weak interaction with AIF and Apaf-1 in bicyclol 100 μ M pretreated HepG2 cells intoxicated with D-GaIN. While HSP70 interacted with Apaf1 and AIF remarkably, but very weak with cytochrome *c*. When RNAi was used to knock down HSP70 expression, the above effects of significantly reduced, suggesting that HSPs induced by bicyclol mainly interacted with the pro-apoptotic proteins and inhibited their activity, and finally suppressed hepatocyte apoptosis.

Caspase-3 can be cleaved and activated by the release of cytochrome *c* from mitochondria to cytosol. It was reported that HSPs mainly functions as an inhibitor of caspase activation. Knock-down of HSP70 induces apoptosis through caspase-3 activation [33]. This phenomenon can be explained by the ability of HSP27 to prevent the formation of the apoptosome and the subsequent activation of caspases[34].

In our results, cultivation of HepG2 cells with 50 mM D-GaIN for 8h caused a significant increase of caspase-3 activity in the cells. Pre-incubation the cells with 100 μ M bicyclol significantly inhibited the activation of caspase-3 induced by D-GaIN. Similarly, RNAi knock down HSP70 and addition of quercetin attenuated this effect of bicyclol, respectively. The results suggested that HSP27 and HSP70 were involved in the inhibitory effect of bicyclol on caspase-3 activity.

Mitochondrial outer membrane permeabilization can activate intracellular stress kinases, such as JNK. MAPK pathways play important roles in apoptosis. Among three MAPKs, JNK is the most important kinase that determines the cell fate to death or survival [35]. Mice lacking either JNK1 or JNK2 are highly resistant to ConA-induced liver failure and show

considerably lower amounts of apoptotic and necrotic hepatocyte death [36]. Recent studies further proposed that down-regulation of JNK activity is a mechanism of HSP27 mediated protection. Overexpressions of HSP27 in stress can definitely play an inhibitory role in JNK signal transduction pathway, and thus blocks cell apoptosis induced by JNK signal transduction pathway [37].

Our study demonstrated that 50 mM D-GaIN caused JNK phosphorylation. The pretreatment of bicyclol 100 μ M suppressed D-GaIN-induced JNK phosphorylation. RNAi knock down HSP70 and addition of quercetin attenuated this effect of bicyclol on JNK phosphorylation, respectively. indicating that HSP27 and HSP70 were involved in the inhibition of JNK activity of bicyclol.

From the above results, it may be seen that bicyclol-induced HSP27 and HSP70 inhibited inflammation and apoptosis of hepatocytes through affecting NF- κ B and JNK signal transduction pathway (Figure 5).

8. Conclusions and Perspective

Liver injury is a pathological change that appears in many liver diseases. Long-term injury of hepatocytes is a pivotal initiating factor that leads to liver fibrosis, and even hepatocarcinoma. In China HBV infection is the main cause of liver disease, so, to develop novel drug with anti-liver injury and anti-hepatitis virus is very important. Through 15 years study, the novel drug bicyclol was developed. It has been proved that bicyclol is highly effective in improving the abnormal liver functions and also effective in inhibiting hepatitis virus replication in hepatitis B and C patients. In animals, bicyclol has multiple pharmacological effects such as anti-liver injury, anti-liver fibrosis, anti-hepatitis virus replication and stimulating protein synthesis of hepatocytes in vivo and in vitro. In this review, we provided new information that bicyclol is a novel inducer of hepatic HSP27 and HSP70 biosynthesis and the induction of HSP27 and HSP70 by bicyclol is the primary molecular mechanism of its protection against liver injury. Figure 5 is a summary of the induction of HSP27 and HSP70 by bicyclol and its role in protection against liver injury.

HSPs have the functions in protecting cells and tissues from injury caused by a variety of physiological and pathological agents and may be used as novel molecular targets for pharmacological and therapeutic interventions. Some drugs and chemicals are known to induce HSPs at toxic doses, which are not feasible in clinical use. It should be very useful to find non-toxic HSPs inducers for the prevention and treatment of various pathological states, such as liver injury, stress ulcers, as well as diseases associated with protein misfolding and protein aggregation. The finding of bicyclol as an inducer of HSP27 and HSP70 is not only a good example of pharmacological induction of HSPs, but also highlights the potential of bicyclol as a therapeutic agent against a variety of stress conditions through induction of HSPs in the future.

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Chapter XVII

Plant Heat Shock Proteins as Molecular Chaperones in Normal and Stress Conditions

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Abstract

Similarly to all other organisms plants are constantly exposed to different environmental abiotic and biotic factors. Some of these agents can exert strongly negative influence upon crop growth. Heat shock protein synthesis appears to be one of the major anti-stress responses conserved among all known organisms on our planet - from bacteria, plants and animals to human, which has unique nuance in plants. Studying and understanding of this defensive mechanism, despite the difficulties in finding appropriate model systems in plants, have great importance for the detection of markers against different kind of stresses and their practical application in genetically transferred crops.

This review summarizes and comments the main part of the researches for different heat shock protein families that can act as molecular chaperones in maintaining the cellular homeostasis. This paper demonstrates the significant role of these proteins in normal and stress conditions in plants, which are discussed in parallel. Classification of heat shock protein families and subfamilies and regulation of heat shock protein expression are presented, and there is information about different kinds of stress inductors in plants. This review explains the understanding for the interaction between different heat shock proteins in one general molecular chaperone network. Additionally, the article discusses thermotolerance and the main participants in this acclimation process.

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Introduction

Stress and Plant Stress Response

Plants are frequently subjected to different biotic and abiotic stresses that adversely affect their growth, development and productivity [2,6]. Abiotic stresses are prevalent in nature and can substantially reduce crop yields (by more than 50%) [20,37]. Among the environmental conditions that cause the most severe damage are drought, high or low temperatures, waterlogging, raised soil salinity, too much or too little light and presence of heavy metals in the soil [2]. Plant responses to different kind of stresses and plant resistance depend on the species belonging, the concrete genotype, the developmental age of the plant, and last but not least, on the intensity and duration of the stress factor. Several unfavourable attacks in combination may provoke a response different from that for an individual applied stress [1,14,39]. Being sessile organisms, plants are constantly exposed to changes in environmental conditions and have developed mechanisms that permit the plant to withstand the stress [19,24,37]. The major tolerance mechanisms in plants can be divided in two groups: (i) conservative mechanisms for defense, such as ubiquitin – dependent protein degradation, ion transporters, osmoprotectants, free-radical scavengers, calcium signaling, programmed cell death and the induction of Hsps; and (ii) unique for plants such as the growth regulators ethylene, jasmonic acid and abscisic acid (ABA), the organization of leaves as photosynthetic organs with the active cells inside and the formation of stress resistant dormant forms like seeds [13,16,37,54].

Plants subjected to extreme environmental conditions exhibit a characteristic set of morpho-anatomical, physiological, biochemical and molecular responses [55]. The present review will focus mostly on the induction of Hsp synthesis as one of the main molecular stress responses.

Molecular Chaperones and Hsps

The role of Hsps in plants

Abiotic stresses usually cause protein dysfunction and lead to altered gene expression in plants [4,20]. It was observed and reported in many crop plants (wheat, maize, pea, soybean) that during heat stress (when temperatures surpass 32-33°C) the synthesis of most normal proteins is repressed and the synthesis of a small set of proteins called Heat shock proteins (Hsp) is initiated [4]. Hsps were first discovered in 1962 by the Italian biologist F. Ritossa, working with *Drosophila melanogaster* [12,18]. After a fortuitous increase of the temperature during an experiment in 1962, he observed considerable changes of the gene activity pattern of the polytene chromosomes in larval salivary glands. Later (in 1974) A. Tissieres identified the newly formed Hsps and described them as a set of proteins whose expression is induced by heat shock [54].

Hsps can be defined as a group of highly conserved polypeptides (among bacteria, plants and animals) which play an important role for survival under both normal and extreme conditions [17,19,20,54]. The comparative study of the Hsp responses in different organisms

has demonstrated that the high conservation is manifested in respect to the molecular mechanisms of gene induction and highly homology among eukaryotes in reference to the major Hsps. According to E.Vierling (1991) Hsps are these proteins that meet the criterion to be heat induced in majority of the cell types in a wide range of organisms. In general, Hsps play crucial role in protecting plants against stress and in maintaining of cellular protein homeostasis during both optimal and adverse growth conditions [20,37,53,54].

Inductors of hsp responses

Many researchers working in the field of Hsps established that not only heat leads to elevation of *Hsp* expression. Extreme factors of environmental conditions such as heavy metals, amino acid analogues, glucose starvation, ethanol, arsenite and a number of other treatments as drought, cold, salinity, oxidative agents, microbial infections, tissue traumas, genetic lesions can affect the synthesis of some (not full complement) or all Hsps in organisms (see Table 1). In these cases, their products (Hsps) can assist in protein refolding during extreme conditions and in recovery of normal protein conformation and can be generally referred to as stress proteins [3,4,17,20]. Additionally, some Hsps are constitutively expressed at significant levels in normal growth conditions because they function to maintain protein homeostasis at particular stages of the cell cycle, after hormonal stimulation or during development in the absence of stress. They are responsible for protein folding, assembly, translocation and degradation in normal cellular processes [4,45,54]. That is way the term HSC (Heat Shock Cognate) was introduced to mark specially these Hsp family members that are expressed in the absence of stress. Therefore, the proteins designated as Hsps are not induced by heat at all, but rather are defined as Hsps according to sequence homology and possible functional similarities. Table 1 shows the most frequent activators of the Hsp expression. For example, it was reported that Hsp70 is synthesized in normal conditions at a specific stage (early S) of the cell mitotic cycle, during mitogenesis and upon other stresses which can be different from high temperatures [45]. Likewise, some members of Hsps can interact with a number of signaling molecules, including nuclear hormone receptors, kinases, cell cycle regulators and cell death regulators, thereby they show regulative activities. With these functions Hsps accompany signal transduction, hormone response and cell death [40].

Regulation of hsp expression

The induction of Hsp encoding genes is controlled by regulatory proteins called heat shock transcription factors (HSF), that recognize a conserved promoter sequence in DNA, called heat shock element (HSE) [5,12]. The HSE in the eukaryotic cell is an inverted repeat of 5-nucleotide bp with sequence 5'-nGAAn-3', presented by five to seven copies in the promoter close to the TATA box. HSFs are expressed constitutively and exist as inactive proteins in the cytoplasm. Stress causes activation of HSFs with oligomerization (the active form is trimer) and translocation to the nucleus, where they bind to their HSE. This process includes changes in the state of HSF phosphorylation (additional phosphorylation was detected under stress conditions).

In higher plants, in contrast to prokaryotes, different heat shock genes are expressed non-coordinately, because they do not form a regulon [37,45]. The peculiarity of plants is the unique complexity of the HSF family, including more than 20 members and the existence of

heat shock-induced forms of HSFs, which play a major role in the modulation of the transcription. Compared to plants the multiplicity of HSFs is significantly smaller in other organisms [54].

Molecular chaperones

Many researches have indicated that the main role of HSPs in plant cell (in normal and stress conditions) involves stabilization of proteins in a particular state of folding and through this mechanism they facilitate important processes as protein folding, transport of proteins across membranes, assembly and modulation of receptor activities. Because all these functions require the alteration or maintenance of specific polypeptide conformations, Hsps have been termed molecular chaperones (polypeptide chain binding –non-native-proteins).

The term “molecular chaperone” was first used by R.A.Laske to describe the role of the nucleoplasmin. It assists in the nucleosome assembly from histones and DNA and does not form part of the nucleosomes. Gradually the group of chaperones has extended and has included many new members. According to Ellis J. (1990) they have been defined as a group of unrelated cellular proteins that mediate the correct assembly of other polypeptides, but they themselves are not components of the final structures. Proteins regarded as molecular chaperones were: nucleoplasmins, chaperonins, Hsps 70 and 90 classes, signal recognition particle, prosequence of subtilisin, prosequence of α -lytic protease ubiquitinated ribosomal proteins, trigger factor, Sec B protein and Pap D protein [56]. At a later stage low molecular weight Hsps and Hsp100 groups were added to this chaperone group after clarifying their role [4].

Today, the molecular chaperone concept includes members of several structurally and genetically unrelated protein families that share the ability to recognize non-native conformations of other proteins and interact with them, without being a component of the final functional structure, thereby facilitate protein folding, targeting and degradation [22,44,50]. They act in preventing aggregation of stressed proteins and also in resolubilizing of aggregates formed under extreme conditions [20]. Chaperone proteins do not covalently bind to their targets [20]. The essential function of molecular chaperones is to prevent the formation of incorrect structures that are nonfunctional biologically and can be result from incorrect interactions between parts of polypeptide chains and other molecules. The natural frontier between molecular chaperones and heat shock proteins is too unclear because many molecular chaperones are stress proteins, many of them were originally discovered as Hsps, thus frequently molecular chaperones are identified with Hsps and authors use the term Hsps/chaperones [20]. But it is important to note that not all chaperones are Hsps and molecular chaperones include the group of Hsps [56]. Apart from the five major families of Hsps, there are other proteins with chaperone functions, such as PDI, PPI and calnexin/calreticulin, which assist in protein folding in the ER. The enzyme PPI catalyzes the isomerization of peptide bonds of proline residues, PDI catalyzes disulfide bond formation in the ER and calnexin/calreticulin assists the folding of glucosylated proteins in the ER. These enzymes called foldases, catalyze the covalent reactions directly involved in protein folding. In this case on the basis of on the definition of molecular chaperones as proteins assisting correct folding without covalent changes the foldases were excluded as chaperones but based

of the main purpose of their activity other authors recognize the foldases as molecular chaperones [20,38].

Table 1. Inducers of heat shock proteins.

Environmental stress conditions	Pathophysiological stress conditions	Normal conditions
High temperature	Bacterial and viral infections	Certain cell cycles
Low temperature	Tissue injuries	Cell differentiation
Drought	Genetic lesions	Hormonal stimulation
Radiation (UV)		
Heavy metals		
Oxidative stress		
Hypoxia		
Salinity		
Chemical substances		
Pesticides		

A molecular model of the Hsp/chaperone structure based on x-ray crystallography of the protein PapD (*E.coli*) was reported which model showed that it contains a large crevice with solvent-exposed hydrophobic sites. It was suggested that exactly these elements design the functional possibilities of Hsps [45].

*Classification of hsp*s

Initially Hsps were considered as gene products whose expression is induced by heat but now they are adopted as molecular chaperones. The genes encoding Hsps are highly conserved and function in all organisms from bacteria to human. These genes and their products are assigned to families on the basis of sequence homology and relatively molecular weight: Hsp100/Clp, Hsp90, Hsp70, Hsp60/Chaperonin and small Hsps (smHsps). In eukaryotes, many families comprise multiple members that differ in inducibility, intracellular localization and function. Table 2 visualizes classification of Hsps according to Wangxia et al. (2004).

Hsp70

Hsp70 family members are the most highly conserved Hsps among the prokaryotes and the eukaryotes and one of the most ubiquitous set of molecular chaperones in the cell compartments. Their molecular weight ranges from 68 to 110 kDa. This Hsp family includes Hsp110 subfamily based on similarities in structural and functional properties irrespective of the great differences in proteins molecular weight [20,21].

Distinct Hsp70 homologues act and are discovered in different part of the cell-cytoplasm, in the lumen of the ER, in chloroplasts and in the matrix of mitochondria. In order to work Hsp70 requires the participation of ATP and co-chaperones such as Hsp40 and GroE. Hsp70 contains highly conserved N-terminal ATPase domain and C-terminal peptide-binding domain [20]. Many researches demonstrated that Hsp70 has essential functions in preventing

aggregation and in assisting of non-native proteins under both normal and stress conditions such as heat, cold, drought, chemical and other stresses. Likewise they are involved in protein translocation processes and in facilitating the proteolytic degradation of unstable proteins [4,9,17,36]. Some family members referred to as HSC70 (70kDa heat shock cognate) are constitutively expressed and play an essential role in folding of nascent polypeptides, refolding of misfolded proteins, and the import/translocation of precursor proteins [20,23]. One example for such HSC70 can be the cytosolic HSC70 (see Table 2) which prevents protein aggregation, assists the folding of *de novo* synthesized polypeptides and maintains the organellar precursor proteins in a competent stage [4]. Other members of Hsp70 family are expressed during environmental stresses and are involved in facilitating refolding of non-native proteins or proteolytic degradation of unstable proteins by targeting the proteins to lysosomes or proteasomes [4,20,21]. There are no established differences between the constitutive and stress induced proteins in their chaperone function [21].

Table 2. Classification of plant Hsps according to Wang Wangxia et al. (2004) with complements

Classes	Representative members	Intracellular localization	Major functions
Hsp70 Subfamily:			Preventing aggregation, assisting folding of nascent polypeptides and refolding of misfolded or denatured proteins, protein translocation, signal transduction and transcriptional activation, thermotolerance
Hsp70/Hsp40	Hsp/HSC70 Hsp70 Bip	Cytosol Chloroplast, mitochondria ER	
Hsp110/SSE	Hsp91	Cytosol	
Hsp60/Hsp10 Chaperonin Subfamily:			Folding, assisting refolding, facilitating assembly (specialized folding machinery)
Group I	Cpn60	Chloroplast, mitochondria	
Group II	CCT	Cytosol	
Hsp90	Hsp90 AtHsp90-1(4) AtHsp90-5 AtHsp90-6 AtHsp90-7	Cytosol Chloroplast Mitochondria ER	Facilitating maturation of signaling molecules (coregulator of signal transduction complexes), genetic buffering
Hsp100/Clp Subfamily:	Hsp100		Disaggregation, unfolding, thermotolerance
Class I	ClpB, ClpA/C ClpD	Cytosol, mitochondria	
Class II	ClpM, ClpN ClpX, ClpY	Chloroplast Chloroplast	
smHsp Subfamily:			Preventing aggregation, stabilizing non-native proteins, thermotolerance
I	Hsp17.6	Cytosol	
II	Hsp17.9	Cytosol	
III	Hsp21 Hsp26.2	Chloroplast	
IV	Hsp22	ER	
V	Hsp23	Mitochondria	
VI	Hsp22.3	Membrane	

Some Hsp70 members are involved in the control of the biological activity of regulatory proteins and act as negative repressors of HSF mediated transcription. It was suggested that the interaction between Hsp70 and HSF prevents the trimerization and binding of HSF to HSE, thereby blocking the activation of heat shock genes by their HSFs. Hsp70 is also involved in the modulation of signal transducers such as protein kinase A, C and protein phosphatase. In this way, Hsp70 might play role in modulating the expression of many genes in signal transduction pathways [20].

