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Macrophage Migration Inhibitory Factor: Cytokine, Hormone, or Enzyme?

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Contents

1 Introduction

Macrophage migration inhibitory factor (MIF) is a widely expressed protein that is secreted in response to inflammatory or hormonal stimuli. Studies with anti-MIF antibodies indicate that neutralization of MIF activity has therapeutic benefits in a number of animal models of inflammatory diseases (Bernhagen et al. 1993; Mikulowska et al. 1997; Lan et al. 1997; Makita et al. 1998; Leech et al. 1998). MIF is postulated to function as a cytokine or protein hormone via a receptor-mediated mechanism, yet a cell surface receptor has not been identified. Structural (Sun et al. 1996; Suzuki et al. 1996b; Kato et al. 1996) and biochemical (Rosengren et al. 1996; Rosengren et al. 1997; Kleeman et al. 1998) studies support an enzymatic function for MIF, yet its physiological substrate also has not been identified. This review describes recent progress in the biology, biochemistry, and structural properties of MIF, and discusses the potential link between the putative active site and cytokine-like properties.

The inhibition of macrophage migration is considered one of the earliest cytokine activities to be identified. This activity was associated with delayed-type hypersensitivity reactions (George and Vaugn 1962) and attributed to a non-dialyzable secretion product from activated T cells (David 1966; Bloom and Bennett 1966). Over the next twenty five years macrophage migration inhibition was found to correlate with general macrophage activation functions such as enhanced cell adhesion, phagocytosis, and tumoricidal activity (Churchill et al. 1975; Nathan et al. 1971; Nathan et al. 1973). These early studies on MIF used conditioned media from activated T cells which contained other proteins (interferon-γ and IL-4) that also exhibited macrophage migration inhibition activity (Thurman et al. 1985; Herriot et al. 1993). Consequently, the biological activities and physiological functions first assigned to MIF are questionable. It was not until 1989, when the cDNA for MIF was cloned that a more rigorous analysis of its biological, biochemical, and biophysical properties could be made (Weiser et al. 1989).

Some aspects of MIF biology are unlike those of other cytokines. MIF has been found in a variety of organs including the pituitary (Bernhagen et al. 1993), pancreas (Waeber et al. 1997), brain (Nishibori et al. 1996; Nishibori et al. 1997; Bacher et al. 1998), kidney (Lan et al. 1996), testes (Meinhardt et al. 1996), and ovaries (Suzuki et al. 1996a; Wada et al. 1997). The protein/mRNA also has been found in early embryos and may have a role in development (Suzuki et al. 1996a). Moreover, homologues of MIF have been identified in species such as *C. elegans* and *A. thaliana*, which would not be expected to express a typical cytokine. In contrast to other cytokines that are synthesized and secreted in response to external stimuli, MIF is constitu-

tively expressed in the cytoplasm and secreted upon appropriate stimulation. In some cells, the protein is localized within secretory granules (Nishino et al. 1995). In others, the mechanism by which it is exported from the cell remains unknown, as MIF does not possess a signal sequence to direct its secretion. These observations suggest that this cytokine has unique properties and may have roles outside the immune system.

MIF possesses a bewildering variety of activities (Table 1). The most unusual activity of this putative cytokine/hormone is its ability to catalyze a number of chemical reactions. In the course of studying melanin biosynthesis, Rorsman and colleagues fortuitously discovered that MIF could catalyze the tautomerization of the non-natural *D*-isomer of 2-carboxy-2,3-dihydroindole-5,6-quinone (*D*-dopachrome) to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Rosengren et al. 1996). In an effort to identify a physiological substrate or ligand for MIF, the same group determined that MIF could catalyze the enolization of phenylpyruvate and the ketonization of *p*-hydroxyphenylpyruvate (Rosengren et al. 1997). Bernhagen and his coworkers recently reported that MIF catalyzes the reduction of disulfides in insulin and small molecular weight substrates via transhydrogenase reactions (Kleeman et al. 1998a). The three-dimensional structure of MIF is unlike any other cytokine (Sun et al. 1996; Suzuki et al. 1996b; Kato et al. 1996),

Table 1. Activities of Macrophage Migration Inhibitory Factor

Cytokine Activities	Reference
Macrophage Migration Inhibition	Weiser et al. 1989
Macrophage Phagocytosis	Onodera et al. 1997
Macrophage Killing of Intracellular Parasites	Juttner et al. 1998
Neutrophil Priming	Swope et al. 1998
Regulation of T cell Growth	Bacher et al. 1996
Inhibition of Natural Killer Cell-Mediated Cell Lysis	Apte et al. 1998
Regulation of IgE Synthesis	Mikayama et al. 1993
Hormone Activities	
Counter-regulation of Glucocorticoid-Induced Cytokine suppression	Calandra et al. 1995
Potentiation of Glucose-Induced Insulin Secretion	Waeber et al. 1997
Inhibition of Inhibin Synthesis	Meinhardt et al. 1996
Catalytic Activities	
D-Dopachrome Tautomerase	Rosengren et al. 1996
Phenylpyruvate Tautomerase	Rosengren et al. 1997
Thiol Protein Oxidoreductase	Kleeman et al. 1998

but bears striking similarity to the global architecture and local active site of two microbial enzymes (Subramanya et al. 1996). These biological and structural observations have raised the intriguing possibility that MIF may have a dual role as a cytokine/hormone and enzyme. The suggestion also has been made that an enzymatic activity may underlie some of the immunological activities that have been described thus far (Swope et al. 1998b; Kleeman et al. 1998). This review discusses the cytokine, hormone, and enzymatic activities of MIF (Table 1), and expands upon the unique structural and enzymatic properties of this protein. For more detailed information on the biological activities of MIF the reader is referred to several excellent reviews (Bernhagen et al. 1998; Metz and Bucala 1997; Bucala 1996).

2 MIF as a Cytokine: Cellular Sources and Effectors

Cytokines play a pivotal role in the regulation of the inflammatory and immune responses due to their effects on leukocytes. These proteins are produced and secreted in response to external insults and act locally on effector cells through autocrine or paracrine mechanisms. Due to the transient nature of their production as well as their localized action, the levels of cytokines in the serum are normally low. These proteins function to regulate the growth, differentiation, and activities of immune cells via receptor-mediated processes. To understand how MIF came to be viewed as a cytokine, it is important to consider both its source of production as well as its function on cells of the immune system.

2.1 Monocytes and Macrophages

For almost 25 years, MIF was considered to be exclusively a T cell product that acted on macrophages. Recent studies have led to the discovery that the macrophage is an important source of MIF during immune reactions (Calandra et al. 1994). High levels of pre-formed MIF are found in unstimulated macrophages and monocytes. MIF is released from these cells upon activation by a variety of pro-inflammatory stimuli such as tumor necrosis factor (TNFα), interferon-γ (IFNγ), lipopolysaccharide (LPS), toxic shock syndrome toxin-1 (TSST-1), streptococcal pyrogenic exotoxin A, and malaria pigment (Calandra et al. 1994; Calandra and Bucala 1996; Calandra et al. 1998). The production of MIF in response to these pro-inflammatory stimuli follows a bell shaped dose-response curve. This suggests that MIF is necessary for initiating an immune response, and that higher concentrations

of pro-inflammatory stimuli or MIF itself, acting through a negative feedback loop, down-regulate MIF production.

MIF activity has had a longstanding association with the delayed-type hypersensitivity (DTH) response. A detailed analysis of the DTH response utilizing RT-PCR, immunohistochemical analyses, and ELISA assays indicated that MIF is present in macrophages and that the macrophage, rather than the T cell, is the major source of MIF during DTH reactions (Bernhagen et al. 1996). To assess the role of MIF in DTH, mice treated with anti-MIF antiserum showed significantly reduced DTH reaction in the classical tuberculin test.

Recent studies support a role for MIF in macrophage activation as was first reported using T cell supernatants. MIF mRNA is up-regulated and protein released in an *in vitro* model of phagocytosis by macrophages (Onodera et al. 1997). Addition of latex beads to macrophages results in a marked increase of MIF release. Increasing concentrations of exogenous recombinant MIF resulted in enhanced phagocytosis of the latex beads by the macrophages. These studies indicate that MIF can regulate macrophage function by both autocrine and paracrine mechanisms. MIF is also very effective in activating macrophages to kill the intracellular parasite *L. major* (Juttner et al. 1998). This effect can be completely blocked by anti-MIF antibody. The MIF-mediated killing of parasites appears to require both TNF-α and nitric oxide. Anti-TNFα antiserum was shown to reduce MIF-mediated macrophage killing of parasites. Macrophages deficient in the TNF receptor p55 (from knockout mice) were unable to destroy parasites in response to MIF. A specific inhibitor of inducible nitric oxide synthase (iNOS), L-N6-(1-iminoethyl)lysine dihydrochloride, also inhibited the antiparasitic properties of MIF. MIF has been shown to induce TNFα secretion and nitric oxide production (when co-stimulated with IFNγ) from macrophages (Herriott et al. 1993; Calandra et al. 1994). It is likely that these cytokines (MIF, TNFα, and IFNγ) act together in a pro-inflammatory loop to activate the macrophage and coordinate host defenses against infection or tissue invasion.

Interestingly, pro-inflammatory molecules are not the only stimuli that induce the release of MIF from macrophages. Physiological concentrations of anti-inflammatory glucocorticoids result in the secretion of MIF, the only cytokine to be up-regulated in this way by glucocorticoids (Calandra et al. 1995). At higher pharmacological concentrations of glucocorticoids, MIF secretion is turned off. The release of MIF by endogenous levels of glucocorticoids leads to a reversal of steroid-induced suppression of cytokine (TNFα, IL-1β, IL-6, and IL-8) synthesis. The observation that MIF is induced by glucocorticoids and, in turn, suppresses glucocorticoid activity has led

Bucala and his colleagues to propose that MIF functions as the physiological counter-regulator of glucocorticoids.

2.2 T Lymphocytes

Activated T cells have been known to be a source of MIF activity since 1966 (David 1966; Bloom and Bennett 1966). The cDNA for the protein was eventually isolated from a lectin-stimulated T cell hybridoma (Weiser et al. 1989). To examine whether MIF displays any autocrine functions on T cells, MIF expression and the effects of anti-MIF antibodies in activated T cells were studied. The most prevalent mechanism of T cell activation is based on stimulation of the T cell receptor (TCR) by antigen presented by the major histocompatibility complex (MHC). This results in secretion of the potent T cell mitogen IL-2 and in up-regulation of the IL-2 receptor, leading to T cell proliferation. Antibodies to the signaling component (CD3) of T cell receptors can also induce T cell activation. Superantigens such as TSST-1 can activate T cells by cross-linking MHC molecules with some T cell receptors (Marrack and Kappler 1990; Kappler et al. 1989). Stimulation of primary T cells with anti-CD3 antibody or TSST-1 was found to induce MIF mRNA and protein secretion (Bacher et al. 1996; Calandra et al. 1998). MIF released by activated T cells could be neutralized with an anti-MIF IgG. Addition of anti-MIF antibodies to stimulated T cells decreased proliferation by 40–60%. For comparison, addition of anti-IL-2 antibodies to neutralize the classical T cell growth factor (IL-2) had a more pronounced effect, decreasing proliferation by 70–75%. Addition of anti-IL-2 and anti-MIF antibodies did not act synergistically (Bacher et al. 1996). Upon further study, it was determined that the reduction in T cell proliferation is likely due to the decrease in IL-2 secretion from activated T cells in the presence of anti-MIF antibodies. It appears, therefore, that T cell activation results in secretion of MIF, which in turn contributes to the secretion of the T cell mitogen IL-2. Interestingly, exogenous MIF has no measurable effect on resting or stimulated T cells.