As mentioned above, one specificity of Hsp70 family is that Hsp110 (one of the major Hsps of eukaryotic cells) is a diverged relative of the Hsp70 family or a subfamily of the Hsp70 based on similarities in structural and functional properties, but with genetical uniqueness [20,29]. The Hsp110 has the ability to prevent protein aggregation and to maintain denatured protein in a soluble (folding-competent state), but with greater capacity compared with Hsp70 and also that their overexpression strongly correlates with the thermotolerance *in vivo* [29]. In plants, synthesis of Hsp110 is more transient than synthesis of other Hsps. It is primarily limited to the first hour of heat stress, therefore Hsp110 are not essential for growth at normal or high temperature but they are necessary (still not sufficient) for thermotolerance [4].

Cytoplasmic Hsp70

It is established that the similarity among eukaryotic cytoplasmic Hsp70 homologues from different species is greater compared to such similarity between Hsp70 homologues in different cellular compartments of a single species. In addition, eukaryotic cytoplasmic Hsp70 show similarity greater than 65% with *E.coli* DnaK protein that makes this protein the most highly evolutionarily conserved protein known. In 1986 Pelham speculated on the functions of cytoplasmic Hsp70s – they play a role in ATP- dependent protein folding and assembly. Later, it was established that these proteins facilitate transport of proteins across membranes by unfolding precursor proteins or maintain them in a form suitable for transport across membranes [4].

Hsp70 in the ER

These proteins demonstrate sequence homology with cytoplasmic Hsp70s along with the ability to bind ATP and nascent peptides, in such a way as to stabilize newly synthesized proteins in the ER until they fold. The Hsp70 located in the ER is also called binding protein (Bip) or glucose regulated protein [4]. ER Bip protein, mitochondrial and chloroplastic Hsp70 proteins are involved in the translocation of precursors proteins as well [20].

Mitochondrial and chloroplast Hsp70

They show greater similarity to the *E.coli* DnaK protein than to eukaryotic cytoplasmic or ER Hsp70 homologues which similarity correlates with the hypothesis of an endosymbiotic origin of these organelles. In all species the mitochondrial and chloroplast Hsp70 are found in the absence of heat stress which suggests that they are essential proteins [4].

Hsp60

The fundamental role of some Hsp70 is determined by their ability to bind to extended, hydrophobic peptide segments and to release them in an ATP-dependent manner in an unfolded conformation. Folding of polypeptides then can occur either spontaneously or with the help of other molecular chaperones called chaperonins [44]. They are a group of a sequence-related proteins found in all organisms and in all mitochondria and plastids examined. Chaperonins are abundant and constitutive molecular chaperones that increase after stress such as heat, and play crucial role by assisting the newly synthesized and translocated proteins to achieve their native forms [56]. There are two types chaperonins established: Cpn60 (or Hsp60) and Cpn10 (or Hsp10). Hsp60 has a subunit molecular weight from 56 to 61 kDa whereas Hsp10 has only 10 kDa [56]. In contrast to molecular chaperones from Hsp70 family, Hsp60 do not directly interact with newly synthesized polypeptides at the ribosome level and form a large structure (like a cage) with two rings of seven subunits, each surrounding a central cavity. The protein-folding intermediate binds to hydrophobic sites lining these cavities. In this way Hsp60 act as a container for the folding of other protein subunits and mediate the assembly of many proteins in an ATP- dependent reaction [56]. This appearance is unusual and makes Hsp60s easy to identify by electron microscopy, while the oligomeric form of Hsp10 is a single ring of seven subunits [57].

Plant chloroplast chaperonins consist of two distinct polypeptides, Cpn60 α and Cpn60 β , which share 50% amino acid identity [34]. The major chaperonin in plant is RBP which is localized in chloroplasts and interacts with Hsp20 (the cofactor of Hsp60) in folding of Rubisco subunits and also facilitates Rubisco assembly. RBP is no homologous proteins in any other eukaryotic cellular compartment. [10,44].

Mitochondrial high-molecular weight Hsp60 complex interacts with newly imported (unassembled) proteins. Release of the folded protein occurs in the second step which requires another component (the homologue of *E.coli* GroES) and ATP. It is surprising that Hsp60 increases more than two - to threefold under stress conditions [4].

smHsps

Small Hsps are the most diverse and abundant group of Hsps synthesized by plants that function as molecular chaperones [21,25,42]. They are found ubiquitously in response to heat and other environmental stresses such cold, drought, salinity and oxidative stress [20,21]. In contrast to Hsp70 and Hsp60, which are present both constitutively and at stresses, higher plants express predominantly (up to 1% of the total proteins) smHsps in response to extreme conditions and only certain smHsps are present in the absence of stress during restricted stages of development [4,27]. Although the role of these proteins has not been demonstrated definitively a number of studies indicate that they are required for normal cellular function [25]. There is published data that especially in wheat some smHsps are detectable at low levels in control tissue, i.e. not only during stress conditions [28]. Likewise, it was suggested that smHsps have a widespread occurrence in optimal growth conditions in seeds and flowers and that their expression is developmentally regulated. These data emphasize the potential role of smHsps during reproduction [46]. Some authors suppose that the high diversification and abundance of plant smHsps probably represent molecular adaptive mechanism to stress that is unique to plants [20,25].

All smHsps have a conserved 90-amino-acid C-terminal domain (highly hydrophilic domain) called the α -crystallin domain, by analogy to the domains from α -crystallin proteins of the eye lens [4,20]. It was observed in wheat smHsps (TaHsp16.9C-I and TaHsp17.8C-II) that N-terminal residues have substrate specificity and contribute to substrate interactions [51].

Plant smHsps are divided into six multigene families and each of them encodes proteins found in a distinct cellular compartments, whereas other eukaryotes have only a single class of smHsps [20,21,27]. Two families are found in the cytoplasm (Class I and Class II), other three are organelle-localized in the mitochondrion, chloroplast and ER and one- in the membrane [20,22]. It was found in pea plants that mitochondrion localized smHsp (Hsp22) can reach 1 to 2 % of total matrix proteins. It is a nuclear-encoded protein de novo synthesized by heat shock in plants [48]. All other eukaryotes contain far fewer smHSPs compared to higher plants and only in these organisms there is evidence for organelle-localized smHsps [4].

Plant smHsps monomers are around 12-40 kDa and *in vivo* form large oligomers with size of 200 to 240 kDa. It is established that these structures are necessary for their chaperone functions [21,25]. Small Hsps have an ability to bind proteins with non-native conformation (probably through hydrophobic interaction in an ATP-independent manner), stabilizing and preventing them from an irreversible aggregation during stress, thereby facilitate their subsequent refolding by ATP-dependent chaperones [19,20,22]. In general, smHsps are not able to refold denatured proteins by themselves and for the realization of their chaperone activity they need co-operation with Hsp70/Hsp40 molecular chaperone system, that requires ATP [25,42]. Thus, smHsps play an important role in plant-acquired stress tolerance and, in particular, for the development of thermotolerance [4,20,27]. For example, aggregation-preventive activity of Hsp18.1 from pea cooperates with the protein-folding activity of Hsp70 system to reactivate heat denatured proteins [22]. Other researchers found that the gene encoding mitochondrial smHsp in tomato (MT-sHSP) enhances thermotolerance in tobacco plants transformed with the above-mentioned gene [49]. Additionally, there are proofs that methionine-rich chloroplast smHsp from tomato plants protects photosystem II during heat stress [47]. Similar expression pattern between *Arabidopsis* and tobacco to combined drought and heat shock stress was described and induction of a unique genetic program upon drought and heat shock combination was observed. These researches established that small molecular weight Hsp18 can be synthesized in tobacco and *Arabidopsis* plants in a strongly elevated level only in cases of combination between withdrawing of water and high temperatures (not during individually applied heat or drought stresses). This fact suggests that Hsp18 maybe acquires stress tolerance especially against mentioned combined stress [1,14].

Hsp90

Proteins of the Hsp90 family are found in the cytosol, the nucleus, the plastids, the mitochondrias and in the ER of eukaryotic cells. They have molecular weight range from 80-94 kDa [20,37]. Hsp90 are chaperones that are engaged in late phases of protein folding-they have to bind and maintain proteins which await final conformational changes making them active as signal transducers [8]. The major role of Hsp90 (see Figure 1) is to manage protein folding but it also participates in signal-transduction networks, cell-cycle control, protein

trafficking and degradation (Hsp90 interacts with the 26S proteasome and plays role in its assembly and maintenance). Hsp90 molecular chaperones require ATP for their functions and act together with Hsp70 and co-chaperones [20]. It is established that typical protein folding requires binding with Hsp70 and only then transferred from Hsp70 to Hsp90 [8].

Hsp90 is distinct from other chaperones because its substrates are signal transduction proteins (steroid hormone receptors and signaling kinases) [8]. This Hsp maintains hormone receptors in specific conformation until the cell receives appropriate receptor-activating signals [4]. Thus, Hsp90 acts as molecular chaperone but unlike typical chaperones, Hsp90s may have specific and long-lived interaction with particular target proteins [37]. In the *Arabidopsis* genome Hsp90 family includes cytoplasmic localized members (from AtHsp90-1 to AtHsp90-4), plastid (AtHsp90-5), mitochondria (AtHsp90-6) and ER (AtHsp90-7) localized members. Hsp90 chaperones are constitutively expressed in plants but their expression increases in response to different kind of stresses [20]. The level of cytoplasmic Hsp90 protein is strongly induced during heat stress, but it is not induced at very high temperatures. Maximal expression Hsp90 occurs at moderate stress [4].

Hsp100

Hsp100/Clp chaperones are members of the large AAA family of ATPases, also known as Clp (caseinolytic protease proteins) and function in protein disaggregation and degradation (see Figure 1) [26]. The family is divided into two classes. The members of Class 1, such as ClpA and ClpB, contain two distinct but conserved ATP-binding domains or NBDs (Nucleotide Binding Domains), whereas the members of Class 2, such as ClpX and ClpY, have a single ATP-binding domain [20,52]. Hsp100/ClpB proteins, presented in higher plants, are typically hexameric rings, and they function as molecular chaperones in cooperation with the Hsp70 and smHsp chaperone systems and play an essential role in thermotolerance [32,52]. The Hsp100/Clp is able to interact with smHsps to resolubilize the aggregated proteins and releases them in a state that can be refolded from the Hsp70 system [19,30,31]. Hsp100/Clp proteins have been reported as constitutively expressed in plants but their expression can be induced by stresses as well [20]. One subfamily ClpB is inducible by heat. The plant Hsp100/ClpB subfamily can be divided into a few subgroups (according to phylogenetic relation or localization), which different members can be involved in response to different stresses. This fact already was reported for *Triticum aestivum*, *Arabidopsis*, soybean, tobacco, rice and maize, whose Hsp101 can be cytosolic or nuclear members of the ClpB family [52]. In all these case Hsp101 is involved in protein disaggregation and/or degradation and prevent protein aggregation and misfolding in a cooperation with Hsp70 chaperone system [20,21,30]. There is genetic evidence for the thermoprotective role of these Hsps [4,20,35].

Development of the thermotolerance

The thermotolerance is one of the major acclimation processes in plants and can be defined as the ability of organisms to survive at lethal high-temperature if they are first subjected to a pretreatment at nonlethal but high temperatures. It is correlated with decreased synthesis of the majority of normal proteins and with increased synthesis of the heat shock proteins that confer thermotolerance [7,33,37]. This protective effect appears to be transient

[45]. Plants respond to high temperature stress by synthesizing an assortment of Hsps derived from different size groups. One of them includes high molecular weight Hsps groups ranging from 68 to 110 kDa (Hsp70, Hsp100, Hsp110), while the other one is represented by low molecular weight Hsps ranging from 15 to 27 kDa /referred to smHsps/. Many researchers found that the low molecular weight Hsps have an essential and adaptive role in thermal tolerance, especially in plants and their synthesis is the most prominent and unique anti-heat mechanism here [39, 41]. There are many proofs for thermoprotective role of smHsps in crops. The example I would like to point out is the thermal tolerant variety wheat called Mustang in which unique Hsps with molecular weight 16, 17 and 26 kDa are established and which Hsps are absent in the more thermal sensitive variety Sturdy [39]. Likewise, it was demonstrated in tobacco that a Class I cytosolic small heat shock protein gives thermal tolerance [43].

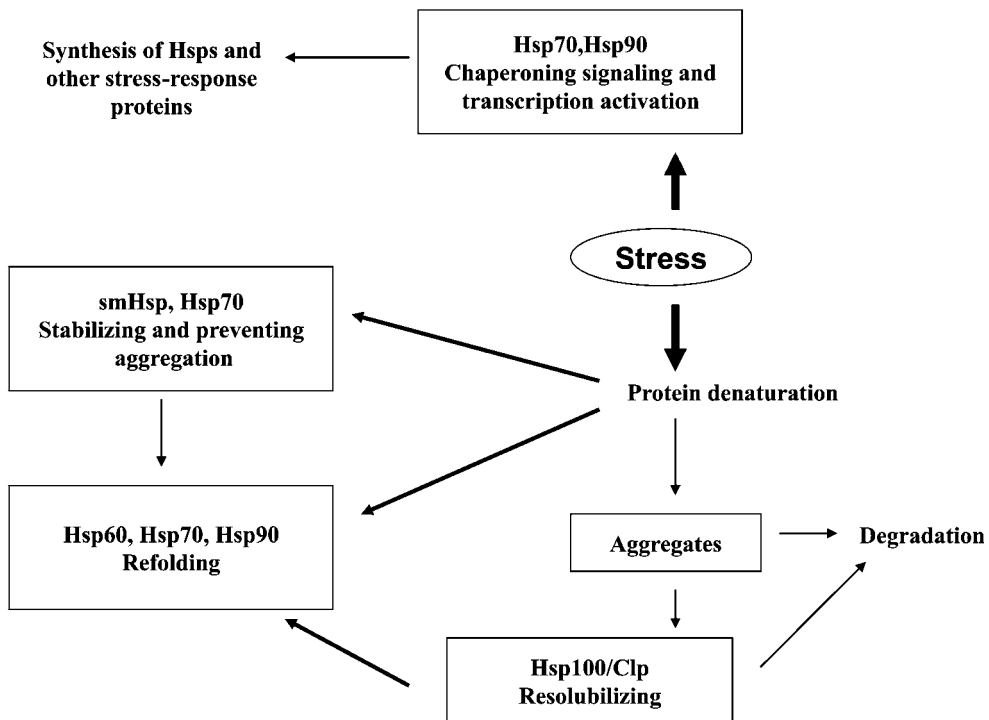


Figure 1. This figure shows the united chaperone network of Hsp families and their functions during abiotic stress in plants. Stress leads to denaturation of structural and functional proteins. Denatured proteins can be stabilized and maintained in a competent state for subsequent refolding, which is realized by Hsp60, Hsp70, Hsp90 families. In cases when non-native proteins are aggregated, new-formed aggregates can be resolubilized by Hsp100/Clp and in one next step- can be refolded by Hsp60, Hsp70 and Hsp90 or degraded by protease. Hsp70 and Hsp90 have also regulatory functions which are expressed under accompanying the signal transduction and transcription activation. That leads to the synthesis of different Hsp members and other stress-proteins.

It should be noted that some of Hsps are necessary but may not be sufficient for thermotolerance in plant cells. It was established that the amount of low molecular weight Hsps enhances repeatedly under heat stress but they alone are not able to refold the newly-

formed denatured proteins. They have to implicate Hsp70 chaperone system in stabilizing the non-native proteins and after that, in many cases, the complex smHsp/Hsp70 together with the Hsp90 chaperone system manage to assist the refolding of non-native proteins [20].

Based on many reports about the relation between Hsp synthesis and provocation of the thermotolerance it would be interesting to evaluate the potential use of Hsps as a biochemical selection criteria in improving plant tolerance in hot environments.

Scheme of Hsp network

The different classes of Hsps do not act independently in the cellular protection and in the maintaining of protein homeostasis. They work in cooperation and sometimes their roles overlap under both normal and stress conditions. Figure 1 shows the simplified scheme of the Hsp network in plant cell according to Wang Wangxia et al. (2004). During different kind of stresses such as drought, heat, cold, salinity, etc. many structural proteins and enzymes undergo a process of denaturation. It was demonstrated *in vitro* that smHsps and Hsp70 bind these non-native proteins and prevent their aggregation, thereby they maintain them in a competent state for subsequent refolding, which is achieved by other group of Hsps including Hsp60, Hsp70 and Hsp90. In case that denatured or misfolded proteins form aggregates, they can be resolubilized by Hsp100/Clp and then refolded by the above-mentioned group of Hsps (Hsp60, Hsp70 and Hsp90) or degraded by proteases (see Figure 1). Some Hsps (such as Hsp70 and Hsp90) can interact with a number of signaling molecules as nuclear hormone receptors and kinases, therefore they also have regulatory roles. Accompanying signal transduction and transcription activation, they lead to synthesis of other members of molecular chaperones and other stress-response proteins [20]. It was shown in *Arabidopsis* that some Hsps are implicated in the antioxidative response in addition to their chaperone function [20].

Conclusion

Maintenance of cellular homeostasis in normal conditions and its recovery after applied stress are crucial for the vitality of plants. This process is even more interesting in plants than in other organisms because crops are immobilized to soil and do not have the possibility to avoid even some of the adverse conditions. Therefore, the understanding for molecular-chaperone function of Hsps as one of the major conservative mechanisms for stabilizing of homeostasis is quite significant for the generation of stress tolerant varieties.

The induction of Hsps is highly conserved response established in all species studied so far. This induction occurs not only during heat shock but also under other environmental stresses and also at certain stages of the normal development of plants. So, all five Hsp families discussed (Hsp70, Hsp90, Hsp100, Hsp60 and smHsp) are essential for the plant existence. In normal conditions Hsp60, Hsp70 and Hsp90 are the most active and they are responsible for protein folding of nascent polypeptides, assembly of functional structures, translocation to different cellular compartments and degradation of misfolded conformations. In extreme conditions the above-mentioned Hsps together with Hsp100 and smHsp can assist in stabilizing the structural (membraneous) and functional (enzymatic) polypeptides and in

following refolding of denatured proteins, resolubilizing of aggregates or degradation of non-native proteins. Special peculiarity of plants is the prominent induction of smHsps, whose diversity and abundance are supposed to be a major molecular adaptive mechanism in plants.

Generally, the individual Hsp from each Hsp family has specific function which is part of the great Hsp network. On the other hand, cross-talk between this molecular chaperone network and other stress-responses mechanisms in plants exists and their joint action and good synchronization provoke stress tolerance in plants. Thus, Hsps are one of the most important molecular anti-stress responses of the sessile plants.

Abbreviations

Hsp-Heat shock protein, ER-Endoplasmic Reticulum, PPI-peptidyl-prolyl *cis/trans* isomerase, PDI-protein disulfide isomerase, HSF-Heat shock transcription factor, HSE-heat shock element, Bip-Hsp70 located in the ER, RBP-Rubisco-binding protein

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Chapter XVIII

Different Mechanisms Responsible for Stress Resistance Operate in the Same Insect Order (Diptera)

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Abstract

Heat shock proteins (Hsps) provide cellular and whole body adaptation of animals to various adverse environmental conditions due to their diverse chaperone properties. Hsp70 is apparently the major player underlying biological adaptation in all organisms studied so far.

Therefore, in our experiments to analyze the patterns of heat shock system firing in different Diptera families we predominantly concentrated on the *hsp70* gene family expression at the transcription and translation levels. We have investigated several species in three families: Drosophilidae, Stratiomyidae and Chironomidae belonging to three suborders (Cyclorrhapha, Orthorrhapha and Nematocera).

All *Drosophila* species studied so far did not reveal any level of Hsps under normal physiological conditions but responded to heat shock (HS) by rapid synthesis of all Hsps. On the contrary, four Stratiomyidae species studied are characterized by extraordinary high concentration of Hsp70 in cells (phenomenon especially pronounced at the larval stage) under normal physiological conditions and hardly detectable in control but inducible synthesis of correspondent RNAs. Such a pattern, which implies high stability of Hsps in species belonging to this family, is probably responsible for wide range of Stratiomyidae species in various habitats, including highly aggressive ones. Preliminary

* These two authors contributed equally to this work.

studies of Chironomidae inhabiting cold running waters (larvae of two species in the subfamily Diamesinae) indicate that representatives of this subfamily exhibit high constitutive level of *hsp70* RNA in cells under normal conditions and seem to lose the ability to respond to heat shock treatment.

The data accumulated suggest different trends in the evolution of molecular adaptations to adverse environmental conditions occurring in the same insect order.

Introduction

The mechanisms of adaptation to unfavorable environmental conditions have long attracted attention of molecular biologists. The range of adaptations is extremely wide and includes behavioral reactions, as well as physiological and molecular-biological mechanisms elaborated in the course of long-term evolution. The range and significance of individual adaptive reactions should differ in different species and under various environmental conditions. A radically new approach to studying the adaptation under the extreme conditions was developed in our laboratory following the description in the 1970s of molecular consequences of the heat shock [1, 2, 3].

After the discovery of heat shock (HS) proteins in *Drosophila* in the middle of 1970s [1], active studies began into the structure and functions of proteins of this class, which were found in practically all studied species: from *Escherichia coli* to man [2]. In addition to heat shock, synthesis of HS proteins (Hsps) can be induced by a number of agents, including hypoxia, various chemicals, etc. [3, 4, 5]. At present, Hsps are classified according to their molecular mass; they are divided into several groups, which include a few homologues with similar molecular masses, structure, and functions. Expression of the genes encoding some Hsps is maintained in most organisms at the normal physiological conditions at a low level or is altogether absent and increases manifold in the presence of stress factors. Hsps encoded by the genes with such a pattern of expression are called inducible. Other genes encoding Hsp-like proteins and homologous to the inducible genes by 50-70% are expressed under the normal conditions at a sufficiently high level. These genes are called constitutive, although their expression may increase under the stress conditions [6, 7].

It was known for a long time that Hsps determine the capacity of cells to adapt to high temperature (up to a certain limit) and other damaging factors. It was shown in the experiments with high temperature effects on cells that preliminary moderate HS allows the cells to endure later a tougher heat shock, which is lethal without preliminary shock. This phenomenon was called inducible thermotolerance [8]. Some experimental data suggest that it develops due to the accumulation Hsps [4, 5]. Artificial increase in the level of Hsp70 provided by constitutive expression of the Hsp-containing vectors transfected into the cells is also accompanied by the formation of a thermoresistant phenotype [9]. On the contrary, inhibition of Hsp synthesis or directed mutagenesis of some genes of the *hsp70* family lead to a decreased thermoresistance of cells [10, 11].

The main function of Hsps in the case of stress is to prevent aggregation and restore the native tertiary structure of denatured cytoplasmic proteins, whose concentration sharply increases with the temperature. Under the normal conditions, some Hsps families, specifically, Hsp70, provide for regular packaging of the polypeptide chain of newly-

synthesized proteins. They also play an important role in the transport of proteins into various organelles, are involved in the regulation of activity of many enzymes, and can regulate such important physiological process as apoptosis [5, 12]. Because of their capacity to bind to hydrophobic regions of denatured and newly synthesized proteins, Hsps and, first of all, Hsp70, were called “molecular chaperones” [13].

It is well known that species differences in heat resistance commonly match geographical (climatic) distribution of the species [14, 15, 16]. On the other hand, species from the same location can differ significantly in the heat shock resistance, and this may allow species to occupy different habitats [14, 15].

Comparative research of molecular mechanisms underlying adaptation to thermal extremes using different species even in the same order is apt to justified criticism [17], which is often completely ignored. Indeed, it is usually difficult to state with certainty that similar molecular mechanisms (e.g. patterns of Hsps expression) observed in the species compared represent an adaptation to thermal extremes that evolved independently in the course of life in thermal environment. Another equally plausible possibility is the presence of genetic background in the independent ancestors of compared species enabling them to invade similar ecological niches and thrive under unfavorable conditions.

Although it has been demonstrated in many studies that Hsp70 exhibits extremely high conservatism both at nucleotide and amino acid levels [18], herein we show that evolution of the structure of the whole *hsp70* cluster can be strikingly different even in related families whose species dwell under conditions that sharply differ in average temperature. Moreover, molecular mechanisms regulating Hsps synthesis and multiple functions of these most interesting proteins in the cells may be also different even in the species of the same order.

High Conservatism is not a Common Feature of *Hsp70* Gene Cluster in Different *Drosophila* Species

Previously we described characteristic differences in the thermoresistance and Hsp synthesis in various organisms including lizards, leishmanias, flies etc. [19-23]. Striking differences observed in Hsp synthesis in response to HS in species of different origin could be due to different molecular mechanisms. It is possible to suggest that thermoresistant species may contain higher copy number of *hsp70* genes as compared to species from cold and temperate climates. However, Southern blot analysis of the genomic DNA has shown that lizard species sharply different in the constitutive level and kinetics of Hsp70 synthesis in response to HS not only preserved the same number of *hsp70* genes but also retained their practically identical structure (Figure 1). It is noteworthy that the species compared belong to different subfamilies diverged millions years ago.

The same is true for *Drosophila melanogaster* Meigen, a cosmopolitan species in which multiple laboratory strains and various geographical populations studied exhibit amazingly uniform number and structure of *hsp70* gene copies [24].

Contrary to this, when studying the *virilis* group of *Drosophila* by Southern blot analysis we have encountered striking differences in the *hsp70* copy number not only between the cosmopolitan high-altitude species *D. virilis* Sturtevant and closely-related northern species

D. lummei Hackman but also between the geographical strains belonging to both the species (Figure 2). Subsequently we have cloned and determined the detailed structure of *hsp70*-containing gene loci [23] in both species studied. Interestingly, strains of the northern thermosensitive species (*D. lummei*) contain significantly less copies than strains of the southern thermophilic species (*D. virilis*). Schematic illustration of the evolution of *hsp70* genes in the *virilis* group is depicted in Figure 3.

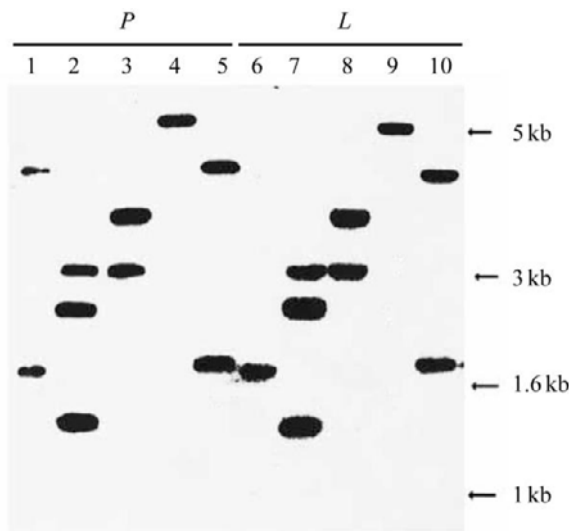


Figure 1. Southern blot analysis of genomic DNA from *Phrynocephalus interscapularis* (P) (lanes 1 – 5) and *Lacerta vivipara* (L) (lanes 6 – 10). Total lizard DNA was digested with *EcoRI* (lanes 1 and 6), *HindIII* (lanes 2 and 7), *PstI* (lanes 3 and 8), *BamHI* (lanes 4 and 9) or *EcoRV* (lanes 5 and 10) and subjected to Southern blot analysis using *Xenopus laevis hsp70*-containing clone as a labeled probe.

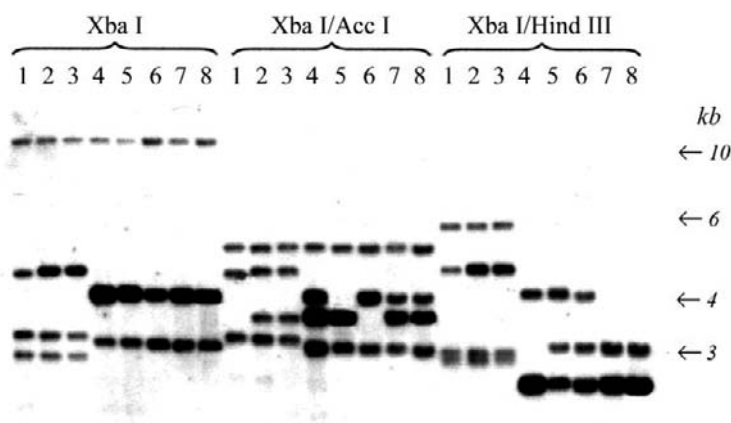


Figure 2. Southern blot analyses of genomic DNA from different *D. virilis* and *D. lummei* geographical strains. Total genomic DNA was subjected to Southern blot analysis using *D. virilis hsp70*-containing clone as a labeled probe. 1 – *D. lummei* 200; 2 – *D. lummei* 202; 3 – *D. lummei* 1102; 4 – *D. virilis* 160; 5 – *D. virilis* 1433; 6 – *D. virilis* T-53; 7 – *D. virilis* 9; 8 – *D. virilis* T-40.

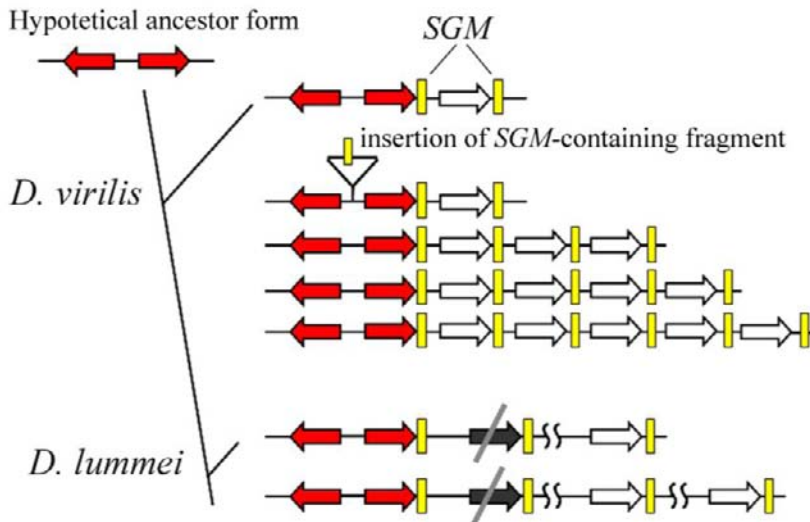


Figure 3. Hypothetical scheme illustrating the evolution of *hsp70* cluster in close species of the *virilis* group of *Drosophila*. The insertion of SGM into vicinity of *hsp70* genes taking place in the presumptive intermediate form resulted in the polymorphism observed in terms of copy number in the species comprising the group. The insertion of SGM-containing 3.6 kb fragment observed in one strain of *D. virilis* is indicated. Pseudogene described in *D. lummei* *hsp70* cluster is shown by crossed black arrow.

Note that the comparison of thermoresistance and Hsps synthesis in response to HS in the geographical strains of *D. virilis* and *D. lummei* did not reveal significant dependence on the temperature of their habitats. All strains of the same species were characterized by similar responses to HS as concerns both thermoresistance and Hsp synthesis irrespective of whether they originated from the southern or northern part of the species range [21].

It is possible that the patterns observed are not universal because in a lowland population of *D. buzzatii* Patterson & Wheeler regularly exposed to high temperature in nature exhibits a lower expression level at a temperature that generates a strong response in the highland population of the same species that rarely encounters extreme temperatures [25].

Role of Mobile Elements in the Evolution of *Hsp70* Gene Cluster in the *virilis* Group of *Drosophila*

In the course of molecular investigation and sequencing of the *hsp70* gene cluster in *D. virilis*, *D. lummei* and other species belonging to the *virilis* group (e.g., *D. montana* Stone, Griffen & Patterson), we have found SGM mobile element [26] at the 3' flanking regions of all copies of the *hsp70* genes comprising the cluster. Moreover, we have detected "fresh" insertion of SGM between inverted copies comprising the *hsp70* cluster in one particular strain of *D. virilis* (Figure 3). This observation favors the conclusion that the process of SGM amplification and transposition is still operating in *D. virilis* at the present time [27]. Ubiquitous occurrence of SGM at the same position in all *hsp70* copies of the cluster in all *virilis*-group species studied enables us to suggest the important role of this mobile element in the evolution of the whole cluster in this group. Probably pairing and unequal crossing

over occurring in functionally insignificant SGM sequences located close to the 3' end of *hsp70* copies represent the molecular mechanism underlying the differences in the *hsp70* copy numbers observed between different geographical strains of the *virilis*-group species and between the sibling species belonging to this group (e.g., *D. virilis* and *D. lummei*) as well. Apparently natural selection subsequently regulates the differences in the *hsp70* copy number depending on the environmental conditions. Characteristically, most of strains of the southern thermophilic species (*D. virilis*) carry significantly more *hsp70* copies than the strains of northern thermosensitive one (*D. lummei*). Figure 3 depicts a scheme illustrating the localization of SGM in the *hsp70* cluster in the two species mentioned above as well as presumptive ancestral arrangement of the cluster.

Role of Transposable Elements in the Regulation of Hsp70 Genes Expression

Note that adaptations to the extreme conditions are far from always achieved by a simple increase in the number of copies of certain *hsp* genes. Several years ago, experiments on transgenic *D. melanogaster* lines with an artificially increased number of copies of *hsp70* genes (twelve instead of usual six) were carried out at the Chicago University [28]. Although such flies were more thermoresistant at some stages, as compared to the control, at other developmental stages, superexpression of *hsp70* could be harmful and increase lethality. It is evident that the number of *hsp* genes and their position in the genome are important adaptive characters and are under the strict control of natural selection. It is also known that a very high homology (practically, identity) of copies of *hsp* genes within the cluster is achieved by means of the conversion mechanism [29].

On the other hand, switching off a part of the *hsp* genes may in some cases be adaptively advantageous, and we described one of the mechanisms providing for such “switching off”. When studying *D. melanogaster* line T originated from a desert in Central Africa, we encountered some unexpected facts. This line was capable of reproducing at 31°C, while other lines of this species become sterile at this temperature. Line T was kept at the St. Petersburg University for many years at 31°C without a noticeable decrease in fertility. Analysis of Hsp70 and correspondent RNA gave quite unexpected results. The level of synthesis of Hsp70 and its RNA was significantly lower in this strain after HS, than in Oregon R [30]. Later it was shown that one copy in the *hsp70* cluster contained a mobile element *Jockey*, which apparently led to its deactivation and reduced synthesis of RNA encoding Hsp70. The arrangement of *hsp70* gene locus in Oregon R and strain T is shown in Figure 4.

It is noteworthy that in other cases as well, long-term cultivation of various *D. melanogaster* lines at an elevated temperature in the laboratory led to switching off some copies of *hsp70* genes due to incorporation of a certain mobile element [31, 32].

Apparently, when flies are under constant conditions of “weak” HS, switching off a part of *hsp70* genes may be adaptively advantageous.