The *in vivo* role of MIF in lymphocyte function during the immune response was examined employing anti-MIF antibodies. Pre-injection of anti-MIF antibody two hours before injection of TSST-1 minimized spleen enlargement and reduced proliferation of splenocytes *ex vivo* (Calandra et al. 1998). The injection of a typical antigen (as opposed to a superantigen such as TSST-1) will normally provoke the production of antigen-specific T cells and antibodies. Treatment of mice with anti-MIF antibodies during and after injection of antigen resulted in marked attenuation of this primary immune response (Bacher et al. 1996). A decrease of antigen-specific IgG and a reduction in antigen-specific proliferation of splenic T cells were ob-

served. A role for MIF in the development of humoral immunity is supported by expression studies with T_H1 and T_H2 T cell subsets. While both of these cell types release a basal level of MIF, secretion is increased only by activated T_H2 clones, which favor antibody responses. The *in vivo* suppression of humoral immunity by anti-MIF antibodies appears to be in direct contrast to the role of MIF in cell-mediated immunity observed in DTH reactions. Further studies are necessary to address this paradox.

The unique relationship between MIF and glucocorticoids is also evident in T cells. Physiological concentrations of glucocorticoids induce MIF secretion from T cells (Bacher et al. 1996). Recombinant MIF overrides the inhibition of proliferation and cytokine synthesis of stimulated T cells treated with high doses of glucocorticoids.

Glycosylation-inhibiting factor, a protein associated with a controversial issue in cellular immunology involving antigen specific T-cell factors (Ishizaka et al. 1996), has the same amino acid sequence as MIF (Mikayama et al. 1993). GIF inhibits N-glycosylation of IgE binding factors. The unglycosylated IgE binding factors are involved in regulating the production of IgE by selectively suppressing IgE synthesis. Interestingly, while many types of cells express and secrete GIF/MIF, only suppressor T cells are reported to produce bioactive GIF. The active form of GIF is speculated to be due to an uncharacterized post-translational modification that occurs only in suppressor T cells. It is known that this modification does not involve phosphorylation or N-glycosylation (Liu et al. 1994). The modification presumably induces an active conformation that is not present in the unmodified protein (Sugie et al. 1997). T helper and natural killer cells are reported to be the only cellular targets of bioactive GIF (Sugie et al. 1997).

2.3 Neutrophils and Eosinophils

The release of MIF and other cytokines in response to pathogens results in an activated immune system and an enhanced inflammatory response. In the early phase of the immune response, neutrophils are induced to migrate to the site of infection and are one of the first cell types to arrive. Although one report has noted the absence of MIF in murine neutrophils (Calandra et al. 1994), we found that MIF mRNA and protein is present in unstimulated human neutrophils (M. Swope and E. Lolis, unpublished data). Moreover, MIF can act by an autocrine mechanism to prime neutrophils for an enhanced respiratory burst upon stimulation with the formylated peptide fMLP (Swope et al. 1998a). Unprimed neutrophils from human blood produce almost no oxygen radicals when treated with the formylated peptides. These peptides, found only in bacteria, are one of the key recognition mole-

cules used by the human immune system to identify microbes. The ability of MIF to deliver a priming signal to neutrophils so that they are mobilized to produce an immediate and robust response in the presence of pathogens suggests that MIF may play an important role in initiating the immune response.

A recent study indicates that MIF is important in eosinophil-related inflammatory disorders such as asthma (Rossi et al. 1998). Unstimulated eosinophils contain significant quantities of MIF in the cytoplasm. Stimulation by phorbol esters or the physiological pro-inflammatory molecules IL-5 or C5a induces secretion of MIF from these cells. Moreover, MIF levels are elevated in the bronchoalveolar lavage fluid of asthmatic patients as compared to controls, suggesting that MIF may play a role in asthma and other pulmonary inflammatory diseases.

2.4 The Eye and Natural Killer Cells

Significant amounts of MIF are expressed in the eye lens (Wistow et al. 1993; Matsuda et al. 1996; Matsuda et al. 1997). The presence of pro-inflammatory molecules in the eye is a paradox because the eye is an immune-privileged site where immune-mediated inflammation is suppressed to prevent damage to ocular tissues. MIF has been proposed to function as an immunosuppressive cytokine that contributes to immune privilege (Apte et al. 1998). Specifically, MIF in the aqueous humor inhibits natural killer (NK) cell-mediated lysis of corneal endothelial and lens epithelial cells, which lack MHC class I molecule and would therefore be vulnerable to destruction by NK cells.

MIF may also have additional non-immune functions in the eye. The expression of MIF correlates with the development of the eye lens, being present in the chicken embryo from 6 to 19 days after fertilization (Wistow et al. 1993). RT-PCR of embryonic chicken lens microdissected into the inner epithelium (enriching for proliferating cells), outer epithelium (enriching for differentiating cells), and differentiated fiber cells revealed a strong association of MIF mRNA expression with differentiating cells. Wistow *et al.* speculate that MIF in the eye may therefore be involved in cell growth and differentiation.

3 MIF as a Hormone: Cellular Sources and Effectors

Protein hormones are induced by physiologic variations, rather than external stimuli, and function to maintain homeostasis. The biosynthesis of hormones normally occurs within endocrine organs, and their cellular targets

are located at distant locations. Consequently, protein hormones are present in the serum at higher concentrations than cytokines.

While these criteria may indicate there are real distinctions between cytokines and hormones, in practice, there appears to be a great deal of overlap between these proteins. For example, protein hormones (such as prolactin and growth hormone) are induced during systemic inflammatory reactions and can have localized immunoregulatory effects (Weigent 1996). Alternatively, some cytokines that down-regulate the immune system or migrate to the bone marrow to function as hematopoietic growth factors can be considered hormones that maintain the homeostasis of the immune system.

3.1 Corticotrophic and Thyrotrophic Cells of the Anterior Pituitary

The hypothalamic-pituitary-adrenal axis plays a central role in neuroendocrine interactions and is critical in mediating the host response to systemic stress. The pituitary is an endocrine organ that secretes follicle-stimulating hormone (FSH), luteinizing hormone, adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), growth hormone, and prolactin. The release of ACTH results in the secretion of cortisol from the adrenal cortex, which acts as a modulator of the systemic stress response and the host response to infection. Cortisol and other glucocorticoids have powerful anti-inflammatory effects. This class of compounds is a mainstay of pharmacological therapy in the treatment of severe inflammatory diseases.

Investigators noted that unlike many other systems in which homeostasis is maintained by both positive and negative regulators, there were no known negative regulators of the anti-inflammatory activities of glucocorticoids. When searching for negative regulators of glucocorticoid anti-inflammatory activities, MIF was found to be secreted from a pituitary cell line upon stimulation with lipopolysaccharide (LPS) (Bernhagen et al. 1993). Analysis of intact pituitary revealed that MIF protein is pre-formed and comprises ~0.05% of total pituitary protein content. In comparison, ACTH and prolactin comprise 0.2% and 0.08%, respectively, of total pituitary protein. MIF is localized within three subtypes of secretory granules in both cortocotropic (ACTH-releasing) and thyrotropic (TSH-releasing) cells: granules with ACTH and MIF, TSH and MIF, or MIF alone (Nishino et al. 1995). The release of MIF from corticotrophic cells is stimulated by the hypothalamic hormone corticotrophin-releasing factor (CRF) in a dose-dependent manner, resulting in a concomitant increase in serum MIF above basal levels (MIF normally circulates at 2–4 ng/ml in human serum) (Nishino et al. 1995). More importantly, the concentrations of CRF needed to stimulate the release of MIF are lower than those needed for release of ACTH, resulting in

the discharge of MIF prior to ACTH. *In vivo* studies in rodents confirm that MIF is secreted from the pituitary during stress or LPS stimulation and results in increased serum levels with an accompanying decrease in pituitary MIF (Calandra et al. 1995). Hypophysectomized mice injected with LPS have no detectable serum MIF at a time that MIF levels are highest in control mice, indicating that the pituitary is the major source of serum MIF during systemic inflammatory responses. Finally, MIF has been shown to potentiate the lethal effects of endotoxemia when co-injected with a sub-lethal dose of LPS (Bernhagen et al. 1993). The increased serum levels of MIF in response to systemic infection or stress suggest that MIF is an important component of the hypothalamic-pituitary-adrenal response.

The observation that exposure of the pituitary to inflammatory stimuli could result in the release of both pro-inflammatory MIF from the pituitary and anti-inflammatory glucocorticoids from the adrenal cortex led to experiments to examine potential biological interactions of these two hormonal mediators. As mentioned previously, Bucala and co-workers determined that MIF is the only cytokine whose production and secretion is stimulated rather than inhibited by physiological concentrations of glucocorticoids. Moreover, the released MIF acts to antagonize the anti-inflammatory effects of glucocorticoids ultimately resulting in increased synthesis of cytokines. Therefore, the intensity of an inflammatory reaction depends on the balance between anti-inflammatory glucocorticoids and pro-inflammatory MIF.

3.2 Pancreatic β Cells

The expression and secretion of MIF from the anterior pituitary suggested that MIF might play a much larger role as a protein mediator within the endocrine system. This is borne out by the presence of MIF mRNA and protein in pancreatic islet β cells. Immunohistochemical studies showed that MIF is present in cells expressing insulin and the glucose transporter GLUT2 (Waeber et al. 1997). Subcellular localization studies also indicate that MIF co-localizes with insulin in secretory granules of the highly differentiated, insulin-producing cell line INS-1. MIF mRNA levels increased in INS-1 cells and in primary β cells in response to 20–30 mM glucose. To probe a possible autocrine function of MIF in islet β cells, recombinant MIF was added to β cells and found to increase (by 140%) glucose-induced insulin secretion. INS-1 cells transfected with MIF antisense cDNA or treated in tissue culture with anti-MIF IgG had the opposite effect: they significantly reduced (by 30–50%) the secretion of insulin induced by glucose. MIF is therefore the first protein mediator to be released by β cells that positively regulates insulin secretion. Bucala and colleagues speculate that in this context, a decrease in

MIF secretion by β cells may contribute to diminished insulin release that is associated with type II diabetes.

3.3 Ovarian and Testicular Cells

MIF is present in reproductive organs of rodents (Meinhardt et al. 1996; Suzuki et al. 1996a). In the female mouse, MIF mRNA has been found in the ovary, oviduct, and uterus. The mRNA levels change in the uterus of the pregnant mouse, suggesting that MIF expression is regulated by endocrinological changes during pregnancy (Suzuki et al. 1996a). MIF has also been found in the Leydig cells of the rat testis (Meinhardt et al. 1996). Neither recombinant MIF nor neutralizing polyclonal anti-MIF antibody has any *in vitro* effect on testosterone biosynthesis, the primary function of Leydig cells. MIF may, nonetheless, be involved in the physiology and regulation of testicular function. Recombinant MIF has been shown to decrease the production of inhibin by Sertoli cells of the testicular seminiferous epithelium. Inhibin is the primary regulator of the gonadotrophic hormone FSH produced by the anterior pituitary. While the physiological role of MIF in reproductive physiology remains to be determined, the observation that MIF is present in reproductive organs of both sexes and affects inhibin biosynthesis suggests that it may play an important role as a regulatory hormone.

4 MIF in Disease

4.1 Infectious Diseases and Sepsis

Gene-transfer experiments and studies with anti-MIF antibodies have been instrumental in identifying diseases in which MIF participates. For example, cytokines (MIF, TNFα, IFNγ, or IL-2) delivered in a sustained fashion by oral administration of an attenuated *S. typhimurium* strain transfected with individual cytokine genes have been used to study their roles in *L. major* infection (Xu et al. 1998). BALB/c mice are normally susceptible to *L. major*, eventually dying from the infection. Prophylactic treatment of mice with the MIF-expressing *S. typhimurium* clone 1 week prior to infection with *L. major* significantly delayed disease progression, demonstrating a protective role for MIF in this animal model of infection. Experiments were also performed to test the therapeutic effectiveness of these cytokines. BALB/c mice were first infected with *L. major* and treated after one week with different combinations of *S. typhimurium* clones expressing MIF, TNFα, IFNγ, and IL-2. Clones expressing a combination of MIF and TNFα provided the best therapeutic benefit – reduced lesion development and parasite burden –

compared to individual treatments or all possible pairings of the four cytokines. This benefit correlated with the expression level of inducible nitric oxide synthase from spleen and mesenteric lymph node cells of infected mice.