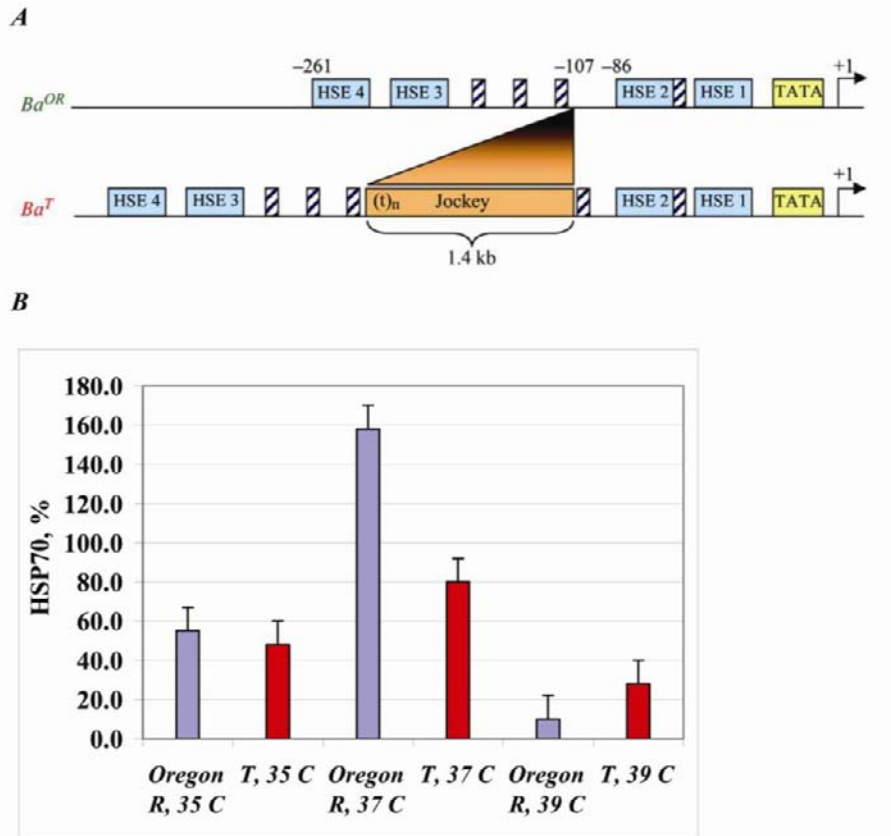


Figure 4. A. Structure of regulatory region of *hsp70Ba* copy in *D. melanogaster* (Oregon R wild type strain) and in strain T (African strain able to live at 31°C). 1.4 kb fragment corresponding to the 3' end of the *Jockey* retroelement is inserted 107 bp upstream of the *hsp70Ba* transcription start site in the T strain. The orientation of *Jockey* is inverted with respect to the *hsp70Ba* gene. The insertion intervenes between HSE 2 and HSE 3, displacing HSE 3 and HSE 4 as well as three GAGA elements shown by hatched columns. B. Levels of inducible Hsp70 concentrations in Oregon R and T strains of *D. melanogaster* depending on the temperature of heat shock treatment.

Regulatory Regions of Heat Shock Genes Represent Hot Spots for Transposon Insertions

Finding the spontaneous incorporation of mobile elements predominantly in promoter regions of the *hsp* genes [31-35] makes them “hot” sites in mutagenesis caused by the transposition of mobile elements. In order to test this hypothesis directly, we used the system developed for studying the incorporation of a construct designed on the basis of *P*-element in the promoter area of low molecular weight *hsp* genes [36]. It is known that *P*-element (the most extensively studied mobile element of *Drosophila*) moves predominantly into the regions of chromosomes located in the neighborhood of preexisting copies. The phenomenon was called “local jumps”. The expression of this construct is dose-dependent and allows distinct detection of new transpositions basing on the eye color. We obtained two *D. melanogaster* lines carrying the necessary marked constructs near locus 87A containing an

inverted repeat of two copies of the *hsp70* gene (Figure 5). Combination of genetic and molecular-biological methods allowed us to analyze the frequency of *P*-element incorporation in the area of *hsp70* genes [37]. To our surprise, all described transpositions of *P*-element in the area of these genes took place exclusively in the nucleosome-free promoter area of *hsp70* genes [38]. Moreover, there were a few “extremely high spots” at the level of specific nucleotides in this area (Figure 5C). Later extensive analysis of multiple natural populations of *D. melanogaster* has demonstrated that the localization of *P*-elements transposed into *hsp70* gene cluster is also restricted to the promoter region and very similar to that described in our “bombardment” experiments [35].

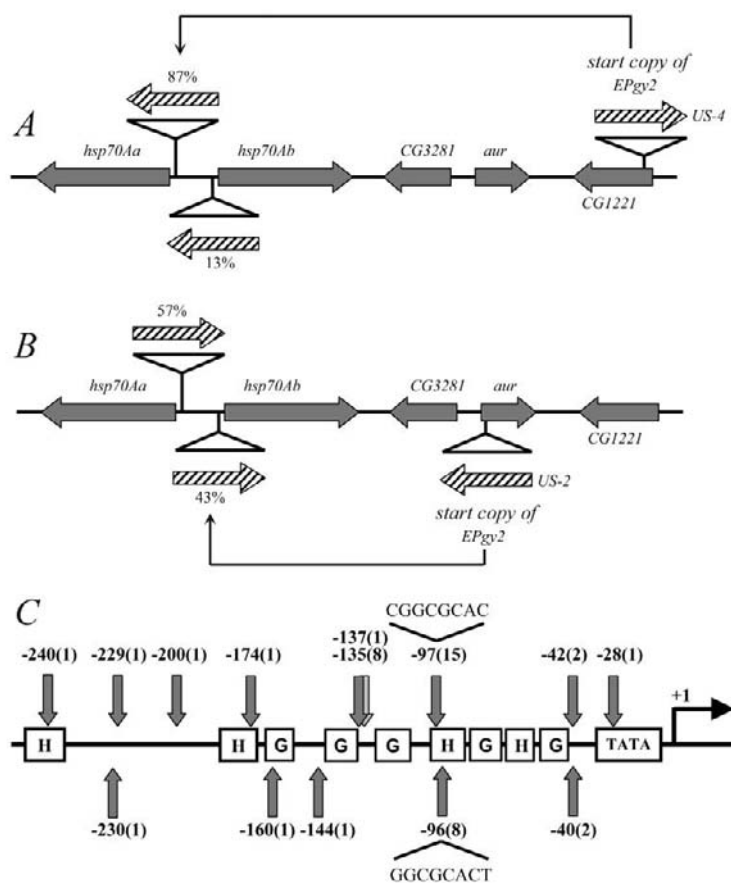


Figure 5. A and B. Organization of *hsp70Aa* and *hsp70Ab* loci in *D. melanogaster*. Localization of the start construction *EPgy2* in strains US-4 (A) и US-2 (B) is indicated by hatched arrows. Arrows show the transcription direction. The frequency of insertions detected in the progeny of both strains in percents is indicated for each strain. All insertions have been found only in the regulatory regions of *hsp70* genes. C. Insertion sites for local transpositions of the *EPgy2* construction into promoter region of *hsp70Aa* and *hsp70Ab* of *D. melanogaster*. Numbers before parentheses refer to nucleotide position relative to transcription start; numbers of independent transpositions into the indicated nucleotide are in parentheses. Approximate positions of heat shock (H) and GAGA (G) elements and the TATA box are indicated. Target site duplications are provided for “hot spots” located at -97 and -96 nucleotides. (Reproduced with permission from Shilova et al. 2006 [37]).

The data accumulated suggest that the promoter regions of *hsp70* genes are indeed “hot” sites for *P*-element transpositions, and we developed a simple scheme for the directed *P*-induced mutagenesis of the promoter regions of *hsp70* genes. It is a challenge to understanding why representatives of other multiple transposable elements TE families existing in *D. melanogaster* genome were rarely detected in the promoters of *hsp*s genes.

Different Evolutionary Trends in the Regulation of Expression of the *Hsp70* Cluster Providing Adaptation to Adverse Environmental Conditions

Protein and DNA studies usually start with *Drosophila* species as we can exploit the knowledge of their sequenced genomes. However, as a next step to assess the generality of patterns observed, one should also investigate non-drosophilids.

In order to compare the regulation of *hsp70* genes expression in *Drosophila* and in the species from nature belonging to the same order (Diptera), we have studied representatives of two families of Diptera other than Drosophilidae, namely, Stratiomyidae and Chironomidae. In Stratiomyidae, we have chosen species with (semi)aquatic larvae that exhibited striking differences in the temperature, mineralization, pH, and in other major characteristics of larval habitats. These species are as follows: *Stratiomys japonica* van der Wulp, whose larvae inhabit a hot volcanic spring of Kunashir Island representing one of the most aggressive environments in the Earth; *S. singularior* (Harris) and *Nemotelus bipunctatus* Loew, whose larvae develop in heated at summer shores and shore pools of salt lakes in the Crimea; and *Oxycera pardalina* Meigen, whose larvae develop in moss at margins of cold springs and streams in the environs of St. Petersburg. In spite of dramatic differences in the average temperature of larval habitats of these species (up to 40°C and higher for immature *S. japonica*, *S. singularior* and *N. bipunctatus*, and 4–10°C for *O. pardalina*) the species studied are characterized by rather similar LT50 (a measure of basal thermotolerance, is the temperature of a 30-min heat shock resulting in 50% mortality) and critical temperatures (i.e., the temperature of HS treatment resulting in less than 1% survival) [39].

In our research we used monoclonal antibody specific to *D. melanogaster* inducible Hsp70 (antibody 7FB) and antibody recognizing entire Hsp70 family including Hsp70, Hsp68 and HSC (antibody 7.10.3). The antibodies were kindly gifted by Susan Lindquist (University of Chicago).

The immunoblotting showed that all species belonging to Stratiomyidae family are characterized by high levels of inducible Hsp70 at larval stages, which does not depend on environmental temperature (Figure 6B). The adults also exhibit significant but lower level of Hsp70 under various thermal conditions. Temperature elevation (HS) may induce the synthesis of *hsp70* mRNA, which is almost undetectable in the individuals under normal physiological conditions (Figure 6A). Interestingly, maximum level of Hsp70 in Stratiomyidae species was observed in 48 hours after HS, while in *Drosophila* species, maximum levels of Hsp70 is usually reached in 6–12 hours after HS. Therefore, we showed that HS induces synthesis of rather stable Hsp70 in Stratiomyidae species. Under normal physiological conditions, all species of this family are characterized by constitutive high levels of Hsp70 in their cells probably due to hardly detectable leakage of respective genes [39]. The difference in constitutive levels of

Hsp70 between larvae and adults is probably due to the fact that larvae live in more aggressive environment in comparison with adults. In contrast to the larvae, adults of Stratiomyidae are not subject to severe conditions of aggressive aquatic environments, and their survival depends to a smaller degree on continuous expression of Hsps [39]. Similar but even more pronounced developmental dichotomy in terms of *hsp70* RNA levels was also observed for Antarctic chironomid midge [40].

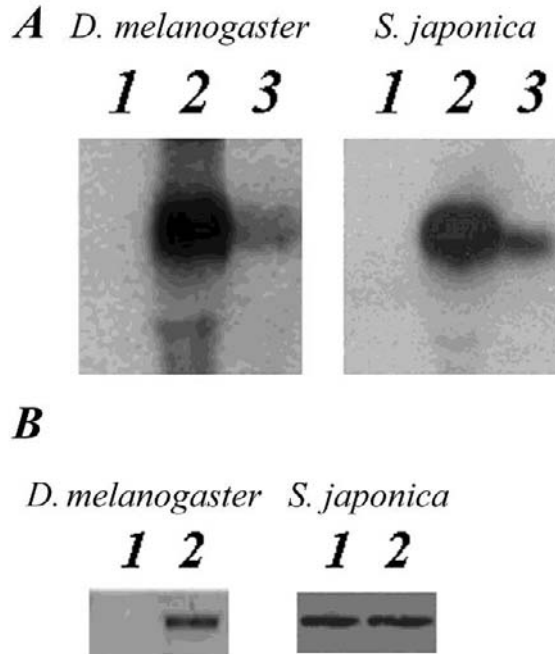


Figure 6. A. Northern blot hybridization of total RNA with 32P-labelled probe in controls 25°C (1), and in response to heat-shock treatments (2 – 37°C for *D. melanogaster* and 42°C for *S. japonica* for 30 min). Levels of RNA after heat shock treatments as in (2) and 24 hours of recovery at 25°C (3). B.. Western blotting of proteins separated by one-dimensional electrophoresis with 7FB antibodies recognizing only the 70-kDa inducible Hsp70 family member (1 – control, 2 – HS 37°C for *D. melanogaster* and 42°C for *S. japonica* for 30 min).

Larvae of two species of the Chironomidae subfamily Diamesinae, *Diamesa* sp. and *Pseudodiamesa* gr. *nivosa*, were collected abundantly from the same cold spring habitat and locality as the larvae of *Oxycera pardalina* (Stratiomyidae). The Diamesinae larvae exhibit highly specific pattern of *hsp70* genes expression. Contrary to all species of other Diptera families mentioned above, in these Chironomidae species we detected high constitutive level of *hsp70* mRNA in the larvae under normal physiological conditions. Moreover, heat shock treatment was ineffective in the induction of Hsp70 at either transcription or translation levels (MS in preparation). These chironomids are specialized inhabitants of cold running waters, as well as most other species of the subfamily Diamesinae. Probably, this group of midges has lost adaptations to higher and variable temperatures typical of other dipterans investigated. Likewise, the larvae of another Chironomidae species mentioned above (Antarctic midge

Belgica antarctica Jacobs belonging to the subfamily Orthoclaadiinae) also exhibit constitutive up-regulation of *hsp* mRNA and exposure to high temperature fails to induce *hsp70* expression [40].

Heat Shock Proteins Synthesis and Thermotolerance in Diptera Species

It is widely assumed that species living under severe aggressive conditions for millions of years may elaborate, in addition to behavioral and morphological adaptations, also molecular-biological mechanisms, which make their normal life and reproduction possible under the extreme conditions. Numerous studies including our results favor the important role of Hsps and in particular the Hsp70 family in thermal adaptation of various animals [14-16; 43-46]. However, it is also clear that in different groups of animals the role of Hsps in thermal adaptation may differ. In our previous studies and above sections we described two different patterns of Hsps expression in the families belonging to Diptera. In all *Drosophila* species, the level of Hsps is not detectable under normal physiological conditions and increases dramatically as temperature rises [3, 41]. On the other hand, in the Stratiomyidae species studied we detected high constitutive level of inducible member of Hsp70 in the cells at normal temperature while HS treatment was able to induce only moderate increase in concentration of Hsp including Hsp70.

We decided to investigate the correlation between the Hsp70 concentration after different HS conditions and thermotolerance in the species belonging to the two families compared.

Comparative analysis has shown that there is a good correlation between the ability to synthesize Hsp70 at extreme temperatures and survival when studying *Drosophila virilis* group species (e.g., *D. virilis* and *D. lummei*) and species belonging to Stratiomyidae family (Figures 7 and 8). On the other hand, it is evident that all species of Stratiomyidae studied including *O. pardalina* dwelling in cold springs significantly exceed species of *virilis*-group in the survival at extreme temperatures (Figure 7A and 8A).

These results imply that although thermal history of the species definitely plays significant role in the adaptation to high temperature conditions, genetic constitution of the ancestor species underlying a specific pattern of Hsps expression and regulation probably plays the major role [39]. It looks like constitutive presence of Hsps in the cells of Stratiomyidae species represents more powerful adaptation to harsh conditions than fast induction of Hsps after temperature elevation typical for *Drosophila*. It is noteworthy that previously we and other authors described constitutive Hsps synthesis in various highly thermoresistant animals including lizards, ants and limpets [42-45].

Conclusion

In our previous studies we have carried out extensive analysis taking various animals from the nature with the aim to describe common molecular mechanisms based on Hsps expression underlying adaptations to high temperature conditions. A wide spectrum of arid zone and desert organisms including *Leishmania*, *Bombyx mori* L., flies, lizards, and mammalian species have been explored [19-23; 30; 42-43; 46-47]. Herein we demonstrate

that in spite of common mechanisms of molecular adaptation to extreme conditions there are striking differences even between the species belonging to the same order and at different

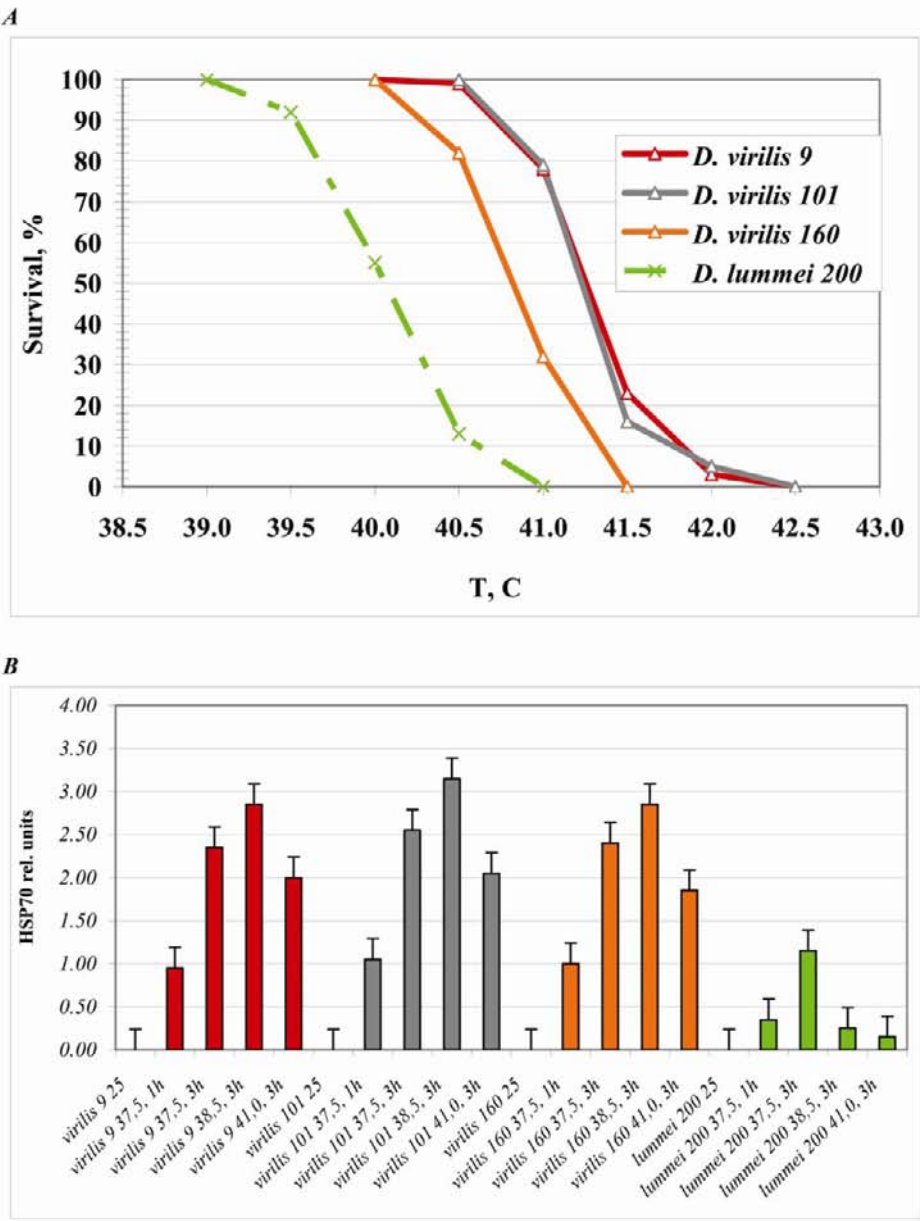


Figure 7. A. Inter- and intraspecific variation of basal thermotolerance (survival in % after HS) in two *virilis* group species after 30-min heat shock treatment. The temperatures of HS are given at the bottom of the figure. B. Concentration of Hsp70 in the species and strains compared due to heat shock conditions. All determinations are for equivalent amounts of total protein and are from immunoblots with 7FB antibody recognizing only the inducible members of Hsp70 family in *Drosophila*. Densitometry and data standardization have been performed as described [21]. Maintenance and heat shock temperatures (25, 37.5 etc.) and *Drosophila* strain numbers (9, 101, 160 etc.) are indicated.

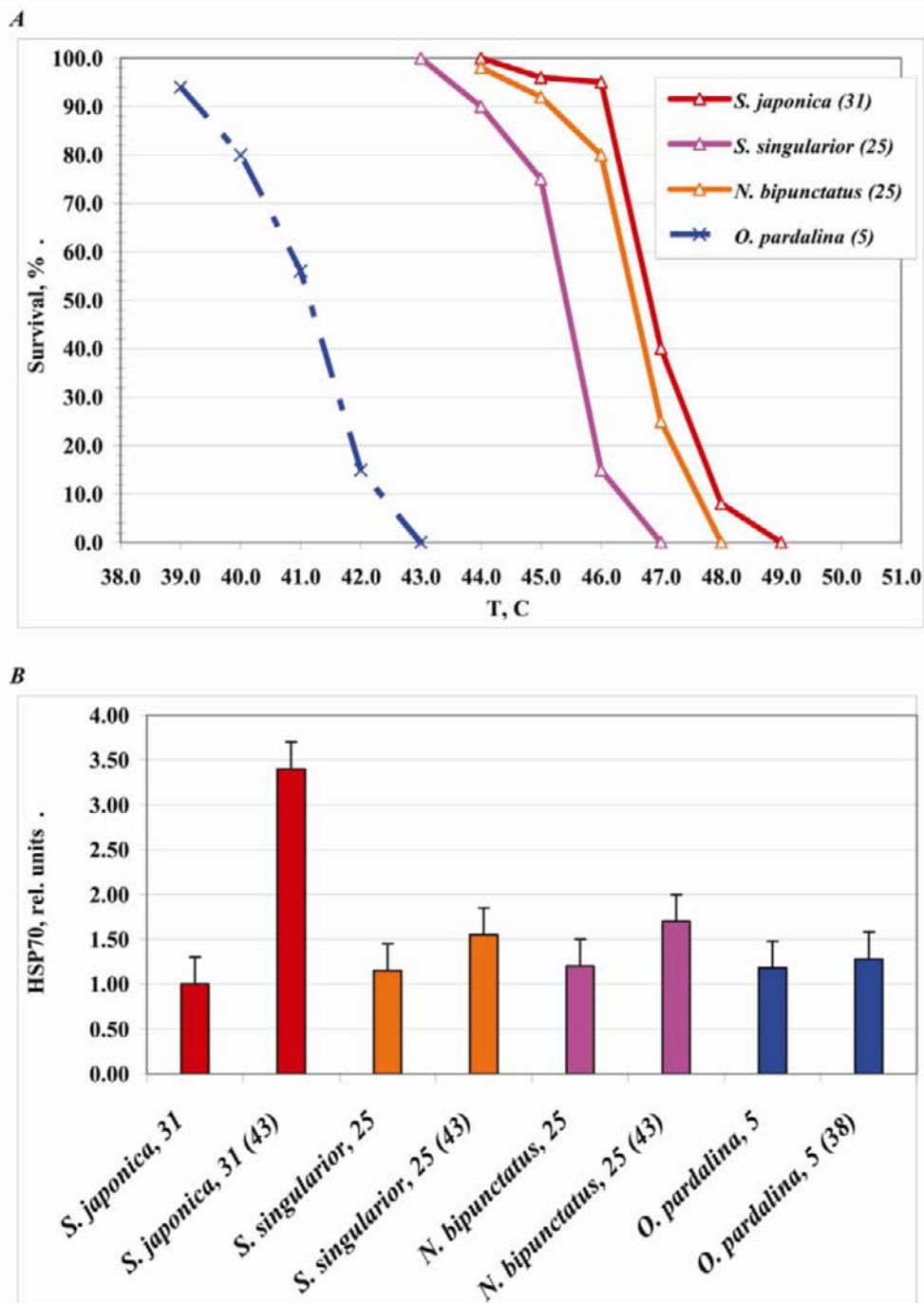


Figure 8. A. Interspecific variation of basal thermotolerance (survival) in Stratiomyidae species after 30-min heat-shock treatment. The temperatures of HS are given at the bottom of the figure. B. Hsp70 concentrations in the larvae of different Stratiomyidae species subjected to different temperatures. Maintenance and heat shock temperatures are indicated. HS temperatures are given in parenthesis.

stages of the life cycle (developmental dichotomy), where such mechanisms operate, as well as in the level of Hsps expression (activation, transcription, translation and stability of RNA or correspondent proteins), where such adaptation is realized.

We believe that long continued large-scale studies of the system of *hsp* genes in species that differ in the temperature of habitats will disclose general patterns of functioning and evolution of this system. The data accumulated will allow reinterpretation of genetic mechanisms, which should be taken into account in developing transgenic animals and selection of farm animals adapted to hyperthermia. The results of our studies may be used in developing approaches for directed mutagenesis of Hsps genes and regulation of functioning of this most important SOS-system of the eukaryotic cell.

Acknowledgments

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Chapter XIX

Bi-Directional Hsp Response in Homeothermic Livestock

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Abstract

Heat shock proteins (Hsp) are considered cellular protective agents against a wide range of stressors; inducible expression of Hsp prevents misfolded proteins from aggregation, interfere with the cell death program and help cells recover and survive.

In normal resting cells Hsp are involved in various "house keeping" missions in the cell, form complexes with client proteins and act as molecular chaperones. This includes protein folding, assembly and translocation between compartments, degradation of misfolded or aggregated proteins, maintenance of the cell cycle, interaction with steroid hormone receptors and signal transduction pathway for steroid hormone).

Over the last few years we have been focusing on various stress stimuli in homeothermic livestock, namely, environmental, nutritional and psychological. Our findings led us to hypothesize that upon stress, the Hsp response may be bi-directional regarding the type of stimulus and the tissue tested. While in some cases Hsp levels increase, in other instances it goes the other way round. In those situations (nutritional stress) where Hsp levels decrease, a concomitant induction of other genes, related to the function of the specific tissue has occurred. This is in marked difference from the classical Hsp response, in which, in parallel with the induction of Hsp the expression of other proteins is silenced.

Introduction

It is well established that various stresses confer hazardous impact on productive and reproductive efficiencies of homeothermic livestock. Among the leading deleterious stimuli,

elevated temperature (Yahav et al, 1997; Yu et al., 2008; Hansen, 2004), transportation (Bao et al., 2008; Snowden et al., 2007; Zhu et al., 2009) and food restriction (Cole and Hutcheson, 1990; Randel, 1990) are considered cardinal in promoting diminished performance in livestock.

Stress challenges protein homeostasis, and results in an increased flux of non-native proteins, which if left unprotected are prone to misfolding and aggregation (Morimoto and Santoro, 1998). The cellular protective traits of heat shock proteins (Hsp) against a wide range of stress stimuli have turned them the focus of enormous number of studies, among which the bulk of agricultural-oriented studies has also been growing.

Heat shock proteins are highly conserved molecules that are present in all eukaryotic and prokaryotic species (Schlesinger, 1990). Heat shock proteins are categorized into several families that are named on the basis of their approximate molecular weight. They are expressed constitutively, making up to 5–10% of the total protein content in healthy growth conditions, but their intracellular concentrations can be several folds increased by insults that induce protein unfolding, misfolding, or aggregation, and a flux of newly-synthesized non-native proteins (Pockley, 2003).

Most Hsp families contain stress-induced members in addition to the constitutively expressed members. Under normal conditions, Hsp function as intracellular molecular chaperones of newly synthesized polypeptide chains, preventing their aggregation during folding and subunit assembly and during the translocation of proteins across sub-cellular membranes to their appropriate cellular compartments (Gething and Sambrook, 1992).

Some Hsp are involved in the clearance of proteins that are improperly folded and proteins that are unfolded as a result of their decreased stability under conditions of cellular stress (for example, oxidation and high temperatures).

The expression of Hsp is primarily regulated at the level of transcription by a family of heat-shock transcription factors (Hsf; Morimoto, 1998), which interact with heat shock elements in the heat shock protein gene promoter regions (Morimoto et al., 1994). In vertebrates, four Hsf have been identified, of which Hsf1 and Hsf2 are ubiquitously expressed and conserved (Nakai and Morimoto, 1993; Sarge et al., 1991). Among the four members of the Hsf family, only the ubiquitous Hsf1 and the avian-unique Hsf3 are considered stress-responsive factors (Nakai and Morimoto, 1993; Nakai et al., 1995; Sistonen et al., 1994). In the absence of stress, Hsf1 exists either in the cytosol or nucleus in an inert monomeric state. Nonactive HSF3 is a dimer that is exclusively cytosolic. Upon heat stress, Hsf1 and Hsf3 are translocated into the nucleus and acquire DNA-binding activity (Nakai et al., 1995).

The term “heat shock proteins” is something of a misnomer, since in addition to raised temperature their expression is induced by various other environmental and pathophysiological conditions (Benjamin and Williams, 1994; Holbrook and Udelsman, 1994; Morimoto and Santoro, 1998; van Eden et al., 2005; Zhu et al., 2009).

It has recently been demonstrated in various mammalian species, including cattle that Hsp70 is present in the extracellular compartment (Kristensen et al., 2004; Fleshner and Johnson, 2005), and that various stressors result in increased levels of plasma Hsp70 (Johnson et al., 2005; Aneja et al., 2006). In spite of observations that extracellular Hsp70 facilitate innate immunity (Campisi et al., 2003; Fleshner and Johnson, 2005; Aneja et al.,

2006), and has stress-associated presence in cattle plasma (Kristensen et al., 2004), the impact of various stresses on its concentrations is yet to be determined.

Over the last few years we have been focusing on various stress stimuli in homeothermic livestock, namely, environmental, nutritional and psychological (Eitam et al., 2009; Shabtay and Arad, 2005; 2006). The current manuscript presents novel results along with published data, to summarize some of our knowledge in Hsp responses of livestock to common stress stimuli.

Material and Methods

Heat Shock Experiments

Twelve mature layer hen (5 months old) of the Leghorn breed participated in the heat shock study. Six chickens served as control group and 6 as experimental group. Experiment was carried out within a temperature-controlled room ($\pm 0.3^{\circ}\text{C}$; 12:12 light-dark cycle, lights on at 6:00 AM). The birds had free access to food and water and could freely move in their individual cages. The experiment started at 8:00 AM at an ambient temperature (T_a) of 24°C ($\text{RH} = 50 \pm 5\%$), which is within the thermoneutral zone of chickens. A copper-constantan thermocouple implanted dorsal to the rectum (Shabtay and Arad, 2005) served to determine, on line, changes in body temperature (T_b). At $T_b = 45^{\circ}\text{C}$ the experiment was terminated and the chickens were immediately sacrificed by decapitation. Tissues were collected and frozen in liquid nitrogen within 1 min from decapitation.

Transportation Stress Experiments

At the age of 4-7 days, 8 Holstein-Frisian bull calves, weighing 41 ± 3 kg, were 2h truck transported from their original dairy farm at Bet Dagan to the nursery in Newe Ya'ar, in two non-dependent transportation events within December 2008. Average ambient temperature of the two events was 19.5°C . From the day of arrival calves were placed together in a 180 m^2 roof-covered yard, and consumed milk replacer (125 g/liter; Top 440, Kofolk, Israel), although having also free access to suckling total mixed ration and water. Milk replacer was supplied to calves using computer-controlled suckling machine, equipped with a real-time algorithm embedded in a software application (Gavish, Israel), to allow individual milk allocations.

Prior to transportation and 1h, 5h and 1d post transportation blood was sampled (see RT PCR section) from the jugular vein and total RNA was isolated to follow changes in Hsp transcription.

Food Restriction Experiments

Six Simmental cows in the third month of gestation were used in this study. Cows were housed in individual pens. After a 2-weeks adaptation period to the individual pens control diet (containing 12% crude protein) intake was measured during one week, at the end of which subcutaneous fat biopsies were sampled under local anesthesia (Esrakain; Lidocaine hydrochloride 2%). Thereafter cows were adapted for a further 2 weeks period to the experimental, low protein diet (6% crude protein). The control and low protein diet were iso-caloric. Cows were maintained on this diet for 3 months, at the end of which subcutaneous fat biopsies were sampled again.

All procedures involving animals were approved by the Israeli committee for animal care and experimentation.

Protein Extraction

Total protein was extracted from brain and liver tissues, of control and Tb=45°C heat-shocked mature hens, as well as from cattle subcutaneous fat. The dissected brain and liver tissues and the fat biopsies were homogenized in ice-cold buffer, containing 0.1 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 20% glycerol (vol/vol), 0.5 mM DTT, 15 µg/ml leupeptin, 1 mM PMSF. Samples were centrifuged for 30 min (4°C, 12,000 rpm), and supernatants were collected, frozen in liquid nitrogen, and stored at -70°C until further analysis.

SDS PAGE and Western Blot Analysis

Whole-cell lysates from the cattle subcutaneous fat biopsies were boiled in sample application buffer containing 2-mercaptoethanol. Equal amounts of proteins (in each lane) were loaded and separated by SDS-polyacrylamide gel (10%), and transferred onto nitrocellulose membrane (Schleicher&Schuell). The membranes were probed with monoclonal anti-actin (Sigma), anti-Hsp70 (Sigma), anti-Hsp90 (Stressgen), anti-C2 and anti C9 proteasomal subunits (a kind gift from Prof. Chaim Kahana), followed by appropriate secondary antibodies. The proteins were visualized by enhanced chemiluminescence.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from liver and brain tissues by TRI REAGENT LS (MRC, Cincinnati, OH, USA), according to the manufacturer's instructions. 5 µg RNA were separated in formaldehyde-agarose gel and transferred onto nylon membrane (Zeta-Probe, Bio-Rad). Chicken Hsp90 and Hsp70 cDNAs were labeled by the extension priming method using [α -³²P]dATP. Membranes were hybridized for 16 h at 55°C, washed with 0.1% SDS in 1 X SSC at 45°C, 50°C, and 55°C, and exposed to X-ray film (Kodak BioMax MS) at -70°C in the presence of intensifying screen.

RT-PCR

RNA was extracted from cattle blood cells using High Pure RNA Isolation Kit (Roche). The concentration of RNA was measured by Nano-Drop (ND-1000) as well as its quality. Quality of total RNA was additionally estimated by non-denaturing agarose gel.

The RNA was stored in -80°C or immediately utilized for RT-PCR reactions using the Verso cDNA Kit (Thermo Fisher Scientific Inc). The T-Personal PCR machine (Biometra) was programmed as follows: 42°C for 60 min for the RT step followed by 94°C for 2 min, and the amplification steps of 94°C for 1 min, 60°C for 40 s, 72°C for 3 min. A master-mix was prepared and aliquoted to test tubes, each of which was amplified for 30 cycles. The RT-PCR reaction in Figure 2 was performed using the following pairs of primers for cattle Hsp90 α : the forward primer ACA AAG GTT ATT CTG CAT CTG A and the reverse primer GGC TTT GTC TTG TTC AGC TC amplified a 338 bp fragment. For actin, the forward AGGCCAACCGTGAGAAGATG and the reverse TGCGGTGGACGATGGAG primers amplified a 780 bp fragment.

Electromobility Shift Assay

Electromobility shift assay (EMSA) was performed as previously described by Mosser et al. (1988). Briefly, 20 μ g brain protein extractions were incubated with a ³²P-labeled double-stranded oligonucleotide (5-CTAGAAGCTTCTAGAAGCTTCTAG-3). The protein-bound and free oligonucleotides were electrophoretically separated by 4% native polyacrylamide gels. The gels were dried and autoradiographed.

Immunocytochemistry

Blood samples from control and Tb = 45°C heat-shocked mature hens were prepared as smears. The smears were air dried and fixed with 4% paraformaldehyde (in PBS). After blocking with 10% normal goat serum in PBS, cells were incubated with 1:500 diluted polyclonal Hsf1. A secondary goat anti-rabbit antibody, conjugated to FITC, was then used to detect the Hsf1 signal. Confocal reconstructions were made by using an MRC-1024, laser confocal scanning microscope (Bio-Rad) with the objective Nikon Plan Apo 603/1.40. DNA was counterstained with 250 ng/ml propidium iodide (PI).

Results and Discussion

Hsp Response to Elevated Temperature in Homeothermic Livestock

Raised ambient temperature is one of the stimuli that challenges protein homeostasis and thus may result in an increased flux of non-native proteins, which if left unprotected are prone to misfolding and aggregation (Morimoto and Santoro, 1998).