While the above experiment probes the role of MIF expression in a localized infection, anti-MIF antibodies have been used to study the role of MIF in systemic infection (Bernhagen et al. 1993; Calandra et al. 1998). The injection of a high dose of LPS or TSST-1 in mice induces all of the symptoms of bacterial septicimia and leads to death. The lethal effects of sepsis (in these animal models as well as in the actual disease in humans) are known to be mediated in large part by the systemic secretion of cytokines (such as TNFα and IL-1β). Co-injection of antibodies or antagonists of these sepsis-mediating cytokines protects mice from LPS-induced lethality (Tracey et al. 1987; McNamara et al. 1993). Similarly, pre-treatment of mice with anti-MIF antibodies results in a dramatic increase in survival (Bernhagen et al. 1993; Calandra et al. 1998).

The effects of sepsis can also be produced in animals by injection of high doses of the pro-inflammatory cytokines TNFα or IL-1β (Tracey et al. 1986, Okusawa et al. 1988). In contrast to the lethal effects of these cytokines, the administration of high doses (10–50 mg/kg) of MIF alone is not fatal. Co-injection of LPS (at a dose that would normally result in 30% lethality) with MIF potentiates the effects of endotoxemia, resulting in the survival of only 10–15% of the mice (Bernhagen et al. 1993). The potentiation of the effects of LPS by MIF suggests that LPS induces the production of a co-factor that, together with MIF, is lethal.

The counter-regulation of glucocorticoid activity by MIF observed *in vitro* is also evident *in vivo*. Early administration of glucocorticoids can protect against the lethal effects of endotoxemia, presumably by suppressing cytokine production. Co-injection of recombinant MIF and glucocorticoids in a mouse model of sepsis abrogated the protective effect of the glucocorticoids (Calandra et al. 1995). These studies demonstrate that MIF is a member of the cytokine network leading to death during endotoxemia. They also suggest that inhibition of MIF activity can increase the therapeutic benefit of glucocorticoid treatment in inflammatory diseases.

4.2 Adult Respiratory Distress Syndrome

Acute respiratory distress syndrome (ARDS) is a life-threatening inflammatory response that occurs in the lungs following acute trauma or sepsis. This condition is mediated by activated neutrophils and results in the breakdown of the endothelial and epithelial membranes of the lung leading to a reduc-

tion in arterial oxygen tension, pulmonary capillary pressure, and leakage of protein-rich fluid into the air space. In addition, patients with ARDS have elevated levels of pro-inflammatory cytokines in the alveolar airspace. In an *in vivo* experiment to study lung injury, anti-MIF antibody reduced the accumulation of neutrophils in the lung (Makita et al. 1998). This effect is believed to be indirect, as MIF is not chemotactic for neutrophils (M. Swope and E. Lolis, unpublished observations). These rats were found to have significantly reduced levels of the neutrophil chemoattractant macrophage inflammatory protein-2/cytokine-induced neutrophil chemoattractant (MIP-2/CINC-3) in bronchoalveolar lavage fluid, suggesting that MIF is involved in the up-regulation of this chemokine during lung injury.

A role for MIF in ARDS is also supported by a recent study with humans. ARDS patients contain elevated levels of MIF in the alveolar airspace due to release by alveolar macrophages (Donnelly et al. 1997). Furthermore, the addition of exogenous MIF to alveolar cells harvested from ARDS patients resulted in increased TNFα and IL-8 production while the addition of anti-MIF antibodies to these cells inhibited their production. Finally, MIF was shown to over-ride the inhibitory effects of glucocorticoids in ARDS alveolar cells. These data suggest that MIF secreted by alveolar macrophages during ARDS could sustain the inflammatory reaction by inhibiting the therapeutic effects of endogenous or pharmacological glucocorticoids.

4.3 Autoimmune Diseases: Rheumatoid Arthritis and Glomerulonephritis

The therapeutic benefits of anti-MIF antibodies on sepsis and lung injury prompted studies into the role of MIF in other inflammatory diseases. The inflammatory and autoimmune disease rheumatoid arthritis (RA) was hypothesized to be subject to the effects of MIF based on the importance of macrophages and T cells, two target cells of MIF, in mediating this disease. In an animal model that closely resembles RA, macrophages process and present collagen type II to T cells resulting in a T cell mediated immune response against collagen that damages joints. Experiments to determine the role of MIF in this animal model of RA have demonstrated that neutralization of MIF with anti-MIF antibodies during the immunization phase delays the onset and reduces the number of mice that develop the disease (Mikulowska et al. 1997). The severity of the disease in those mice that do develop arthritis, however, is undiminished relative to control mice. Nor does administration of anti-MIF antibodies after the disease has developed have any significant effects. The physiological mechanism by which MIF is involved in this autoimmune disease remains to be determined. However,

the study by Mikulowska et al demonstrated that anti-MIF reduced the production of IgG2a antibodies, a Th1-mediated isotype suggested to be highly arthritogenic. MIF may therefore be involved in the up-regulation of IgG2a during an immune response.

In a second rodent model (rat adjuvant arthritis) of rheumatoid arthritis, MIF was absent from the synovium of normal rats (Leech et al. 1998). However, significant levels were present a few days after injection of heat-inactivated *M. tuberculosis* to induce arthritis. The appearance of MIF in the synovium preceded the clinical symptoms of arthritis. Moreover, rats with arthritis had elevated levels of MIF in the serum. Neutralization of MIF by a high, non-toxic dose of anti-MIF antibodies had profound effects on the course of the disease. Intraperitoneal injection of the antibodies every 3 days (from 0 to 12 days after adjuvant injection) suppressed all clinical symptoms of arthritis in 5 of 6 rats on day 13, a time at which all control rats had developed arthritis. Treatment with a lower dose decreased the effect, but still reduced the severity of arthritis. These studies indicate that induction of MIF is an important pro-inflammatory event in the induction of arthritis, and inhibition of MIF activity can ameliorate the symptoms of this disease.

The role of MIF was investigated in a second autoimmune disease that also is mediated by macrophage infiltration and accumulation. A rat model of accelerated antiglomerular basement membrane glomerulonephritis was used to determine the role of MIF in the onset and severity of the disease (Lan et al. 1997). MIF up-regulation in the kidney had been observed during development of rat glomerulonephritis (Lan et al. 1996). This disease has an early phase characterized by deposition of antibody and complement followed by infiltration of neutrophils, and a progressive phase characterized by macrophage and T cell infiltration. Renal injury during the progressive phase is believed to be mediated by IL-1β production from macrophages because treatment with IL-1 receptor antagonist suppresses tissue injury (Lan et al. 1995; Lan et al. 1993). Pre-treatment of animals with anti-MIF antibodies had no effect on the early phase, but significantly inhibited the progressive phase of the disease (Lan et al. 1997). This effect was due to a combination of reduced infiltrating macrophages and T cells, decreased activation of remaining infiltrating macrophage and T cells, and inhibition of IL-1β and iNOS expression in both the infiltrating macrophages and kidney cells.

5 Enzymatic and Structural Properties of MIF

5.1 Enzymatic Activities

While most of the studies described above are consistent with a cytokine function for MIF, the catalytic activities and three-dimensional structure are inconsistent with a role as a cytokine. The first report of a catalytic activity

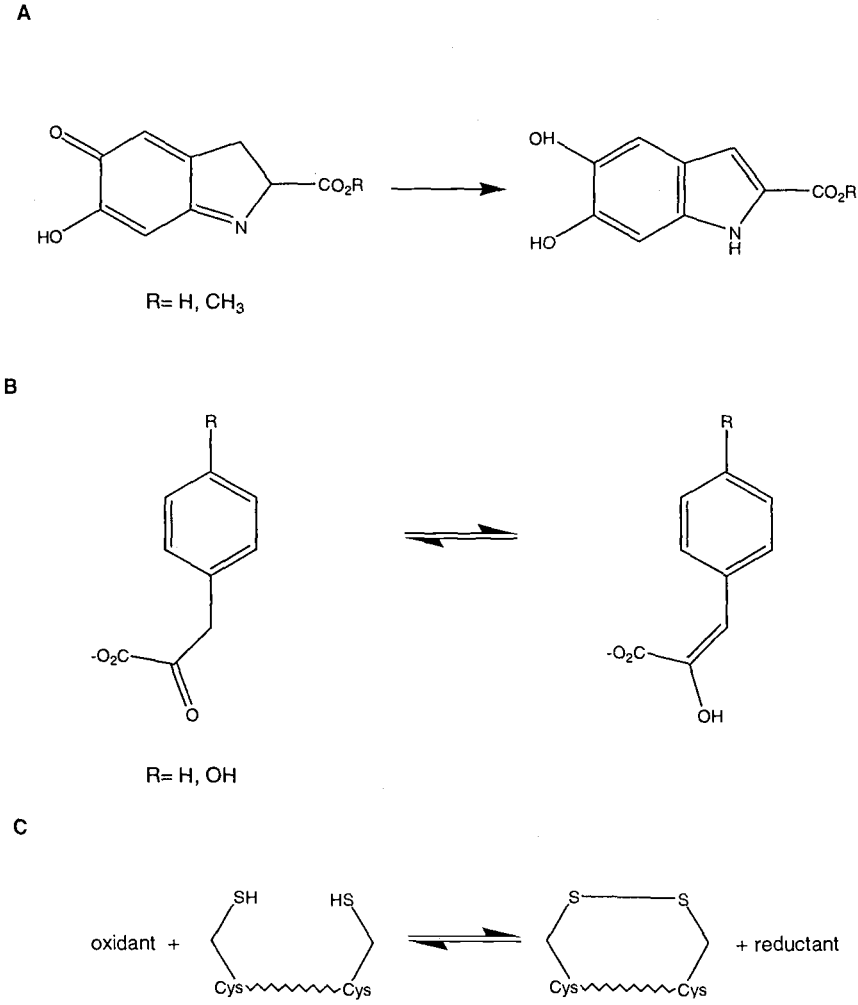

Fig. 1. Chemical reactions catalyzed by MIF. (**A**) *D*-dopachrome tautomerase (Rosengren et al. 1996). (**B**) Phenylpyruvate tautomerase (Rosengren et al. 1997). (C) Thiol-protein oxidoreductase (Kleeman et al. 1998a; Kleeman et al. 1998b)

for MIF was the result of a search to identify novel glutathione S-transferase isozymes (Blocki et al. 1992), but this was eventually shown to be an experimental artifact (Muhlhahn et al. 1996; Swope et al. 1998b). MIF has also been reported to possess three additional catalytic activities: *D*-dopachrome tautomerase (Rosengren et al. 1996), phenylpyruvate (and hydroxyphenylpyruvate) keto-enol isomerase (Rosengren et al. 1997), and thiol-protein oxidoreductase (Kleeman et al. 1998a) (Fig. 1).

The late stages of the melanin biosynthetic pathway involves the enzymatic conversion of 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by a membrane-localized enzyme. The natural substrate for this reaction is the *L*-stereoisomer. In the course of studying the enzymatic reaction in cultured melanoma cells, a catalytic conversion to DHICA was observed when the non-physiological *D*-dopachrome was used as a control substrate. In contrast to the reaction of the natural isomer which is associated with a membrane protein, the catalytic activity for *D*-dopachrome was present in the cytoplasm. Two proteins were eventually isolated that were responsible for the catalytic conversion of the non-physiologic substrate: MIF and a novel protein provisionally called *D*-dopachrome tautomerase. MIF and *D*-dopachrome tautomerase share 27% sequence identity (Zhang et al. 1995).

In an attempt to identify natural ligands for MIF, the phenylpyruvate tautomerase activity was discovered (Rosengren et al. 1997). In this reaction, MIF catalyzes the keto-enol isomerization of both *p*-hydroxyphenylpyruvate and phenylpyruvate. These molecules are products of phenylalanine and tyrosine degradation and were observed in studies more than 40 years ago (Knox and Pitt 1957). Although MIF can act as a phenylpyruvate tautomerase, neither hydroxyphenylpyruvate nor phenylpyruvate is proposed to be the true physiological substrate for MIF because the measured K_M values are too high in comparison with the reported physiological concentrations (Rosengren et al. 1997; Deutsch 1997). It should be noted, however, that K_M values are not good indicators of the physiological relevance of substrate-enzyme pairs. Additional studies are required to address the significance of the phenylpyruvate tautomerase activity of MIF.

Analysis of mammalian MIF sequences revealed a conserved CXXC sequence motif. This same sequence has been shown to be part of the active site for thiol-protein oxidoreductases such as thioredoxin (Takahashi et al. 1996), protein disulfide isomerase (Puig et al. 1994), and DsbA (Zapun et al. 1994). The oxidoreductase activity in these enzymes is dependent on the formation and reduction of a disulfide bridge between the cysteine residues in the conserved sequence motif. Based on these observations, MIF was assayed and found to catalyze the reduction of disulfides in insulin and 2-

hydroxyethyldisulfide. One paradox with this activity is that glutathione, which has been shown to have weak, if any binding to MIF (Muhlhahn et al. 1996), can serve as a co-substrate to provide the reducing equivalents (Kleeman et al. 1996a; Kleeman et al. 1996b).

5.2 Three-Dimensional Structure

We determined the three-dimensional structure of human MIF by X-ray crystallography (Sun et al. 1996a). Additional X-ray and NMR studies were independently reported by others (Suzuki et al. 1996b; Kato et al. 1996; Muhlhahn et al. 1996). All high resolution studies found MIF to be a trimer of identical subunits with overall dimensions of 35 Å x 50 Å x 50 Å. The MIF monomer contains two antiparallel α helices and six β strands, four of which form a mixed β sheet (Fig. 2A). Three monomers associate to assemble an α/β structure consisting of six α helices surrounding three β sheets that form a barrel with a solvent accessible channel (Fig. 2B). The tertiary structure of the MIF monomer is deceivingly reminiscent of the IL-8 dimer and

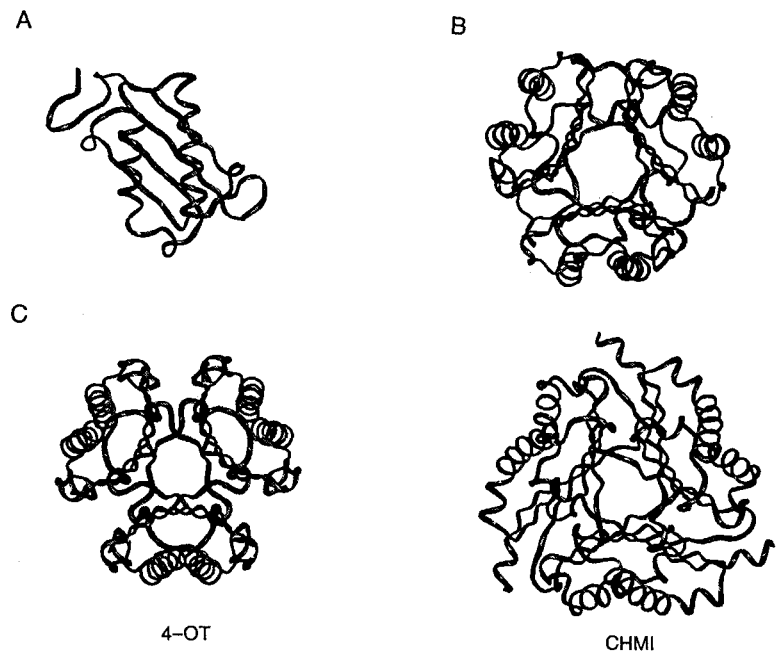

Fig. 2. Three dimensional ribbon structure of (A) the MIF monomer, (B) the MIF trimer, (C) the 4-oxalocrotonate tautomerase (4-OT) hexamer and the 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) trimer

MHC. However, the quaternary structures and the topology (the sequential arrangement of the secondary structures) of the monomers for these three proteins are totally different. Suzuki et al. noted a striking structural similarity of MIF to two microbial enzymes, 4-oxaocrotonate tautomerase (4-OT) and 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) (Fig. 2C) (Subramanya et al. 1996; Suzuki et al. 1996b). Chorismate mutase from *B. subtilis* is more distantly related to MIF and is not considered to be a member of the evolving superfamily (Chook et al. 1994; Murzin 1996). Both 4-OT and CHMI catalyze reactions in a pathway that converts aromatic hydrocarbons to intermediates which can enter the citric acid cycle (Hajipour et al. 1993). CHMI is a trimer with a central barrel of β sheets, while 4-OT is a hexamer of a single subunit consisting of 62 amino acids. Two monomers of 4-OT associate to form a dimer similar in structure to the MIF monomer. Three of these dimers form the hexamer with structural similarity to the MIF trimer. Although 4-OT, CHMI, and MIF share similar three-dimensional structures, no substantial sequence homology exists among the proteins.

The structural similarity of MIF to 4-OT and CHMI extends to the active site. Both 4-OT and CHMI possess unusual enzymatic mechanisms in that the N-terminal amine functions as a catalytic base. In most proteins, the N-terminus (as well as C-terminus) protrudes from the protein and is usually flexible. In 4-OT and CHMI, the N-terminal residue is a proline (after the initiating methionine is cleaved) and sits at the base of a hydrophobic pocket (Subramanya et al. 1996). MIF also has a proline as its N-terminal residue, which is found in a hydrophobic cleft. The importance of the N-terminal proline in MIF is highlighted by multiple sequence alignment of all MIF homologues (Swope et al. 1998a). The N-terminal proline is among only 11 residues (10% of the sequence) that are invariant (Fig. 3A). Display of the other invariant residues on the three-dimensional structure of MIF shows that many of these residues cluster around the N-terminal proline and illustrates the evolutionary pressure to preserve this site (Fig. 3B). The residues do not form part of the hydrophobic core of the protein, but rather are solvent accessible. The solvent accessible cleft with the N-terminal proline at its base resembles a catalytic active site that could accommodate small molecule substrates.

The catalytic activities of MIF, the remarkable structural similarity to microbial enzymes, and the pattern of invariant residues prompted further studies to more fully characterize the putative active site of MIF. The substrates phenylpyruvate, *p*-hydroxyphenylpyruvate, and *D*-dopachrome were useful probes in this regard. As an initial experiment to determine the resi-

A

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                *     **                          **                    *
  Human MIF  PMFIVNTNVP RASV.PDGFL SELTQQLAQA TGKPPQYIAV HV.VPDQLMA GGSSEPCAL
  Bovine MIF PMFVVNTNVP RASV.PDGLL SELTQQLAQA TGKPAQYIAV HV.VPDQLMT GGSSEPCAL
    Rat MIF  PMFIVNTNVP RASV.PEGFL SELTQQLAQA TGKPAQYIAV HV.VPDQLMT RGTSDPCAL
  Murine MIF PMFIVNTNVP RASV.PEGFL SELTQQLAQA TGKPAQYIAV HV.VPDQLMT SGTNDPCAL
Chicken MIF  PMFTIHTNVC KDAV.PDSLL GELTQQLAKA TGKPAQYIAV HI.VPDQMMS GGSTDPCAL
   Human DT  PFLELDTNLP ANRV.PAGLE KRLCAAAASI LGKPADRVNV TVR.PGLAMA SGSTEPCAQ
     Rat DT  PFVELETNLP ASRI.PAGLE NRLCAATATI LDKPEDRVSV TIR.PGMTLL NKSTEPCAH
  Arab. MIF  PCLNLSTNVN LDGVDTSSIL SEASSTVAKI IGKPENYVMI .VLKGSVPMS GGTEDPAAY
  C. el.MIF  PMVRVATNLP NEKV.PVDFE IRLTDLLARS MGKPRERIAV EIAAGARLV. GATHDPVTV
     Bm MIF  PYFTIDTNIP QNSI.SSAFL KKASNVVAKA IGKPESYVSI HVNVGGQAMV GGSEDPCAV
     Wm MIF  PYFTIDTNKP QDSI.SSAFL KKAPNVVPKA .GKPESYVSI HVN.GGQPMV GGSEDPCPV