We tested the Hsp response in liver, brain and blood tissues of $T_b = 45^\circ\text{C}$ heat-stressed chickens, at the level of Hsf activation and mRNA transcription. As seen in Figure 1, increased T_b resulted in the activation of Hsf in the brain tissue (Figure 1A), and promoted Hsp90 α mRNA transcription (Figure 1B). Similarly, mature layer hens responded to increased T_b by a significant induction of liver Hsp70 mRNA (Figure 1B). Avian erythrocytes contain chromatin-condensed nucleus that is considered inert in terms of protein synthetic activity (Morimoto and Fodor, 1984). The data presented in Figure 1C reflect the capability of avian Hsf to translocate to the nucleus upon heat challenge. Physiologically, this capability is of great significance since the blood tissue, due to its thermoregulatory role in dissipating heat from the body, is exposed to temperature fluctuations and should thus be able to protect its “respiratory proteins” from denaturation.

Figure 1A

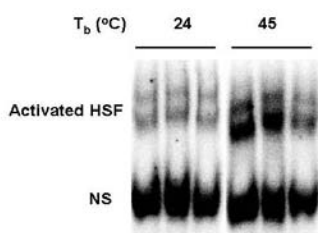


Figure 1B

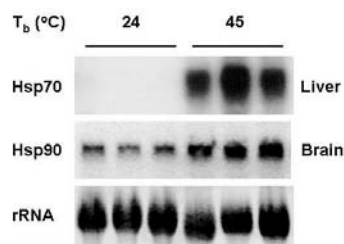


Figure 1C

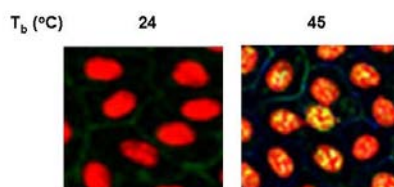


Figure 1. Hsp response of mature Leghorn layer hens under control ($T_b = 45^\circ\text{C}$) and heat shock ($T_b = 45^\circ\text{C}$) conditions. (A) Hsf–DNA-binding activity detected by EMSA. EMSA was performed with a [^{32}P]HSE oligonucleotide and whole brain tissue extracts. (B) Northern blot analysis of Hsp70 and Hsp90 in liver and brain tissues, respectively, detected by chicken Hsp70 Hsp90 α cDNA as a probes. Ribosomal RNA (rRNA) levels were used to compare the amounts of loaded RNA between lanes. Each lane represents a different individual. (C) Confocal microscopy images of control and $T_b = 45^\circ\text{C}$ blood cells. Blood smears were fixed, treated with RNase, and stained with PI for DNA visualization (red) and with polyclonal Hsf1 β , followed by a secondary antibody conjugated to FITC (green). The yellow appearance is a result of merge between green and red and indicates the translocation of Hsf1 into the nucleus. Please note the typical granular appearance of Hsf in the nucleus.

Hsp responses to increased T_a have been extensively studied in chickens (Shabtay et al., 2005; Shabtay and Arad, 2006; Yahav et al., 1997; Yu et al., 2008). Generally, it can be concluded from these studies that, exposure of chickens to high ambient temperature promotes an elevated expression of Hsp.

Lactating dairy cows are particularly sensitive to environmental hyperthermia because metabolic heat load, as a result of milk synthesis, is proportionate to production levels.

The involvement of Hsp in improving thermotolerance of bovine mammary epithelial cells, promoted by administration of prostaglandin A1, was tested by Collier and collaborators. Their study revealed that thermotolerance could be extended by the addition of PGA1 to the culture media by a vast induction of Hsp 70 gene expression (Collier et al., 2008).

Hsp Response to Transportation in Pig and Cattle

Farm animals are routinely transported as a common management practice in the pig and beef industries. The transport of animals is regarded as an acute physical stressor stimulating an associated psychological response (Van de Water et al., 2003). Transportation stress affects many aspects of health, production, and welfare of piglets (Pérez et al. 2002; Gosálvez et al. 2006; Mota-Rojas et al. 2006) and cattle (Muggli- Buckham Sporer et al., 2008; Cockett et al., 1992; Gupta et al., 2007). From both animal welfare and economical points of view it is necessary to control and minimize stress-inducing factors during transport of pigs and cattle.

Many studies dealt with various components (i.e. cortisol, acute phase proteins, immune responses) of the stress response in transported livestock, however, just few, mainly in pigs (Bao et al., 2008; Zhu et al., 2009), looked at the involvement of Hsp. Transported piglets are susceptible to acute heart failure and sudden death syndrome, leading to considerable economic losses. In this regard, Bao et al. (2008) reported an attenuation of Hsp in the heart, in response to 6 h road transportation. In accordance with this study, Zhu et al. (2009) found more than 60% reduction in cardiac Hsp60 of piglets, following 2 h transportation. Most interestingly, the authors report a tissue-specific Hsp response; in addition to the decrease in cardiac Hsp, a significant increase and non-changed levels of Hsp60 were detected for the stomach, liver and kidney, respectively.

In cattle, transportation stress is one of the leading factors that when combined with infectious agents, may promote the development of bovine respiratory disease (BRD, Yates, 1982). BRD is the most costly disease in feedlot cattle all over the world (Snowder et al., 2007; Thompson et al., 2006). The incidence of BRD following transportation of feedlot calves has been associated with alterations in immune function (Blecha et al., 1984; Buckman Sporer et al., 2007; Gupta et al., 2007; Yagi et al., 2004).

In the present paper, we report an increased expression of Hsp90 α mRNA in leukocytes of 2 h truck transported young calves (Figure 2). Similar induction was observed also for Hsp60 and Hsp70A1A (data not shown). It is noteworthy, however, that not all individuals respond to transportation in this manner; we observed individuals which lacked the capability to induce Hsp upon transportation (data not shown). In this regard, elevation of Hsp in transported calves may confer cytoprotection in 2 ways: 1) Intracellular - As recently reported by Bao et al. (2008). 2) Extracellular – secreted Hsp have been implicated with recruitment of the immune response similar to LPS (Pockley, 2003). Accordingly, calves which are capable to induce and secrete Hsp may induce immune responses to combat BRD-promoting pathogens.

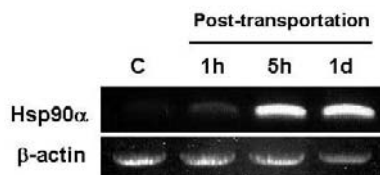


Figure 2. Expression of Hsp90 α mRNA in leukocytes of young Holstein-Friesian bull calf, pre-transportation (C) and 1h, 5h and 1d post-transportation, as detected by RT-PCR. β -actin levels were used to detect equal amounts of loaded RNA.

Hsp Response to Food Restriction in Cattle

Many of the studies on caloric restriction (CR) were done in laboratory animals in the context of the anti-aging action of CR. The anti-aging effect of CR can be viewed as nutritional stress, since the reduced caloric intake by the animal seems to evoke metabolic response for survivability. Studies of this kind interestingly demonstrated that in hepatocytes, CR could provide cytoprotection against heat challenge by activating conserved resources, such as the Hsp machinery, to withstand stress (Heydari et al., 1993). Other kind of studies non-related to anti-aging, also report the induction of Hsp in response to CR, particularly in various regions of the rodent brain (Lee et al., 2000).

Food restriction effect on performance is sometimes secondary; especially when the primary effect is thermal stress which results is a reduction in feed intake. Both factors, however, may increase disease susceptibility (Franci et al., 1996).

To the best of our knowledge, Hsp response to CR in productive tissues of homeothermic farm animals received little attention. Generally, dietary restriction in livestock is not an initiative operation of farmers. However, in many countries all over the world, beef cattle herds are grown under extensive regime. The implications of this regime in habitats such as the Mediterranean ecosystem, is an imbalance in nutritional availability throughout the year. Indeed, free grazing beef cattle may face a reduction in the nutritional quality of forage through its chemical ingredients, digestibility and metabolized energy, especially during the hot and dry seasons (Aharoni et al. 2004; Brosh et al. 2004). Poor quality feed may deleteriously affect reproduction (Randel 1990), and through its adverse effect on milk production, also interfere with calves' weaning success. Thus, beef cows may serve a good model to test the effect of CR on the stress response in productive or energy preserving tissues. In cattle, CR may be achieved directly, by reducing the metabolizable energy, or indirectly, by manipulating the protein content of the diet. In a recent study (Eitam et al., 2009), we showed that prolonged low-energy diet promoted cell-specific Hsp response in gestated and lactating beef cows; whereas significant increase of Hsp90 but unchanged levels of Hsp70 proteins were observed in white blood cells, the expression of Hsp70 in mammary-derived epithelial cells was markedly attenuated.

In parallel with the attenuation of Hsp70, an increase in the expression of α_{s1} -casein and fatty acid binding protein 3 (FABP3) were measured.

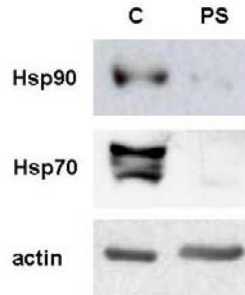


Figure 3. A typical Hsp response in the subcutaneous fat tissue of gestating beef cows fed prolonged (3 months) low protein diet (PS, 6% crude protein), as detected by Western blot analysis. Actin levels were used to detect equal amounts of loaded protein. C – cows fed control diet (12% crude protein).

As mentioned earlier, challenging cattle with low protein intake may lead to a similar metabolic effect as attained by direct caloric deprivation. In the present study we report the outcome of low protein intake in terms of Hsp expression in the subcutaneous fat tissue. As seen in Figure 3, gestating beef cows responded to prolonged low protein intake by a similar Hsp attenuation to that observed by Eitam et al. (2009). Again, the Hsp attenuation was accompanied by a marked increase in the expression of adipocyte fatty acid binding protein (a-FABP, data not shown).

We postulate that as Hsp70 and Hsp90 assist newly synthesized proteins to attain their native states, regulates protein degradation and are important components of cellular networks, their attenuation in both described CR experimental systems reflects a decrease in the turn-over of self proteins in favor of energy producing factors, related to the metabolic state of the body and the function of the tissue examined. This postulation is partially supported by other results which demonstrate the attenuation of proteasome subunits in response to low protein intake by beef cows (data not shown).

Conclusions

The present manuscript reviews the current knowledge exists in the field of “Hsp responses of homeothermic livestock to common stress stimuli”, using novel results along with previous studies from our and other labs. Although Hsp are induced by many environmental, patho-physiological and psychological stimuli, in cell cultures, poikilotherms and lab animals, there are instances in which their expression is attenuated.

One example for such attenuation is the cardiac Hsp60 of transported piglets. Another example is the direct and indirect CR administrated to beef cows. Here, it leads to decreasing levels of Hsp70 and Hsp90 in energy producing tissues, not necessarily correlated to the overall Hsp response of the body, but in parallel with an increase in energy producing genes. The concept that emerges from the current manuscript is that, in marked difference from the classical Hsp response, in which, in parallel with the induction of Hsp the expression of other proteins is silenced, in producing tissues of homeothermic livestock (fat, mammary gland) exposed to CR, energy producing genes may increase at the cost of attenuated Hsp levels.

This Hsp attenuation may be the result of altered metabolic set point, reflecting a decrease in the turn-over of self proteins in favor of producing factors.

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Chapter XX

The Chaperonin Containing T-Complex Polypeptide: Do Monomeric Subunits Have Discrete Individual Functions?

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Abstract

The chaperonin containing T-complex polypeptide (CCT) is the major cytosolic chaperonin in eukaryotes and has been estimated to interact with up to 15% of all cellular proteins. The CCT holoenzyme is a hexadecameric molecule comprised of two copies each of eight discrete subunits, each of which shares partial homology to the others, as they are thought to have evolved from a single common ancestor gene. CCT has principally been implicated in the folding of cytoskeletal proteins such as tubulin and actin, and has been noted as an important factor in a variety of cellular processes, including cell proliferation, embryogenesis, ciliary biogenesis, etc.

The majority of studies on CCT function have thus far focused on the 16-mer holoenzyme; however, an increasing body of evidence suggests that the individual subunits of CCT may have independent function and significance. This evidence derives from several different types of observations: the effects of naturally occurring (or experimentally-induced) mutations in individual CCT subunits, the differential patterns of expression and localization displayed by various CCT subunits, and direct

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experimental demonstration of individual CCT subunit physiology in a capacity without the CCT aggregate enzyme.

Multiple spontaneous and induced mutations in CCT subunits have been identified that lead to discrete phenotypes: in CCT-epsilon (causing mutilating sensory neuropathy with spastic paraplegia in humans), in CCT-delta (causing the mutilated foot phenotype in rats), and in CCT-gamma (leading to the *no tectal neuron* phenotype in zebrafish, interfering with eye development). Investigations on expression of CCT subunits have demonstrated that not all subunits are coordinately expressed, and that some subunits localize within cells as monomers or as microcomplexes much smaller than the 16-mer CCT enzyme. In some instances a specific biological activity has been imputed to a single CCT subunit. The CCT-eta has been found to be a biological partner for the soluble guanylyl cyclase, affording it a role within nitric oxide signaling, and CCT-alpha has been shown to preferentially inhibit the polyglutamine-mediated toxicity of the huntingtin protein. This seemingly disparate collection of observations together suggest that individual CCT subunits may have particular physiological roles apart from the CCT holoenzyme.

Introduction

The chaperonin containing T-complex polypeptide (CCT), also known as TCP-1 Ring Complex (TRiC), is the major cytosolic chaperonin in eukaryotes and has been estimated to interact with up to 15% of all cellular proteins. The structure of the CCT holoenzyme is unique among chaperonins; it consists of two rings each comprised of eight discrete subunits: alpha, beta, gamma, delta, epsilon, zeta-1, eta, and theta (zeta-2, a variant of zeta, is highly expressed only in testis). The molecular weight of the complete assemblage is approximately 900 kD. CCT has especially and principally been implicated in the folding of cytoskeletal proteins such as tubulin and actin by both biochemical and genetic analysis; in the case of actin, for example, CCT alone (in the presence of ATP) is sufficient for the generation of native product [1], and reduction of CCT in a colon carcinoma cell line results in decreased native actin and alterations in cytoskeletal organization [2].

The CCT protein has been implicated as an important factor in a variety of cellular processes, including embryogenesis, ciliary biogenesis, cell proliferation and locomotion. Although its role as a chaperonin has been best elucidated in its interactions with the cytoskeletal proteins actin and tubulin, it has also been found to assist in the folding of multiple other proteins, including cyclin E [3], myosin [4], transducin [5], and the Von Hippel Lindau tumor suppressor [6] among others. Alteration of CCT expression thus has the potential to impose pleiotropic secondary effects on cells.

Null mutation of CCT subunit genes in yeast is uniformly lethal, further highlighting the importance of this molecule [7,8], considered an essential protein. Although each CCT subunit arises from a separate gene, the subunits do share some homology (~30% at the amino acid level) which indicates that all derive from a common ancestor gene [9,10]. This cross-subunit homology is greatest in the ATP-binding sites of the subunits (equatorial domains), and diminishes substantially in the regions that bind to substrate proteins (apical domains). Within a subunit, however, there is substantial sequence homology across species even within the apical domains [1].

A large amount of study has been devoted to understanding the mechanisms by which the CCT holoenzyme engages and re-folds target substrates. This process, dependent on ATP hydrolysis, involves conformational changes within the CCT holoenzyme itself, not just within its substrates. There is a growing body of evidence that different substrate proteins are preferentially engaged by different CCT subunits within the hexadecameric aggregate molecule; for example, alpha-actin has been found to bind CCT-delta and either -beta or -epsilon [11], whereas beta-actin has been shown to bind to CCT-alpha, -beta, -epsilon, and -theta subunits [12]. Tubulin also appears to engage with CCT through specific subunits [13]. Given the highly ordered and unique structure of the CCT hexadecamer, perturbations in one subunit may well allosterically affect another; indeed, there is evidence that the lack of any individual subunit will result in near abrogation of CCT activity altogether.

This complex interaction between the multiple components of the CCT holoenzyme renders difficult evaluation of the unique contributions of any specific CCT subunit. More difficult still is an assessment of what roles CCT subunits may play in monomeric form, dissociated from the CCT compound molecule. Still, several lines of evidence suggest that CCT subunits do have important biological functions without hexadecameric CCT; these include the distinct phenotypes rendered by mutations in individual CCT subunits, the differential expression and localization patterns seen with various CCT subunits, and some direct experimental evidence of heterodox CCT subunit biochemical activity clearly unrelated to the CCT holoenzyme.

Variable Phenotype with Mutation of CCT Subunits

Mutations in three CCT subunit genes have been identified that lead to distinct neurodegenerative phenotypes. The first of these to be discovered was in the *mf* (mutilated foot) rat, a well-characterized animal model for human hereditary sensory neuropathies that clinically displays ataxia, insensitivity to pain, and foot ulceration, with pathological findings of markedly decreased sensory ganglia and fibres. Lee et al. [14] conducted a genome-wide scan on *mf* rats crossed with an unaffected strain using polymorphic microsatellite repeat loci. They were able to first narrow the responsible locus to an ~ 0.7 cM region on chromosome 14, then determined that the *Cct4* (delta) gene was responsible by direct sequencing of cDNAs of candidate genes within the region. They identified a single substitution of adenine for guanine at nucleotide 1349 that resulted in a substitution of tyrosine for a highly conserved cysteine at amino acid 450. This suggested that disulfide bonding involving this subunit, either intramolecular or with a partner molecule, might be disrupted.

A second CCT subunit mutation has been found in an actual human hereditary neuropathy. Bouhouche et al. [15] investigated a consanguineous Moroccan family with four patients affected with mutilating sensory neuropathy associated with spastic paraplegia. They first mapped the disease locus to a 25 cM interval, but, aware of the *Cct4* mutation leading to *mf*, were then able to focus on the *Cct5* (epsilon) gene within that interval. They identified a missense mutation in the coding region of CCT-epsilon, with a guanine replacing adenine at nucleotide 492, resulting in arginine substituting for a highly conserved histidine residue.