                ***         *              *
  Human MIF  CSLHSIGKIG GA.QNRSYSK LLCGLLAERL RISPDRVYIN YYDMNAANVG NNSTFA*..
  Bovine MIF CSLHSIGKIG GA.QNRSYSK LLCGLLTERL RISPDRIYIN FCDMNAANVG NGSTFA...
    Rat MIF  CSLHSIGKIG GA.QNRNYSK LLCGLLSDRL HISPDRVYIN YYDMNAANVG NGSTFA*..
  Murine MIF CSLHSIGKIG GA.QNRNYSK LLCGLLSDRL HISPDRVYIN YYDMNAANVG NGSTFA*..
Chicken MIF  CSLYSIGKIG GQ.QNKTYTK LLCDMIAKHL HVSADRVYIN YFDINAANVG NGSTFA*..
   Human DT  CSISSIGVVG TARDNRSHSK HFFEFLTKEL ALGQDRILIR FFPLESWQIG IGTVMTFL.
     Rat DT  LLISSIGVVG TAEQNRSHSA SFFKFLTEEL SLDQDRIIIR FFPLEPWQIG KGTVMTFL.
  Arab. MIF  GELVSIGGLN AD.VNKKLSS AVSAILETKL SVPKSRFFLK FYDTKGSFFG NGATL....
  C. el.MIF  ISIKSIGAVS AE.DNIRNTA AITEFCGKEL GLPKDKVVIT FHDLPPATVG NGTTVAEA.
     Bm MIF  CVLKSIGCVG PK.VNNSHAA KLYKLLADEL KIPKNRCYIE FVDIEASSMA NGSTLLG..
     Wm MIF  CVLKSIGCVG PK.VNNSHAE KLYKLLADEL KIPKNRCYIE SVDIEASSMA NGSTFG...
```

B

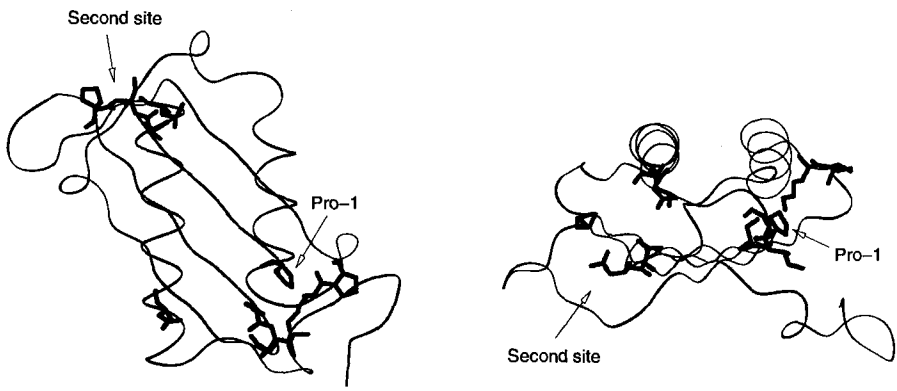

Fig. 3. (A) Multiple sequence alignment of MIF homologues. Amino acid residues that are invariant are indicated by an asterisk (*). DT, *D*-dopachrome tautomerase; Arab, *Arabidhopsis thaliana*; C. el., *Caenorhabditis elegans*; Bm, *Brugia malayi*; Wm, *Wuchereria bancrofti*. (B) Invariant residues of MIF. The atoms of the invariant residues are shown in the context of the ribbon diagram of the MIF monomer. The two orientations are related by a 90° rotation along horizontal axis

dues that interact with the substrates, we titrated *p*-hydroxyphenylpyruvate into ^{15}N-labeled MIF and monitored the change in chemical shift for each residue in a heteronuclear quantum correlation (HSQC) NMR experiment. The result from this experiment indicated that the N-terminal proline and

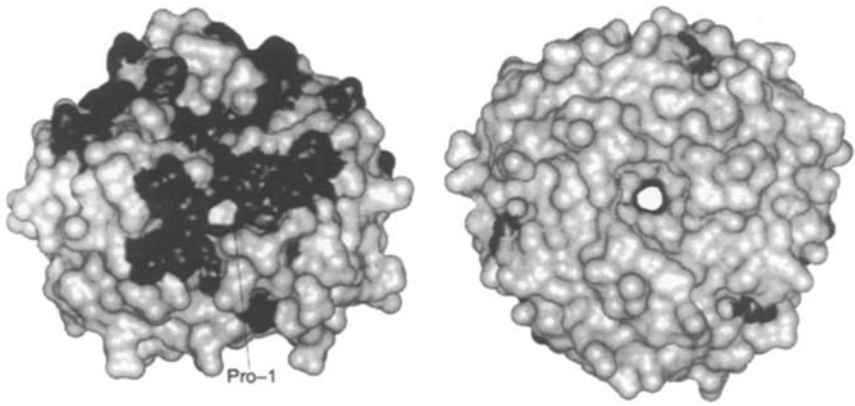

Fig. 4. Residues that are perturbed by the presence of *p*-hydroxyphenylpyruvate (HPP). The solvent accessible surface of two orientations of the MIF trimer is shown. The orientation on the right shows the solvent accessible channel coincident with the 3-fold molecular axis. This view is rotated 125° along the horizontal axis relative to the orientation on the left. MIF residues with perturbed chemical shifts from a ^{1}H-^{15}N HSQC NMR spectrum in the presence of excess of HPP are drawn in black, and Pro-1 of a single subunit is indicated (Swope et al. 1998a)

many of its surrounding residues were perturbed upon addition of *p*-hydroxyphenylpyruvate (Fig. 4). This experiment also provided evidence that the catalytically active form of MIF is comprised of a trimer, as two residues adjacent to the N-terminus that are perturbed by *p*-hydroxyphenylpyruvate are from another subunit (Swope et al. 1998a). We have now embarked on crystallographic studies between MIF and *p*-hydroxyphenylpyruvate to study the interactions between these two molecules at atomic resolution and decipher the structural source of catalysis.

Further evidence for the importance of the N-terminal proline in the catalytic activity of MIF has been provided by a number of labs including our own (Bendrat et al. 1997; Swope et al. 1998a; Stamps et al. 1998). Deletion or replacement of the N-terminal proline abrogates the catalytic activity of MIF (Bendrat et al. 1997; Swope et al. 1998a). Treatment of MIF with 3-bromopyruvate, an irreversible inhibitor of 4-OT, also inactivates MIF catalytic activity (Stamps et al. 1998). The 3-bromopyruvate is covalently attached to a site within an 11 residue amino terminal fragment, presumably Pro-1. While the importance of the N-terminus in the catalytic activity is certain, the actual mechanistic role for the proline remains to be resolved. Bendrat et al. suggest that Pro-1 may serve as a general acid catalyst based on modeling of the dopachrome substrate in the active site. Other studies indicate that Pro-1 can function as a general base (Swope et al. 1998a; Stamps et al. 1998).

For the nitrogen atom of a proline to serve as a catalytic base, the lone pair electrons on the nitrogen must be available for proton abstraction. For this requirement to be met, two criteria must be satisfied. The proline must be present at the amino terminus – as it is for 4-OT, CHMI, and MIF – so that the electrons are not involved in resonance stabilization of a peptide bond. The pKa of the secondary amine of the proline, which is normally greater than 9 (Stivers et al. 1996), also must be significantly lowered such that the proline remains uncharged at physiological pH. To investigate the possibility that the N-terminal proline of MIF functions as a catalytic base, its pKa was determined. We used NMR spectroscopy to directly titrate the N-terminal proline. The ^{15}N chemical shift of the proline is easily resolvable from all other resonance peaks. Direct measurement of this chemical shift as a function of pH reveals a pKa of 5.6 ± 0.1 (Swope et al. 1998a), almost 4 pH units lower than the pKa of a proline amide (Stivers et al. 1996). Whitman and co-workers determined the pKa of MIF by use of k_{cat}/K_M profiles as well as irreversible inactivation by the active site-directed inhibitor 3-bromopyruvate as a function of pH (Stamps et al. 1998). These studies measure the pKa values of the uncomplexed protein and substrate (or inhibitor). The pKa based on the pH dependence of k_{inact}/K_I for irreversible inhibition and of k_{cat}/K_M for the enolization of phenylpyruvate are 5.7 ± 0.2 and 6.0 ± 0.1, respectively, in reasonable agreement with the pKa of Pro-1 determined directly by NMR. As with 4-OT and CHMI, the hydrophobic environment at the N-terminus is believed to be responsible for the reduced pKa of the amine. Formation of a positively charged amine in this environment is disfavored in the absence of a negatively charged counter-ion. Careful inspection of the three-dimensional structure of MIF provides an alternative explanation for the reduced pKa. An electrostatic potential map reveals a region of positive potential arising from Lys-32 and Lys-66 that surrounds the N-terminal proline (Swope et al. 1998a). These two lysines sit at the entrance of the cleft. Formation of a positive charge in a region of positive potential without a neutralizing counter-charge is energetically unfavorable. The relative contribution of the hydrophobic pocket and the positive electrostatic potential on the reduced pKa of the N-terminal proline remains to be determined.

The thiol-protein oxidoreductase activity is far more difficult to understand in the context of the three-dimensional structure. Bernhagen and his co-workers present a series of experiments that can be interpreted in terms of disulfide bond formation between Cys-57 and Cys-60 (Kleeman et al. 1998a). This disulfide is required for the oxidoreductase activity. Mutation of Cys-60 (but not Cys-57) to serine abolishes the oxidoreductase activity. The lack of catalytic activity for this mutant correlates with the absence of bio-

logical activity as measured by a macrophage-mediated killing assay of *L. major* parasites. In the crystal structure of MIF, Cys-57 and Cys-60 are buried in the core of the protein and do not form a disulfide bond. The absence of a disulfide is not due to a reducing environment as no reducing agents were present during purification and crystallization of the protein (Sun et al. 1996b). It is interesting to note, however, that Cys-57 and Cys-60 are in close proximity to a second site containing invariant residues (Fig. 3B).

6 Conclusions and Future Directions

While much has been learned about MIF and its role in disease, there are large gaps in our understanding of what this protein does and how it does it. Our knowledge regarding the physiological role of MIF is based predominantly on inhibition studies with anti-MIF antibodies. It has not been possible to address the physiological function of MIF by studying MIF knockout mice as attempts to create $MIF^{-/-}$ mice have been unsuccessful thus far (Kobayashi et al 1988). Ultimately, homologous recombination to generate $MIF^{-/-}$ mice and/or mice with temporal or cell-restricted MIF mutations will allow the physiological role of this protein to be studied. The presence of MIF homologues in *C. elegans* and *A. thaliana* offers alternative model organisms for studying the physiological function of MIF.

At the molecular level, the structural biology of this protein has outpaced the mechanistic studies required to understand how this protein functions. Questions are being asked regarding the roles of residues in catalysis or in receptor binding without having identified either a natural substrate or cell surface receptor involved in MIF biology. This reflects both the difficulty in identifying substrates or receptors for enzymes or cytokines/hormones, respectively, and the power of the structural approach to infer function (in this case, an enzymatic activity) from structural relationships among proteins. Until we identify a physiological substrate and/or a receptor for MIF, we will not be able to achieve a comprehensive understanding of how this protein works. Nor will we be able to resolve the dilemma of whether MIF is an enzyme or a cytokine/protein hormone.

The potential link between MIF cytokine activities and the invariant catalytic site is also unclear. Bucala and colleagues report that a catalytically inactive Pro-1 mutant is still capable of overriding the inhibition of TNFα production by glucocorticoids (Bendrat et al. 1997). We have reported that a catalytically inactive Pro-1 mutant of MIF has substantially reduced capacity to prime neutrophils (Swope et al. 1998a). The relationship between the catalytic and cytokine activities is more than an academic question. The

answer to this question has implications for the discovery and development of small molecule inhibitors that mimic the effects of anti-MIF antibody. There are many examples of small molecule inhibitors that target enzyme active sites, but very few that target the receptor-binding surface of cytokines. Enzymatic catalysis occurs at a localized site within a protein surface. Binding between a cytokine/protein hormone and receptor occurs over a large surface area with the participation of many residues (de Vos et al. 1992). Consequently, it is easier to identify small molecules that bind within the active sites of enzymes rather than small molecules that disrupt the large surface areas involved in cytokine-receptor interactions. If the N-terminal site is actually involved in the biological activity of MIF, then it should be fairly straightforward to identify small molecule inhibitors that bind to this site. Indeed, some low molecular weight compounds with micromolar inhibition constants have already been identified (Swope et al. 1998b; Stamps et al. 1998). Such small molecules may be useful as lead compounds to treat the diseases associated with MIF.

The possibility that MIF may catalyze an enzymatic reaction *and* bind to a cell surface receptor would not be unique. Over the last fifteen years, other proteins with "dual functions or mechanisms" have been identified (Table 2). Of particular note is the recruitment of a variety of enzymes that serve as structural proteins in the eye (Piatigorsky and Wistow 1989). These proteins are encoded by one gene, yet have two entirely different functions. The genes for these proteins are subject to two distinct selective pressures: to maintain the active site required for catalysis and to maintain the sites required for protein-protein interactions necessary for the optical properties of the lens. In light of the constitutive expression of MIF in the cytoplasm, it remains possible that this protein has an intracellular enzymatic function and a separate extracellular function when released under inflammatory or hormonal stimuli. The sites involved in each of these functions may arise from different regions of the protein surface. In this regard, it is interesting that the invariant residues cluster at two distinct sites (Fig. 3B).

For some proteins in Table 2, the enzymatic and cytokine activities are mediated by a single site on the molecular surface. The molecular mechanism involving the catalytic and cytokine activities for thrombin are best characterized. Thrombin functions to promote clot formation by producing fibrin and activating a G protein-coupled receptor to induce platelet aggregation. The proteolytic site of thrombin functions in both fibrin formation and activation of the receptor. Thrombin cleaves fibrinogen to fibrin and also cleaves a portion of the N-terminal region of the receptor to unmask a tethered ligand that induces self-activation (Vu et al. 1991).

Table 2. Enzymes with Non-enzymatic Functions or Mechanisms

Protein	Enzymatic Activity	Receptor	Cytokine or Non-ezymatic Activity	References
ε-Crystallin	Lactate dehydrogenase	None	Structural protein	Piatigorsky and Wistow 1989
τ-Crystallin	Enolase	None	Structural protein	Piatigorsky and Wistow 1989
Thrombin	Protease	Thrombin receptor	Platelet aggregation	Vu et al. 1991
Angiogenin	Ribonuclease	170 kDa, 49 kDa	Angiogenesis	Shapiro et al. 1989
GPI/AMF	Glucose-6-phosphate isomerase	AMF receptor	Cell motility	Chaput et al. 1988; Watanabe et al. 1996
Cyclophilin	Cis/trans proline isomerase	Unknown	Neutrophil chemotaxis	Sherry et al. 1992; Wu et al. 1992
FKBP	Cis/trans proline isomerase	Unknown	Eosinophil chemotaxis	Leiva and Lyttle 1992
NAP-2	Heparinase	CXCR2	Neutrophil chemotaxis	Hoogewerf et al. 1996
Factor Xa	Protease	EPR-1	Cytokine upregulation	Altieri and Stamnes 1994

The molecular components involved in angiogenin activity have not been well characterized, but the angiogenic activity of this protein also appears to require receptor binding and chemical catalysis (Shapiro et al. 1989). In contrast to thrombin, the uncharacterized cell surface receptor for angiogenin functions to transport the protein into the cell (Moroianu and Riordan 1994a). Angiogenin possesses a nuclear localization sequence that allows it to enter the nucleus (Moroianu and Riordan 1994b). Once there, angiogenin presumably degrades RNA to induce its biological activity, as RNAse-deficient mutants of angiogenin do not harbor angiogenic activity (Shapiro et al. 1989).

For the remaining proteins, the relationship of the catalytic and cytokine activities to the physiological function is less clear. The glycolytic protein

glucose-6-phosphate isomerase "moonlights" as a secreted hormone which binds with high affinity to the autocrine motility factor receptor (Chaput et al. 1988; Watanabe et al. 1996). A small molecule inhibitor of GPI blocks the interaction with the cell surface receptor indicating that the catalytic activity and receptor binding surface form an overlapping site (Watanabe et al. 1996). Similar observations have been made for cyclophilin and FKBP, two cis-trans proline isomerases that possess chemoattractant activities. In both cases, specific inhibitors of the cis-trans proline isomerization reaction block their chemoattractant activities (Sherry et al. 1992; Xu et al. 1992; Leiva and Lyttle 1992). NAP-2 is one of many chemokines that bind glycosamine proteoglycans and activate a specific G protein-coupled receptor (Cerretti et al. 1993). NAP-2 also possesses heparinase activity (Hoogewerf et al. 1996). Proteoglycans have been found to play a role in the biological activity of some chemokines, but the biological significance of the heparinase activity of NAP-2 is not yet known. The serine protease factor Xa is not only involved in the proteolytic coagulation cascade, but also binds to a specific cells surface receptor with high affinity and regulates T cell activation (Altieri and Stamnes 1994). The role of the proteolytic activity in this function is unknown. It remains to be seen whether MIF and these proteins represent a new class of biological molecules that possess dual mechanisms (receptor binding and chemical catalysis) or dual functions to mediate their physiological activities.

Postscript

After submission of this review but prior to its publication, a number of studies on the immunoregulatory and catalytic roles of MIF were reported that are worth noting. Basophils and mast cells can be added to the list of cells that constitutively express MIF at high levels (Chen et al. 1998). The high level expression in these cells implicates MIF as one of the key mediators of basophil and mast cell function in host defense. Further studies are required to address this role.

The gene for MIF finally has been disrupted in mice (Bozza et al. 1999). The mice appear to develop normally and have no organ abnormalities based on gross examination and histopathological analysis. The initial phenotypes of these $MIF^{-/-}$ mice support the conclusions of some *in vivo* studies with anti-MIF antibodies. Upon treatment with a high dose of LPS (or *S. aureus* enterotoxin B in conjunction with D-galactosamine), the mice exhibited signs of endotoxemia, but were remarkably resistant to its lethal effects compared to $MIF^{+/-}$ and wild-type mice. The $MIF^{-/-}$ mice have a marked reduction in plasma levels of TNF-α during endotoxemia, which may partly explain their ability to survive what would normally be a lethal

challenge. In an alternate model of gram-negative infection, *P. aeruginosa* were instilled into $MIF^{-/-}$, $MIF^{+/-}$, and wild-type mice. The infection was cleared from the lungs much more efficiently in the $MIF^{-/-}$ mice than in the control mice. Moreover, the $MIF^{-/-}$ mice had reduced levels of neutrophils in the bronchoalveolar lavage, which agreed with a previous study of the effect of anti-MIF antibodies on LPS-induced lung inflammatory disease (Makita et al. 1998).

A number of recent studies have focused on the catalytic properties of MIF. In an attempt to identify physiological substrates of the *D*-dopachrome tautomerase activity of MIF, the oxidized catecholamines 3,4-dihydroxyphenylaminechrome, epinephrinechrome, and norepinephrinechrome were found to be converted by MIF to 5,6-dihydroxyindole, 3,5,6-trihydroxyindole-1-methylindole, and 3,5,6-trihydroxyindole, respectively (Matsunaga et al. 1999). These products are precursors of neuromelanin, a pigment found in neurons and glial cells proposed to function as a sink for toxic metabolites of catecholamine biosynthesis. Consistent with this hypothesis, an inverse relationship has been found between neuromelanin content and neurological degeneration in Parkinson's disease. It is therefore possible that MIF contributes in the detoxification of these quinones in the brain.

Finally, we and others have been successful at determining crystal structures of small molecule ligands complexed to MIF. MIF has been co-crystallized with a substrate[1] and an inhibitor[2] of the phenylpyruvate tautomerase activity. Both studies find that the ligands interact with Pro-1, Lys-32, Ile-64, Tyr-95, and Asn-97. Pro-1 is positioned to function as a catalytic base. Interestingly, there is no functional group that polarizes the α-carbonyl of the substrate to weaken the adjacent C-H bond for transfer by Pro-1. The structures suggest a catalytic mechanism for this reaction, but the absence of a polarizing functional group also suggests that this substrate may not be a physiological substrate of an MIF-mediated enzymatic activity.

Acknowledgements

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Voltage-Dependent Calcium Channels: From Structure to Function

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Contents

Abbreviations used

HVA – high-voltage-activated
LVA – low-voltage-activated
DHP – dihydropyridine
PAA – phenylalkylamine
BTZ – benzothiazepine
HEK – human embryonic kidney
HP – holding potential
RyR – ryanodine receptor
SNAP – synaptosome-associated protein
ER – endoplasmic reticulum
VAMP – vesicle-associated membrane protein
SNARE – synaptic core complex
AID – alpha subunit interaction domain
BID – beta subunit interaction domain
IC_{50} – half-maximal inhibition concentration
AKAP – A-kinase anchoring protein
PKC – protein kinase C
FHM – familial hemiplegic migraine
EA – episodic ataxia

Voltage-activated calcium channels regulate the intracellular calcium concentration and contribute thereby to calcium signalling in numerous cell types. These channels are widely distributed in the animal kingdom and are an essential part of many excitable and non-excitable mammalian cells.The opening of these channels is primarily regulated by the membrane potential, but is also modulated by a wide variety of hormones, protein kinases, protein phosphatases, toxins and drugs. Site-directed mutagenesis has identified sites on these channels, which interact specifically with other proteins, inhibitors and ions. This article will focus on these recent developments. The older findings have been summarized in several excellent reviews (Striessnig et al. 1993; Hofmann et al. 1994; Catterall 1995; De Waard et al. 1996a).

A Subunit Composition of the Calcium Channel Complex and Genes

A.I.1 Subunit Composition of High-Voltage-Activated (HVA) Channels

HVA calcium channels are heterooligomeric complexes of five proteins from four genes (Fig. 1): the α_1 subunit, which contains the binding sites for all

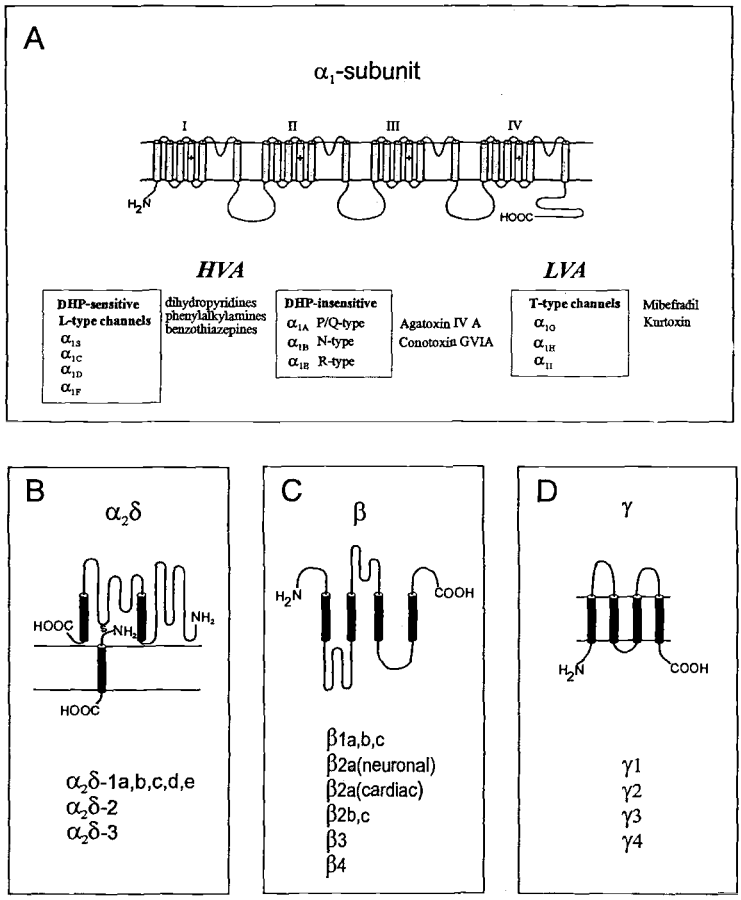

Fig. 1. Proposed structures of the calcium channel subunits. (A) Membrane topology of the pore-forming α_1 subunit, molecular diversity of the α_1 genes and pharmacological properties of the different classes. HVA, high voltage activated; LVA low voltage activated. (B), (C) and (D) Putative structures and genes of the accessory $\alpha_2\delta$, ß and γ subunits. Small letters indicate splice variants