This substitution occurs in the equatorial region of the CCT molecule wherein resides the ATP-binding domain, suggesting that an alteration in ATPase activity may result in the mutated phenotype.

Perhaps the most interesting CCT subunit mutation occurs in the zebrafish system. Matsuda and Mishina [16] induced a mutation in zebrafish using trimethylpsoralen that manifested with retinotectal neuronal abnormalities and small eyes (no tectal neuron; *ntn*). They then used representational difference analysis to isolate the genome region responsible for the mutation, and identified a 143 bp deletion in the zebrafish cDNA for *Cct3* (gamma), resulting in a complete loss of the ATPase motif and a frameshift of the remaining sequence. Thus, CCT-gamma mutation also leads to a significant neurodegenerative phenotype, but one markedly distinct from that seen with mutation in CCT-delta or -epsilon, an observation itself suggestive that there may be a discrete function for CCT-gamma.

Since the CCT-gamma mutation occurs in a very well-defined and accessible developmental system, the investigators were able to more precisely examine the nature of the defect caused by the *ntn* mutation in CCT-gamma. They noted that *ntn* mutants showed no detectable differences in body patterning or neurogenesis at 30 hours post-fertilization (hpf), despite the fact that CCT-gamma begins to show strong expression at 12 hpf and despite numerous morphogenetic changes involving cell proliferation, cellular locomotion, and body segmentation in this interval. Rather, *ntn* phenotypes manifested first at ~ 2 days post-fertilization, and then were spatially restricted to the retina and tectum [16]. This highly specific and limited phenotype is suggestive of a unique and discrete biological function for the CCT-gamma subunit; although a secondary effect on the CCT holoenzyme as a possible mechanism is not formally excluded, it is arguable that disruption of such a ubiquitous and multivalent protein throughout development might be expected to result in a much more widespread and severe phenotype. It is fascinating to note that the *ntn* mutation, comprised of a deletion and frameshift, would appear to be a null mutation, but obviously is not lethal to the organism.

Variable Expression of CCT Subunits

If the only functional importance of CCT subunits was as participants in the complete hexadecameric CCT holoenzyme, it might be supposed that regulation of subunit expression would somehow be coordinated so as to maintain an optimal (and equivalent) stoichiometric ratio. Indeed, an early report suggested that all subunits were in fact thusly co-regulated: Kubota et al. [17] examined the constitutive level of CCT subunit expression in a wide spectrum of mouse tissues and cells using both Western and Northern blot analyses. They found that, although there was significant variation in the absolute level of expression of CCT among different tissues, the expression patterns were very similar among the eight subunit species. They concluded that “it is likely that expression levels of the eight different subunits are tightly co-regulated to maintain a constant ratio of these subunits.”

However, subsequent reports from a variety of experimental systems have identified multiple instances where CCT subunit expression appears to be differentially regulated, implying that specific subunits may be more critical to certain cellular processes than others,

thereby suggesting alternative and individualized functions for CCT subunits. Yokota et al. [18], examining CCT subunit expression in FM3A mammary carcinoma cells, noted that M phase arrest with colcemid specifically resulted in markedly decreased levels of CCT-alpha, -delta, and zeta-1. These subunits returned to their normal levels of expression upon release from the arrest; the other five subunits remained constant throughout. Cyrne et al. [19], working in the ciliated protozoan *Tetrahymena*, found that the CCT-eta and -gamma mRNAs are co-regulated during ciliary biogenesis and sexual reproduction. Himmelsbach et al. [20], investigating CCT expression in a plant system (maize mesocotyl), found a light dependent reduction of CCT-epsilon but not CCT-alpha.

Our own laboratory has observed differential expression of CCT subunits during integumentary wound healing in fetal and adult rabbits. CCT-eta was first recovered as a gene product specifically underexpressed in healing fetal wounds by differential display [21], and this pattern was confirmed by both semi-quantitative limiting dilution RT-PCR and subsequently by quantitative real-time RT-PCR. No other CCT subunit message was found to share this fetal wound-specific decrease, but the CCT-alpha subunit was found to be specifically reduced in healing adult wounds [22], raising the possibility that changes in CCT subunit expression may play an important role in distinguishing the markedly different phenotype of (scarless) fetal wound healing from (schirrhous) adult wound healing. Interestingly, CCT-eta mRNA was actually found to be transiently elevated in a rabbit model of corneal wound healing following ultraviolet radiation, although no other subunits were examined in that study [23].

Although the trans- and cis-acting factors governing CCT subunit gene expression have only been partially examined thus far, reports to date do not suggest identical regulatory mechanisms across subunits. The genes encoding the eight major CCT subunits have been cloned and sequenced in mouse, and transcriptional start sites determined. The majority carry possible Sp1 binding sites in the 5' flanking region, and all do contain potential heat shock element (HSE) sequences. However, promoter/reporter gene constructs tested in HeLa cells resulted in disparate levels of reporter activity from the various subunit promoter sequences examined [17]. Moreover, it is becoming increasingly clear that different subunits are indeed controlled by different transcriptional mechanisms: Yamazaki et al. have demonstrated that CCT-theta gene expression is under the control of Ets domain transcription factors (in HeLa cells) [24], whereas the CCT-alpha promoter was found to contain cis-acting sequences that are acted upon by the Staf family zinc-finger transcription factors ZNF143 and ZNF76, also in HeLa cells [25]. Although it remains possible that these varying transcriptional control mechanisms can somehow converge to a coordinated subunit expression, it seems more likely that they have developed to allow for individual control of subunit expression in response to specific physiological exigencies that require particular CCT subunits. This again implies functions for CCT subunits beyond simply component pieces of the CCT aggregate protein.

Variable Subcellular Localization of CCT Subunits

Another line of evidence suggesting individualized functions for CCT subunits apart from the hexadecameric enzyme is the observation by multiple groups that not all CCT

subunits co-localize within the cell identically. If the biological importance of individual CCT subunits was limited to their role in the CCT holoenzyme, it would be metabolically useless for the cell to compartmentalize said subunits discrepantly, strongly implying that such compartmentalization is of utility to the cell.

Roobol et al. [26] observed such subunit-specific compartmentalization in the ND7/23 cell line. Using immunocytochemistry they demonstrated that CCT-alpha was abundant at the leading edge of neurite growth during neuronal differentiation of these cells, but that CCT-beta, -gamma, and -epsilon remained in a largely perikaryal cytoplasmic distribution. Roobol and Carden [27] found a similar pattern in mouse P19 embryonal carcinoma cells, which can be induced to take on neuronal characteristics by retinoic acid: CCT-alpha was significantly more abundant in P19 neurites than other CCT subunits. In addition, they found that CCT-alpha and -gamma (but not other subunits) were selectively localized to the nucleus as well. Moreover, using double immunofluorescent technique to simultaneously assay for pairs of CCT subunits, they detected cytosolic foci which stained positive for only one of the subunits probed, pointing to a heterogeneous subcellular distribution of CCT subunits even within the cytoplasm.

Other investigators in other systems have also noted differential subcellular localization of CCT subunits. Casalou et al. [28] examined multiple CCT subunit species in exponentially growing (and taxol- and colchicine-treated) *Tetrahymena*. They found that CCT-alpha and -epsilon but not CCT-eta were associated with the insoluble (pellet) fraction on cellular extraction in both growing and treated cells, and in the case of CCT-epsilon this association was increased by taxol or colchicine treatment. They surmised that “the differential association of some CCT subunits with the insoluble fractions that contain the ciliate cortex structure, basal bodies and cilia suggests that the different CCT subunits may have distinct roles in cells either when acting as free subunits and/or as part of oligomeric structures.” Coghlin et al. [29] identified two CCT subunits, beta and epsilon, as differentially expressed in colorectal adenocarcinoma through comparative proteomic analysis versus normal colorectal mucosa. In directly investigating the expression of these subunits by immunohistochemistry, they observed that CCT-beta exhibited strong cytoplasmic staining in the adenocarcinoma cells, but that CCT-epsilon demonstrated both strong cytoplasmic and nuclear staining, a distinction suggesting potentially discrete physiological roles for these subunits in these transformed cells.

Direct Evidence of Biologic Activity of Individual Subunits

In a limited number of instances individual CCT subunits have been demonstrated to have specific physiological effects; in a very few cases a specific biochemical activity outside of the CCT hexadecameric complex has been documented. These data are the strongest indication to date that these individual polypeptides have various and diverse roles to play in cell physiology without the CCT holoenzyme.

Several lines of evidence implicate the CCT-delta subunit, already familiar as the cause of mutilated foot, in other cellular processes, specifically apoptosis and RNA polymerase binding to an HIV long terminal repeat sequence. Zilkha-Falb et al. [30] used differential

display to identify gene products specifically up-regulated during dopamine-induced apoptosis of chick sympathetic neurons in culture. One of the recovered gene products, on full-length cloning and sequencing, was found to be the chick homologue of CCT-delta. Increased expression of CCT-delta on dopamine treatment of the cultured cells was directly confirmed at both the RNA and protein levels. Overexpression of the CCT-delta subunit alone (via a cytomegalovirus promoter-driven expression vector in transient transfection) accentuated the dopamine-induced apoptosis of the neuronal cells. Conversely, inhibition of CCT-delta expression (via a specific RNAi) protected the neuronal cells from dopamine-induced apoptosis.

Wu-Baer et al. [31], working in HeLa cells, used protein purification techniques to isolate a group of three cellular cofactors that associated with the trans-acting factor TRP-185 and increased its ability to bind to the TAR RNA sequence in the long terminal repeat of HIV. One of these cofactors, on microsequencing, was designated “stimulator of TAR RNA-binding proteins (SRB);” a full-length clone for SRB from a HeLa cDNA library revealed that SRB has highest homology to chaperonin proteins, and corresponds specifically to the CCT-delta subunit. Electrophoretic mobility shift assays demonstrated that direct addition of purified SRB (CCT-delta) facilitated binding of both TRP-185 and also RNA polymerase II to TAR RNA target sequence, representing a distinct biochemical activity for CCT-delta not reliant upon any other CCT subunit nor on the CCT complex as a whole.

The CCT-alpha subunit also evidences an independent and unique biological function, specifically with regard to the processing of peptides containing expanded polyglutamine repeats, misfolding and aggregation of which are the underlying pathophysiological mechanisms for Huntington’s disease and other neurodegenerative disorders. Tam et al. [32] examined the conformation of the expanded polyglutamine tract from huntingtin in yeast cells mutant for CCT4 (and thereby also deficient in CCT holoenzyme activity). They found significant aggregation of the huntingtin peptides (often into a single large inclusion body), indicating that the CCT complex is indeed important in modulating this biology. Interestingly, on supplying each individual CCT subunit complementarily to the mutant yeast cells in turn, they found that only CCT1 (alpha) and CCT4 (delta) were able to alleviate this phenomenon, the latter possibly by enabling reconstitution of the CCT holoenzyme, but the former (CCT-alpha) by a distinct and holoenzyme-independent activity. They were subsequently able to demonstrate direct interaction between the apical region of CCT1 and the expanded polyglutamine polypeptide, whereas no such interaction was found with the apical regions of CCT3 (gamma) or CCT7 (eta). They then further showed that, in neurons expressing polyglutamine-expanded huntingtin, overexpression of the single CCT1 subunit (but not CCT7) is able to both reduce huntingtin aggregation and reduce the cellular toxicity of said aggregation. These data indicate that, while the CCT hexadecameric enzyme is a significant factor in huntingtin processing (also demonstrated by Kitamura et al.[33]), the monomeric CCT-alpha subunit has an independent ability to regulate this process as well.

CCT-eta offers another example of a CCT subunit with a biochemical function clearly separate from its role as part of the CCT holoenzyme. Hanafy et al. [34], prospecting for binding partners to the soluble guanylyl cyclase (sGC), the major intracellular mediator of nitric oxide (NO) signaling, identified CCT-eta as such a protein through yeast two-hybrid screening. They were then able to co-immunoprecipitate sGC and CCT-eta directly, and they

found that sGC and CCT-eta displayed a highly similar pattern of expression in rat brain. They were able to directly inhibit the Vmax (but not the EC50) of NO-stimulated sGC by co-incubation with purified CCT-eta; the N-terminal region of CCT-eta appears essential to mediate this inhibition. In vivo overexpression of CCT-eta in a stably transfected BE2 neuroblastoma cell line similarly inhibited sodium nitroprusside induction of sGC activity. Thus, the solo CCT-eta subunit is a biological partner and inhibitor of soluble guanylyl cyclase in addition to its role within the CCT holoenzyme.

The experiments and evidence recapped above- circumstantial and direct, observational and mechanistic, genetic and biochemical- comprise an emerging body of evidence for the multifarious importances of the individual constituent subunits of the CCT holoenzyme. While it remains problematic to dissect the exact nature of the physiologic effects attributable to CCT subunits in monomeric and hexadecameric form, further studies on this complicated set of molecules will doubtless continue to clarify the multiple ways they interact with each other and with other specific biological partners.

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Chapter XXI

Evidence for *In Vivo* Phosphorylation of Hsp26 from *Saccharomyces cerevisiae*

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Abstract

Hsp26 from *Saccharomyces cerevisiae* is represented by a 24-mer oligomer with the overall organization resembling that of a hollow globular sphere, which possesses co-chaperone activity. It functions in the recovery of misfolded proteins and prevent aggregation, but its *in vivo* role in protein homeostasis remains unclear. sHsps from some organisms are known to be posttranslational regulated by phosphorylation, where those covalent modifications regulate function and quaternary structure. Global analysis studies of yeast phosphoproteome identified phosphorylated peptide sequences in Hsp26. To study the *in vivo* phosphorylation of yeast Hsp26, the gene encoding Hsp26 was overexpressed from a multicopy plasmid using its own promoter. Hsp26 was purified from stationary phase cells to homogeneity by a procedure already described in our lab. The purification method used consisting of three steps: ethanol precipitation, sucrose gradient ultracentrifugation, and heat inactivation of residual contaminants produced native Hsp26 protein. The purified Hsp26 was shown to be phosphorylated in its serine-peptides. Hsp26 was resolved in four isoforms, displaying the same molecular masses but different isoelectric points. A MALDI/MS analysis of the isoforms led to the identification of a phosphopeptide 37-QLANT(p)PAK-44 at the N-terminus of Hsp26. If the isoforms represent multiple phosphorylated forms of Hsp26 is an open question.

Keywords: Hsp26, isoforms, *in vivo* phosphorylation, MALDI/MS, *Saccharomyces cerevisiae*

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Introduction

Yeast Hsp26 belongs to the phylogenetically conserved family of small heat shock proteins (sHsps). They share a C-terminal domain of 80-100 amino acids with sequence homology to the major eye lens protein α A-crystallin [1]. On the other hand, their N-terminal extremity differs in sequence and length, leading to proteins with molecular masses of 12–42 kDa in different organisms [2]. Almost all sHsps assemble into large oligomeric complexes of 4 to 40 subunits of up to 800kDa [3, 4]. Some sHsps such as those from plants form assemblies with well-defined stoichiometries, whereas other sHsps, including the mammalian proteins, form oligomers in a range of sizes [5,6] with dynamic and variable quaternary structures showing subunits that can freely and rapidly exchange between oligomers [7-9]. The crystal structure of an archaeal sHsp [10] and the cryo-electron microscopy reconstruction of α B-crystallin [8] and yeast Hsp26 [11] revealed that their overall organization is that of a hollow globular sphere. A variation of this scheme is the three-dimensional structure of wheat Hsp16.9, which assembles into a dodecameric double disk, where each disk is organized as a trimer of dimers [12]. The activation of Hsp26 under heat shock coincides with the destabilization of the oligomer and appearance of dimers. Those results led to generally accept notion that dissociation might be a requirement for the chaperone mechanism of Hsp26 [47]. Studies of mammalian cells indicate that phosphorylation of sHsps may be an important regulator of their function. The mammalian sHsps are phosphorylated via a mitogen-activated protein kinase pathway at three conserved R-X-X-S sites [13]. Phosphorylation of mammalian sHsps results in a dissociation of the sHsp oligomer from approximately 400 kDa to less than 70 kDa [14] or from 200 and 250 kDa to 150 and 125 kDa [15], respectively, depending on the method used for molecular size estimation. sHsp phosphorylation is proposed to affect interaction of sHsps with actin and, in some cases, altering also thermotolerance [16, 17, 15]. Human [33], mouse [34], rat [35], *Drosophila melanogaster* [32] and Chinese hamster [36], among sHsps have shown to be phosphorylated *in vivo*. Despite the importance and widespread occurrence of phosphorylation as a regulator mechanism of protein activity, identification of sites of protein phosphorylation is still a challenge, even when performed on highly purified protein. Recently, methodologies have arisen that should characterize phosphoproteins from a whole cell lysate. Those methodologies identified five phosphorylated peptides on Hsp26 (40, 45) displaying the sequences 37-QLANT(p)PAK-44, 89-RS(p)VAVPVDILDHDNNYELK-108, 160-RVIT(p)LPDYPGVDADNIKA-176, 202-KIEVSS(p)QES(p)WGN-213, 202-KIEVSSQES(p)WGN-213. Here we investigated the *in vivo* phosphorylation of Hsp26 using a preparation purified to apparent homogeneity as judged by SDS-PAGE. The yeast small heat shock protein Hsp26 was from stationary phase cells using a reproducible procedure previously described in our laboratory. The presence of phosphopeptides in Hsp26 sequence was tested by western-blotting using a monoclonal anti-phosphoserine antibody as a probe and also by resolving the pure protein in 2D-electrophoresis combined to MALDI/MS analysis. Findmod search for post-translational modifications using tryptic peptide masses from Hsp26 was performed in order to find threonine/serine-containing phosphopeptides. Candidate protein kinases for phosphorylation of target peptide sequences within Hsp26 and the physiological significance of N-terminus phosphorylation were also discussed.

Materials and Methods

Yeast Strain and Growth Conditions

Cells from *Saccharomyces cerevisiae* strain W303-1A (*Mata ade2-1 trp1-1 leu2-3,2-112 his3-11,15 ura3 can1-100*) harboring the episomal plasmid pL19 containing the *HSP26* gene were grown in YNB-glucose medium (0.67% Yeas Nitrogen Base without amino acid (Difco), 2% glucose and 0.01% of appropriate auxotrophic requirements) at 28°C, on an orbital shaker operated at 160rpm to ensure aeration. Growth of culture was followed by turbidity measurements at 570 nm.