known calcium channel blockers, the voltage-sensor, the selectivity filter and the ion-conducting pore; the intracellularly located ß subunit; the $\alpha_2\delta$ subunit, a disulfide-linked dimer, and the transmembrane γ subunit (for details see: Striessnig et al. 1993; Hofmann et al. 1994; Catterall 1995; De Waard et al. 1996a). The γ subunit is specifically expressed in skeletal muscle, but recently additional γ subunits have been detected in the retina, brain and other tissues (Letts et al. 1998; Klugbauer et al. 1999c).

A.I.2 Subunit Composition of Low-Voltage-Activated (LVA) Channels

The exact subunit composition of the LVA channels is unknown. Three α_1 subunits have been identified which induce large T-type current after expression in Xenopus oocytes and in HEK cells in the absence of additional subunits (Perez-Reyes et al. 1998a, b; Cribbs et al. 1998; Klugbauer et al. 1999b). Elimination of the four known ß subunits in neurones of the nodose ganglion and in a neuroblastoma cell line by transfection with antisense oligonucleotides did not affect the size or voltage-dependence of the T-type current (Lambert et al. 1997; Leuranguer et al. 1998). Wyatt and coworkers (1998), who overexpressed the $\alpha_2\delta$-1 and the neuronal $ß_{2a}$ subunits in undifferentiated NG108-15 cells, reported that the T-type current is affected by the $\alpha_2\delta$-1 but not by the $ß_{2a}$ subunit. However, the $\alpha_2\delta$-1or $\alpha_2\delta$-3 subunits did not modulate the T-type current when coexpressed with the α_{1G} subunit (Lacinová et al. 1999). LVA channels are possibly composed of a single α_1 subunit protein which contains the voltage-sensor, the selectivity filter, the ion-conducting pore as well as the binding site for the T-type channel blockers mibefradil and kurtoxin.

A.II Genes

A.II.1 The α_1 Subunit

Most of the prominent features of the calcium channel complex can be assigned to the α_1 subunit. The α_1 subunit contains the ion conducting pore, the selectivity filter of the pore, the voltage sensor and the interaction sites for the ß subunits, the ßγ subunits of G proteins, the $\alpha_2\delta$ subunit, the calcium channel blockers and activators. Nine individual genes have been identified for the α_1 subunit, which are homologous to each other and encode proteins of predicted molecular masses of 212 to 273 kDa. They belong to the same multigene family as voltage-activated sodium and potassium channels and share a common ancestral protein with them. Hydrophobicity analysis of the α_1 subunits predicts a transmembrane topology with four homologous repeats, each containing five hydrophobic putative α helices and one amphiphatic segment (Fig. 1).

An early evolutionary event separated the α_1 subunits into the electrophysiologically distinct low-voltage-activated (LVA) and high-voltage-activated (HVA) calcium channels, which share less than 30% sequence identity. The two LVA genes G and H induce T-type currents in the absence of additional subunits (Perez-Reyes et al. 1998a; Cribbs et al. 1998). A third LVA channel, an α_{1I}, has been identified (Perez-Reyes et al. 1998b). An event

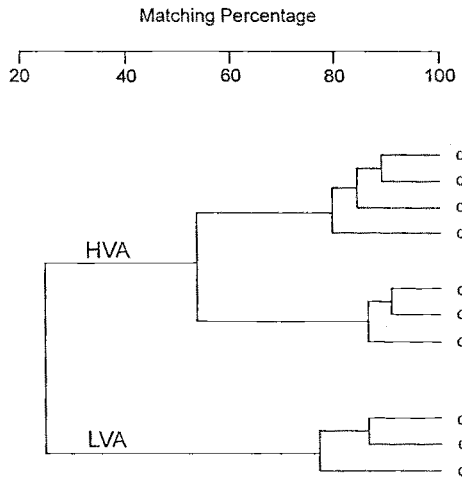

Fig. 2. Phylogenetic comparison of the α_1 subunits based on the primary structure alignment of the membrane spanning regions. Matching percentage was calculated using the program CLUSTAL

which occurred later separated the HVA-channels again in two subfamilies, the four (C, D, F, S) dihydropyridine (DHP)-sensitive and the three (A, B, E) DHP-insensitive calcium channels. The A, B and E genes are expressed almost exclusively in neuronal tissues. Both groups share about 50% identical amino acids, whereas the amino acid identity of the individual members of each subfamily is generally over 60% (Fig. 2).

The native currents of the HVA calcium channels have been subdivided into five distinct classes by biophysical and pharmacological criteria: L (long-lasting)-type, P (Purkinje)-type, N (neither L nor T channel)-type, Q-type and R (remaining)-type channels. The P-, Q-, N- and R-type currents have been mainly identified in neuronal and endocrine tissues, whereas L-type current (α_{1C}, α_{1D}, α_{1S}, α_{1F}) have been found in skeletal, heart and smooth muscles, in fibroblasts and kidney, but also in neuronal and endocrine tissues. N-type current is mediated by α_{1B} channels and is blocked specifically by ω-conotoxin GVIA (100–500 nM) and MVIIC (> 100 nM). P-type current is mediated by α_{1A} channels and is blocked by the funnel web spider toxin ω-agatoxin (< 10 nM) and ω-conotoxin MVIIC (5–30nM) (Sather et al. 1993; Zhang et al. 1993; McDonough et al. 1996). The Q -type current, which may be mediated by α_{1A} channels, is blocked by ω-conotoxin MVIIC (> 100 nM) and ω-agatoxin IVA (> 10 nM) (Sather et al. 1993; Zhang et al. 1993). L-type channels are readily blocked by the three groups of classical calcium channel blockers, i.e., the dihydropyridines, phenylalkylamines and benzothiazepines. The functional role of L-type channels is rather diverse. In skeletal muscle they are crucial for excitation-contraction (e–c) coupling, which does not require an influx of calcium through the channel (Rios et al. 1992). In

heart, they are necessary for the generation of electrical impulses and for the initiation of contraction in atrial and ventricular muscle and in the smooth muscle they are involved in tension development. In neurons, L-type channels provide the calcium for activation of small conductance, calcium-activated K^+ channels (Marrion et Tavalin 1998). They do not participate in neurotransmitter secretion, a process which is linked in many neuronal cells to N- and P/Q-type channels. R-type channels (α_{1E}) have been identified by cloning as a major neuronal calcium channel (Niidome et al. 1992; Soong et al. 1993; Schneider et al. 1994).

A.II.1.1 The L-Type α_1 Channels

The class S α_1 gene

The complete cDNA sequence of the class S gene was originally cloned from rabbit skeletal muscle (Tanabe et al. 1987). Two isoforms of this calcium channel type can be identified in rabbit skeletal muscle: a 212 kDa polypeptide equivalent to the full length calcium channel transcript and a smaller 190 kDa protein, which is derived from the full length product by posttranslational proteolysis. This short form represents about 95% of the total α_{1S} calcium channel protein (De Jongh et al. 1991) (Fig. 3a).

The class C α_1 gene

The class C gene is expressed in heart and smooth muscle and in endocrine and neuronal cells. The human gene for the α_{1C} subunit is localized to the distal region of chromosome 12p13 (Schultz et al. 1993). The gene spans about 150 kb and is composed of 44 invariant and more than 6 alternative exons (Soldatov 1994). The α_1 subunit of the cardiac ($\alpha_{1C\text{-}a}$; Mikami et al. 1989) and smooth muscle ($\alpha_{1C\text{-}b}$; Biel et al. 1990) calcium channel differ only at four sites and share 95% identical amino acids. Molecular analysis showed that the alternatively spliced exon 8, which codes for the IS6 segment, is differentially expressed in cardiac and vascular smooth muscle and is responsible, in part, for the different DHP sensitivity of the cardiac and vascular smooth muscle L-type current (Welling et al. 1997). Two additional splice forms have been cloned from rat brain (Snutch et al. 1991) and human heart (Schultz et al. 1993). The brain clone is identical with the $\alpha_{1C\text{-}b}$ sequence but lacks the insert in the repeat I/II loop and contains a 3 amino acid insert in the repeat II/III loop and a 20 amino acid extension at the carboxy terminus. The human heart clone has a short amino terminus (start at Met 60 of the $\alpha_{1C\text{-}a}$) and an insert of 71 amino acids after Ser 1643 of the $\alpha_{1C\text{-}a}$ clone.

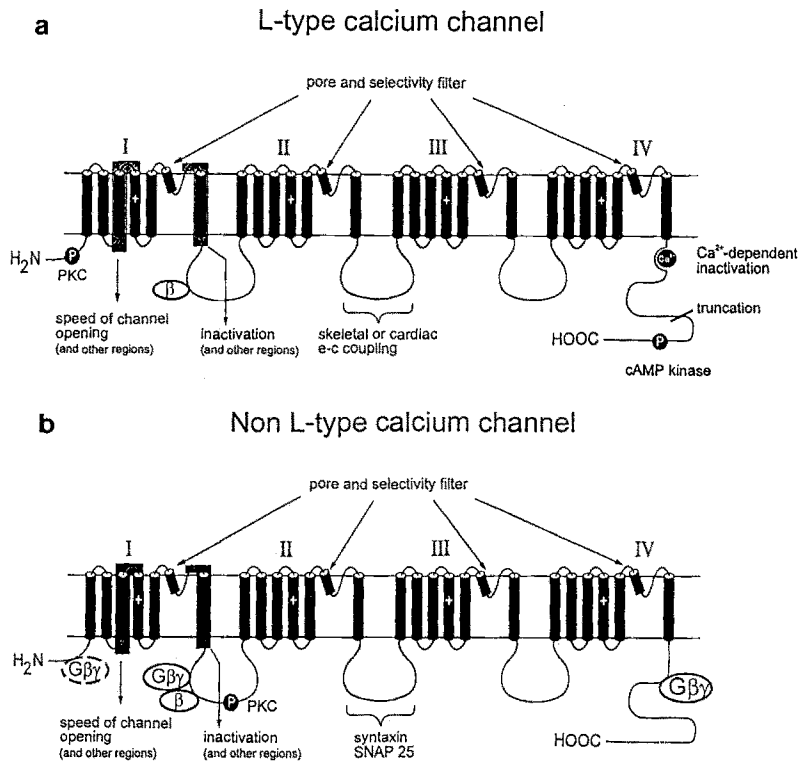

Fig. 3. Suggested topology of the L-type (**a**) and non L-type (**b**) calcium channel α_1 subunits. The putative transmembrane configuration is based on the hydrophobicity analysis of the primary structure. The amphipathic segment that forms the voltage sensor of the channel is indicated by a +. The channel pore and selectivity filter is formed by a short α–helical segment and the connecting loop between this region and the sixth transmembrane segment. Grey boxes indicate regions involved in activation or inactivation kinetics. (P) indicates sites for cAMP kinase or protein kinase C (PKC). e–c coupling, excitation-contraction coupling; ß, binding site for the calcium channel ß subunit; Gßγ, interaction sites with G protein ßγ subunit; Ca^{2+}, Ca^{2+} dependent inactivation. Syntaxin and SNAP25, binding sites for synaptic membrane proteins

The class D α_1 gene

The cDNA of the class D gene was isolated from neuronal and endocrine tissues and represents a neuroendocrine-specific L-type calcium channel (Williams et al. 1992b; Seino et al. 1992). Expression of α_{1D} cDNA in different host cells resulted only in a small dihydropyridine sensitive inward current,

indicating that the native channel may contain an additional, so far unknown subunit.

The class F α_1 gene

Analysis of the locus for the incomplete form of X-linked congenital stationary night blindness (CSNB2) identified mutations in a new L-type calcium channel α_1 subunit as the cause of the disease (Strom et al. 1998; Bech-Hansen et al. 1998). The gene for the α_{1F} subunit is localized at Xp11.23. The F channel shows a 55–62% overall amino acid sequence identity with other L-type calcium channel α_1 subunits. Apparently, this channel is expressed specifically in the retina and required for optimal night vision.

A.II.1.2 The Non L-Type α_1 Channels

The class A α_1 gene

Transcripts of the class A channel are present at high levels in the mammalian brain and peripheral nervous system (Mori et al. 1991; Starr et al. 1991). Because the α_{1A} transcripts are expressed in many neurons shown to possess P- and Q-type channels and because the properties of α_{1A} exhibits similarities to both of these channels (Stea et al. 1994), the class A cDNA is referred to as P/Q-type calcium channel (Fig. 3b).

The class B α_1 gene

The class B gene has been cloned exclusively from brain (Williams et al. 1992a; Dubel et al. 1992; Fujita et al. 1993). Expression studies using dysgenic myotubes or *Xenopus* oocytes revealed that α_{1B} induced a barium current which is inhibited by low concentrations of ω-conotoxin GVIA (Fujita et al. 1993; Williams et al. 1992a). These results identify the α_{1B} channel as the neuronal N-type calcium channel. The α_{1B} subunit also binds ω-conotoxin GVIA with high affinity (Dubel et al. 1992) at the extracellular side (Ellinor et al. 1994). Chimeras between the α_{1B} and α_{1A} subunit indicated that each repeat contributes to the binding pocket with the pore region of repeat III being the most important determinant (Ellinor et al. 1994).