Purification of Hsp26

Hsp 26 was purified from stationary phase cells (1.7 mg (dry wt)/ml) as described previously (18) Protein concentration was determined according to the procedure of Bradford using bovine serum albumin as the standard. Concentration of the purified Hsp26 was determined by measurement of absorbance at 280nm. Conversion of absorbance at 280nm to milligram of protein per milliliter was performed using $Abs_{0.1\%} = 0.856$ (estimated by ProtParam tools at www.expasy.org/tools/protparam.html from the primary sequence of Hsp26).

Gel Electrophoresis

Purified Hsp26 (100µg) was precipitated with trichloroacetic acid in presence of sodium deoxycholate [19] 1976 (2). For SDS-PAGE, the pellet was solubilized in 100µL of sample buffer (0.25M Tris-HCl pH6.8, 0.72M β-mercaptoethanol, 10% glycerol and 7mM SDS), heated for 3 min at 100°C and fractionated on a 10 or 12.5% SDS-polyacrylamide gel [20]. For two-dimensional electrophoresis, trichloroacetic acid-precipitated Hsp26 was suspended in rehydration buffer (8M urea, 2% triton X-100, 2% IPG buffer and bromophenol blue). Isoelectric focusing (IEF) was carried out at 20°C in the Multiphor™ II System (Amersham Biosciences) using a immobilized non-linear pH gradient (pH 3-10) in a 18cm strip, which was loaded with 400µg of protein using the rehydration method. Second dimension was carried out at 15°C in an 8-18% gradient SDS-polyacrylamide gel after soaking the immobilized pH gradient strips into SDS-equilibration buffer (50mM Tris-Cl pH8.8, 6M urea, 30% glycerol, 2% SDS and bromophenol blue). Gels were stained with Coomassie Brilliant Blue G 250 [21-23].

Western-Blotting Assay

Polypeptides fractioned on 12.5% SDS-polyacrylamide gel were electrophoretically transferred onto nitrocellulose membranes by one hour at 5V and constant current at 200mA

by 60 min. After overnight blocking with skimmed milk, the membranes were probed with anti-Hsp26 IgG purified by protein A [24], for 2h at room temperature. After washing, the membranes were incubated with secondary goat anti-rabbit IgG antibody conjugated to horseradish peroxidase for one hour at room temperature [25, 26]. The blots were developed with diaminobenzidine and H_2O_2 plus CoCl_2 to enhance sensitivity. Phosphorylated serine residues in the purified yeast Hsp26 was detected by using mouse monoclonal anti-phosphoserine antibody (Sigma) and anti-mouse IgG conjugated to alkaline phosphatase as a secondary antibody. Blots were developed with NBT/BCIP in AP buffer [27, 28]. Sodium fluoride (NaF), a phosphatase inhibitor, was added to the blocking agent to increase the signal for phospho-specific antibody [29].

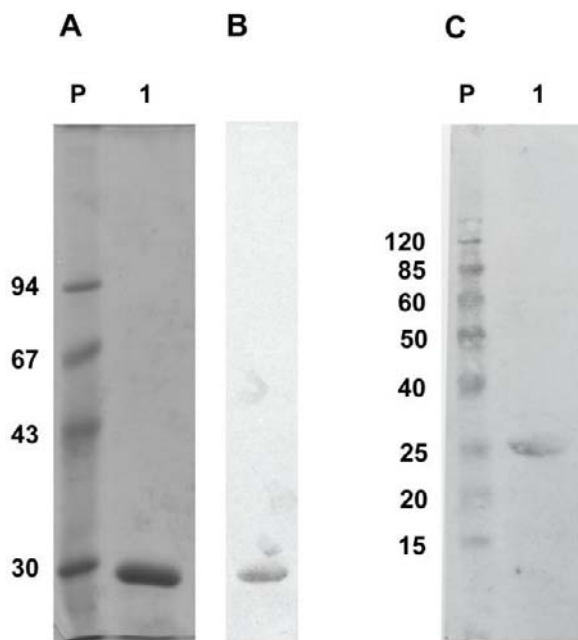
MALDI-MS Analysis

Spots revealed by Coomassie Brilliant Blue in IPG-2D-PAGE were excised from the gel and transferred to nylon membranes. Immobilized proteins (0.2-1 μg) were digested with trypsin and the masses of the resulting peptides were determined by MALDI/MS (Matrix Assisted Laser Desorption/Ionization Mass Spectrometry) at the SWISS - 2D SERVICE (University Hospital of Geneva - Genève - Switzerland). As internal calibrant, were applied 100fmol of bradykinin ($\text{MH}^+ = 1060.57$) and ACTH clip ($\text{MH}^+ = 2466.702$). The peptides masses obtained were used for searching protein databases using ProFound (<http://65.219.84.5/service/prowl/profound.html>). Putative occurrence of post-translational modifications was searched with FindMod at www.expasy.org/tools/findmod/ [30-32].

Results and Discussion

Recently, we have purified yeast Hsp26 to apparent homogeneity from stationary-phase cells harboring the *HSP26* gene cloned in the multicopy plasmid pL19. The over-expressed protein was purified by a combination of three procedures consisting of ethanol precipitation treatment, linear sucrose density gradient ultracentrifugation and heat inactivation of the residual contaminants (18). The resultant Hsp26 preparation was analyzed on a 10% SDS-PAGE and displayed a unique band of 26kDa (Figure 1A). The purified polypeptide was transferred onto nitrocellulose membrane and treated with a specific rabbit anti-Hsp26 polyclonal antibody [24], confirming the identity of Hsp26 (Figure 1B).

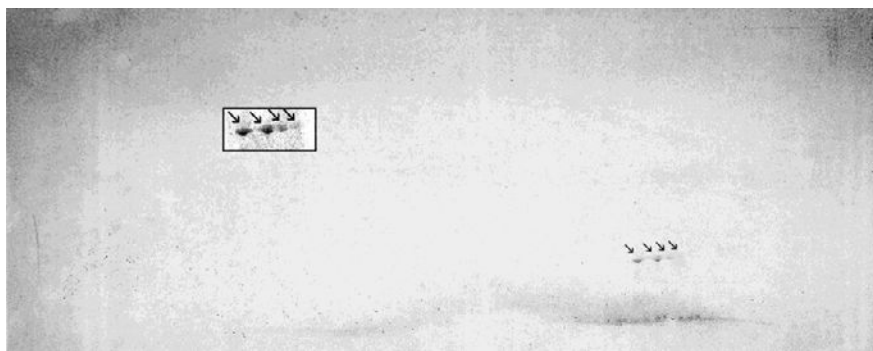
To confirm the *in vivo* phosphorylation of Hsp26 at the stationary growth phase, yeast Hsp26 was resolved on 12.5% SDS-PAGE, blotted onto nitrocellulose membrane and probed with a monoclonal anti-phosphoserine antibody in presence of 50mM NaF. As showed in Figure 1C, a unique strong band was visible in the position correspondent to yeast Hsp26, indicating that the purified protein could be in its phosphorylated form as a result of the incorporation of phosphate(s) group(s) in serine residue(s). A very faint band was observed if NaF was omitted from assay (result not shown).



Note: **A** - Purified Hsp26 (lane 2) was analyzed on 10% SDS-PAGE stained with Coomassie blue. Molecular weight markers are shown on the left (lane 1). **B** - Western-blotting analysis of the purified Hsp26 using a polyclonal specific anti-Hsp26 antibody as a probe (lanes 1 and 2). **C** - Western-blotting analysis of the purified Hsp26 using a monoclonal anti-phosphoserine antibody as a probe (lane 2). Pre-stained molecular weight markers were loaded on lane 1. The arrow indicates Hsp26.

Figure 1. SDS-PAGE and Western-blot analysis of the Hsp26 from *Saccharomyces cerevisiae*.

To assess if Hsp26 could exist in multiple forms, the purified protein was fractionated on 2D-electrophoresis and stained with Coomassie Blue. Four spots with distinct acidic pI, but with similar molecular masses were observed (Figure 2), which vary in relative abundance from left (the more abundant, more basic isoforms) to right (the less abundant, more acidic isoforms) and could represent different phosphorylation states for Hsp26: the more basic isoforms representing the unphosphorylated protein, and the three others consisting of isoforms mono, doubly or triply phosphorylated, respectively. A previous report, have already demonstrated that Hsp26 purified from exponentially growing yeast cells (with a multicopy plasmid containing *HSP26* under control of its native promoter) exposed to 37°C for 90min and analyzed by bidimensional electrophoresis displayed three Hsp26 isoforms, which were supposed to be generated by differential phosphorylation [37]. However no additional effort was performed to assess this hypothesis.

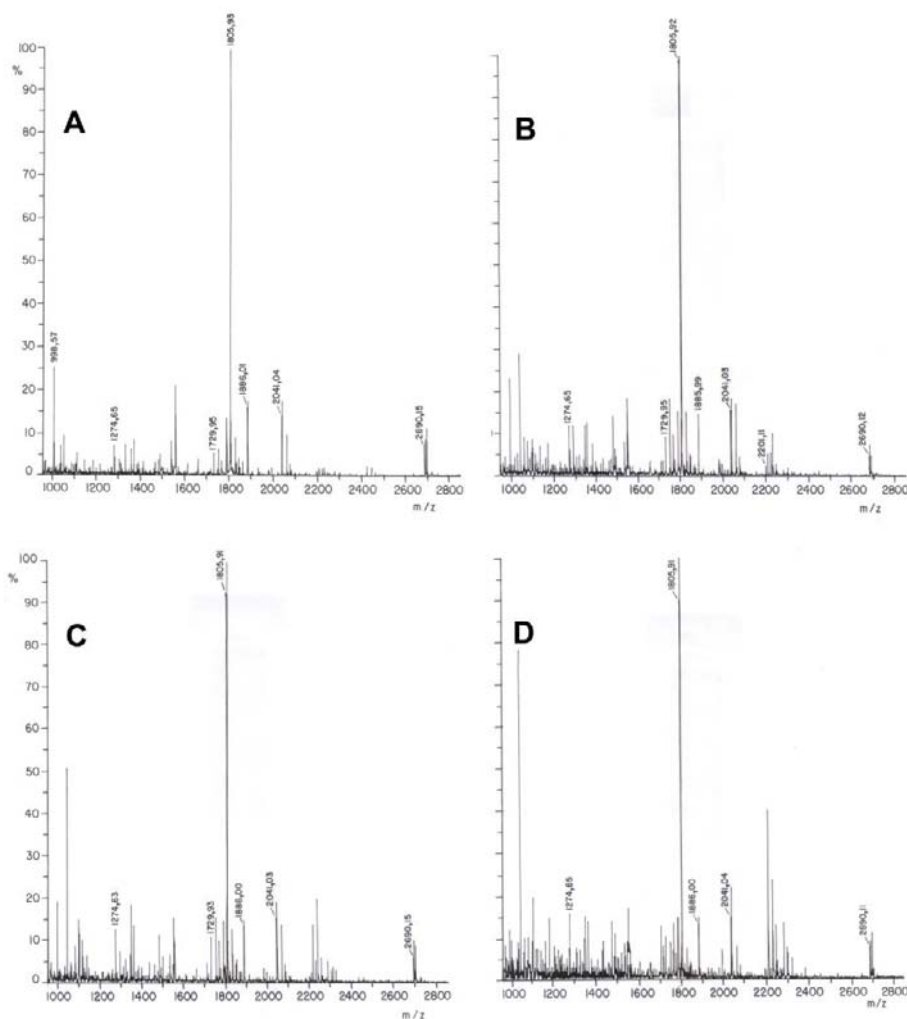


Note: Purified Hsp26 was fractionated by two-dimensional electrophoresis using an 18cm immobilized non-linear pH gradient (pH 3-10) in the first dimension and an 8-18% gradient SDS-PAGE in the second dimension. Gel was stained with Coomassie brilliant blue. The insert shows a magnification of the region displaying the protein spots.

Figure 2. 2-D electrophoresis of Hsp26.

In order to determine if the four 26kDa spots were originated by differential post-translational modifications acting on Hsp26 or if represent residual contaminant proteins, they were excised from the gel, electrophoretically transferred onto nylon membranes, digested with trypsin and the masses of resulting peptides were determined by MALDI/MS [38]. Very similar MALDI/MS footprints were obtained (Figure 3, panels A, B, C, and D), indicating that all four proteins share the same identity. Analysis of the peptide masses using ProFound showed that the four spots corresponded to the yeast Hsp26. At least 06 monoisotopic peptide masses were matched within ± 0.5 Da error to predict tryptic peptides expected from yeast Hsp26, covering 39.4% of its sequence. FindMod searching for posttranslational modifications using tryptic peptide masses from Hsp26 isoforms found a threonine-phosphopeptide of 998.57 Da present in the most basic polypeptide (Figure 3 A) and absent in the other isoforms. The phosphopeptide was identified as 37-QLANTPAK-44 and seems to be phosphorylated in T41 since the mass of the correspondent phosphorylated peptide was missed in the MALDI/MS spectra. Additional efforts to identify additional putative phosphorylated peptides were performed using those molecular masses obtained by mass spectroscopy that did not matched to Hsp26, but none phosphopeptide was found. Peptide masses corresponding to those phosphopeptides already identified during phosphoproteome global studies [40, 45] 89-RS(p)VAVPVDILDHDNNYELK-108, 160-RVIT(p)LPDYPGVDADNIKA-176, 202-KIEVSS(p)QES(p)WGN-213, 202-KIEVSSQES(p)WGN-213 were absent from the mass spectra obtained. This fault is not unusual since the phosphate group is believed to cause an effect on the ionization of phosphorylated peptides in mass spectroscopy, resulting in decreased signal intensity for phosphorylated peptides in the presence of non-phosphorylated peptides, i.e., an ion suppression phenomenon [39]

However the four isoforms of Hsp26 remained unsolved. If the isoforms were generated solely by phosphorylation we could speculated, based on their isoelectric points, the existence of an unphosphorylated, a monophosphorylated, a doubly and a triply phosphorylated isoforms, and the QLANT(p)PAK peptide present in all of them.



Note: The four 26kDa (from more basic to more acidic, figure 2) Coomassie-stained spots were excised from the 2D-electrophoresis gel, digested with trypsin and the resulting tryptic fragments were analyzed by MALDI-MS. As internal calibrants were used 100fmol of bradykinin and ACTH clip.

Figure 3. MALDI-MS spectra of the four 26kDa spots resolved on the 2D-PAGE.

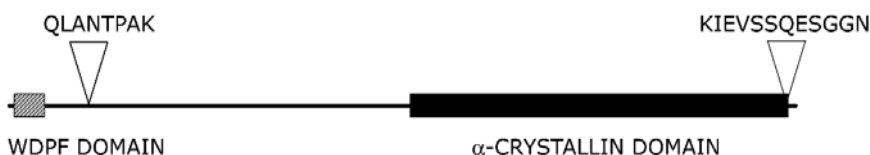
Analysis of the primary sequence of Hsp26 using NetPhosK 1.0 [41] at www.cbs.dtu.dk/services/NetPhosK/ predicted that T41 in QLANTPAK is a putative phosphorylation site for cdk5, a cyclin-dependent protein kinase closely related to yeast pho85 (56% identity and 72% similarity at the primary amino acid level) [42] (Huang et al., 1999). S207 located in peptide 202-KIEVSSQESWGN-213 was predicted to be phosphorylated by a member of the ATM protein kinase family and/or by the related protein kinase DNAPK. In yeast these kinase protein families include rad3p, Mec1p, Tel1p, Tor1p and Tor2 [43]. S210, on the other hand, was marginally ranked as a putative substrate for casein kinase I. The low score obtained and the fact that casein kinase I is a phosphate-directed kinase acting on proteins previously phosphorylated by other kinases [44] (Gross and

Anderson, 1998) makes improbable that this protein kinase could be the physiological kinase acting on S210, once KIEVSSQES(p)WGN is present at the native Hsp26. Note worthily, ATM protein kinase, DNAPK and pho85 are stress-responsive kinases.

```

SFNSPFEDFF DNINNEVDAF NRLLEGGLR GYAPRRQLAN TPAKDSTGKE 50
VARPNNYAGA LYDPRDETLD DWFDNDLSLF PSGFGFPRSV AVPVDILDHD 100
NNYELKVVPV GVKSKKDIDI EYHQNKQIL VSGEIPSTLN EESKDKVKVK 150
ESSSGKFGRV ITLPDYPGVD ADNIKADYAN GVLTLTVPKL KPQKDGKNHV 200
KKIEVSSQES GGN

```



Note: **A.** Yeast Hsp26 sequence (SwissProt accession number P15992) is depicted with the phosphorylated peptides (in bold) and functional domains WPDF at N-terminal region and α -crystallin domain (at the C-terminal region). **B.** Schematic representation of Hsp26 displaying its functional domains and phosphorylation sites.

Figure 4. Location of the phosphorylated peptides in the primary sequence of yeast Hsp26.

The significance of Hsp26 phosphorylation is not clear. QLANTPAK sequence is located near the WPDF motif found in N-terminus of Hsp26 (figure 4) and other sHSPs, which is believed to be responsible for the multimerization of those proteins [33]. On the other hand, KIEVSSQESWGN is placed proximal to the α -crystallin domain, responsible for dimerization [33]. Thus, phosphorylation of these peptides in Hsp26 could potentially regulate the association-dissociation of oligomers and modulated its chaperone activity. In hamster Hsp27, the phosphorylation of the Ser90, but not of the Ser15, is responsible for the dissociation of the 700kDA oligomer to dimers, decreasing its chaperone activity. In *Saccharomyces cerevisiae* [11], it was observed a temperature-induced shift from oligomers to dimers on the Hsp26 structure, with a concomitant increase in its chaperone activity, differently from the described for other sHSPs. If phosphorylation of Hsp26 has an effect on the association-dissociation of Hsp26 oligomer and/or changes Hsp chaperone activity is an open question.

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