The class E α_1 gene

The sixth gene has been cloned from rat, rabbit and human brain libaries (Niidome et al. 1992; Soong et al. 1993; Williams et al. 1994; Schneider et al. 1994). Initially, this channel was characterized as a LVA T-type channel (Soong et al. 1993). However, later studies showed (Williams et al. 1994;

Schneider et al. 1994) that the expressed α_{1E} channel has the activation and inactivation kinetics of a HVA neuronal channel. The human and rat α_{1E} currents have some properties in common with the R-type currents observed in cerebellar granule cells (Ellinor et al. 1993; Randall and Tsien 1997).

A.II.1.3 The Low Voltage-Activated α_1 Channels

The class G, H and I genes

The recently cloned class G and H α_1 subunits are LVA calcium channels, which have the basic electrophysiological characteristics of T-type channels (Perez-Reyes et al. 1998a; Cribbs et al. 1998). The G gene localizes to human chromosome17q22 and is expressed strongly in brain and less abundantly in heart. The expressed channel has a single channel conductance of 7.7 pS in 115 mM Ba^{2+}. The Ca^{2+} current through expressed α_{1G} is blocked half maximally by Ni^{2+} at 1.1 mM. The mibefradil block is slightly voltage dependent with IC_{50} values of 0.4 μM and 0.1 μM at holding potentials (HPs) of −100 mV and −60 mV, respectively (Klugbauer et al. 1999b). The H gene localizes to the human chromosome 16p13.3 and is expressed strongly in kidney, at intermediate levels in heart and at low abundance in brain. The expressed channel has a single channel conductance of 5.5 pS and is blocked by mibefradil with an IC_{50} of 1.4 μM at HP −90 mV (Cribbs et al. 1998). The expressed α_{1G} subunit is inhibited with high affinity by the scorpion toxin "kurtoxin" (Chuang et al. 1998).

A.II.2 Auxiliary Subunits of the Calcium Channel

A.II.2.1 The $\alpha_2\delta$ Subunit

The skeletal muscle $\alpha_2\delta$-1 subunit is a highly glycosylated membrane protein of 125 kDa (Ellis et al. 1988). The protein is posttranslationally cleaved to yield a disulfide-linked α_2 and δ protein (for older literature see Hofmann et al. 1994; Catterall 1995; De Waard et al. 1996a). The δ part anchors the α_2 protein to the α_1 subunit via a single transmembrane segment, whereas the α_2 protein is localized extracellularly. This membrane topology of the $\alpha_2\delta$ subunit was confirmed and further refined (Wiser et al. 1996; Gurnett et al. 1996, 1997; Felix et al. 1997). Recent evidence suggests that part of the $\alpha_2\delta$-1 subunit still binds to the plasma membrane after deletion of the transmembrane segment of the δ protein (Brown and Gee 1998). Extensive splicing of the $\alpha_2\delta$ subunit results in at least five different isoforms, which are expressed in a tissue-specific manner (Angelotti and Hofmann 1996). Two additional

$\alpha_2\delta$ genes, i.e., $\alpha_2\delta$-2 and $\alpha_2\delta$-3 have been identified recently (Klugbauer et al. 1999a). The primary structures of the novel $\alpha_2\delta$-2 and $\alpha_2\delta$-3 subunits are about 50% and 30% identical to the $\alpha_2\delta$-1 subunit, respectively. Northern blot analysis indicates that $\alpha_2\delta$-3 is expressed exclusively in brain, whereas $\alpha_2\delta$-2 is found in several tissues including heart and $\alpha_2\delta$-1 is expressed ubiquitously. In situ hybridization of mouse brain sections showed mRNA expression of $\alpha_2\delta$-1 and $\alpha_2\delta$-3 in the hippocampus, cerebellum and cortex, with $\alpha_2\delta$-1 strongly detected in the olfactory bulb and $\alpha_2\delta$-3 in the caudate putamen. The number of putative glycosylation sites and cysteine residues, hydropathicity profiles and electrophysiological character of the $\alpha_2\delta$-3 subunit are similar to those of the $\alpha_2\delta$-1 subunit, if expressed together with the α_{1C} and cardiac $ß_{2a}$ subunit (Klugbauer et al. 1999a). In general, coexpression of the $\alpha_2\delta$-1 subunit with α_1 and β subunits shifts the voltage-dependence of channel activation and inactivation in a hyperpolarizing direction, accelerates the kinetics of current activation and inactivation and increases the current amplitude (Singer et al. 1991; De Waard et al. 1995a; Gurnett et al. 1996, 1997; Bangalore et al. 1996; Felix et al. 1997; Qin et al. 1998b; Klugbauer et al. 1999a). Some inconsistencies in reported results can be accounted for by the experimental conditions, as various expression systems (Xenopus oocytes or mammalian cell lines), different charge carriers (Ba^{2+} or Ca^{2+}), different splice variants of the $\alpha_2\delta$-1 subunit, different α_1 (α_{1C}, α_{1A}, α_{1E}) and β (β_1, β_2, β_3 or β_4) subunits were used. Detailed analysis of the effects of the α_2 and δ proteins suggests (Gurnett et al. 1996, 1997; Felix et al. 1997) that the extracellular α_2 protein enhances current density and the affinity for the DHP isradipine, whereas the transmembrane segment of the δ protein interacts with repeat III and some additional parts of the channel (Gurnett et al. 1997). Changes in the channel kinetics are associated with the expression of the δ protein.

The mechanism whereby $\alpha_2\delta$ modulates the conductance of α_1 is not clearly understood. The increase in current density can be partly accounted for by improved targeting of expressed α_1 subunits to the cell membrane (Shistik et al. 1995). The effects of the coexpression of the $\alpha_2\delta$ subunit on time course and/or voltage dependence on current activation and inactivation also suggests a specific modulation of channel gating. In the presence of the $\alpha_2\delta$-1 subunit, the open probability of the channel is enhanced without a change in the mean open time (Shistik et al. 1995), and the amount of charge moved during channel activation increases (Bangalore et al. 1996; Qin et al. 1998b). This increase in charge movement was coupled to an increased and an unchanged maximal conductance, when the L-type α_{1C} calcium channel (Bangalore et al. 1996) and the neuronal α_{1E} channel (Qin et al. 1998b) were used, respectively. Shirokov (1998) reported that $\alpha_2\delta$-1 speeds up the trans-

fer of the α_{1C} channel into a slowly inactivated state and slows down its recovery from inactivation. These changes in channel gating may underlie the observed effects on the inactivation of the whole cell current.

The anticonvulsant drug gabapentin binds stereospecifically with high affinity (K_D 37.5 nM) to the $\alpha_2\delta$-1 subunit (Gee et al. 1996; Brown and Gee 1998). Through C-terminal deletion mutagenesis of the δ polypeptide, a segment was identified (residues 960–994) that is required for correct assembly of the gabapentin binding pocket. These residues are outside of the putative transmembrane segment of the δ protein. The pharmacological significance of this high affinity binding site is not clear. Block of HVA calcium channels has not been observed or has been reported to occur at 1–10 μM gabapentin (Taylor et al. 1998; Stefani et al. 1998).

A.II.2.2 The ß-Subunit

The ß subunits are intracellularly located proteins ranging from 50 to 72 kDa. Four genes – $ß_1$, $ß_2$, $ß_3$ and $ß_4$ – have been identified (Ruth et al. 1989; Hullin et al. 1992; Perez-Reyes et al. 1992; Castellano et al. 1993) which give rise to several splice variants. A primary structure alignment of ß subunits revealed that all share a common central core, whereas their N- and C-termini and a part of the central region differ significantly. Coexpression of a ß subunit with various α_1 subunits increases peak current (Singer et al. 1991) most likely by increasing the number of functional surface membrane channels and by facilitating channel pore opening (Neely et al. 1993; Josephson and Varadi 1996; Kamp et al. 1996). With the exception of the rat brain $ß_{2a}$, all other ß subunits accelerate channel activation and inactivation and shift the steady state inactivation curve to hyperpolarized membrane voltages (Singer et al. 1991; Wei et al. 1991; Hullin et al. 1992; Castellano et al. 1993). All four ß subunits combine with the neuronal α_1 subunits (Scott et al. 1996; Liu et al. 1996; Ludwig et al. 1997; Pichler et al.1997; Volsen et al. 1997; Vance et al. 1998). The brain expression of the $ß_4$ subunit increases about 10 fold between postnatal day 2 and maturity, during which time it associates with N- and P-type channels (Vance et al. 1998). In lethargic mice, ataxia and seizures are associated with mutation of the $ß_4$ subunit (Burgess et al. 1997). The lethargic phenotype could be caused by the persistence of an immature N-type calcium channel coassembled with the $ß_{1b}$ subunit (McEnery et al. 1998). In contrast to neuronal calcium channels, the skeletal and cardiac muscle calcium channels are associated apparently exclusively with the $ß_{1a}$ and cardiac $ß_{2a}$ subunits, respectively (Ruth et al. 1989; Ludwig et al. 1997; Qin et al. 1998a).

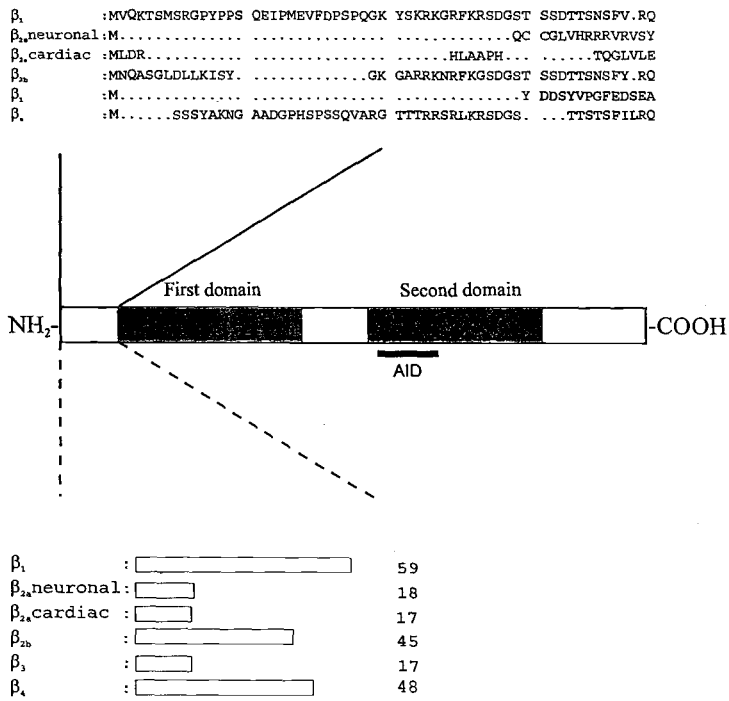

Fig. 4. Comparison of the primary structures of calcium channel ß subunits. (**Top**) Sequence alignment of the four different ß subunit genes and their splice variants. (**Middle**) Scheme of organization of the ß subunit. The regions of high and low degrees of sequence conservation are shown in grey (first domain, second domain) and white, respectively. AID, interaction site with the calcium channel α_1 subunit (**Bottom**) relative length of amino terminus

Differential splicing of the primary transcripts of $ß_1$ results in the expression of at least three isoforms (Ruth et al. 1989; Pragnell et al. 1991; Williams et al. 1992b) (Fig. 4). $ß_{1a}$ is exclusively expressed in skeletal muscle together with the α_{1S}, $\alpha_2\delta$-1 and γ_1 subunits, whereas the other two isoforms of $ß_1$ were identified in brain and spleen (Powers et al. 1992). Deletion of the $ß_1$ gene in mice leads to perinatal lethality (Gregg et al. 1996). The absence of the $ß_1$ subunit lowers the concentration of the α_{1S} subunit in skeletal muscle and impairs thereby e-c coupling. Coexpression of the brain splice variant $ß_{1b}$, but not the skeletal muscle $ß_{1a}$ variant, together with the α_{1S}, $\alpha_2\delta$-1 and γ_1 subunits has been reported to induce measurable inward current in oocytes suggesting that this specific splice variant has significant effects on the properties of the skeletal muscle calcium channel (Ren and Hall 1997).

The ß$_2$ gene is expressed abundantly in heart and to a lower degree in aorta, trachea, lung and brain (Biel et al. 1991), whereas the ß$_3$-specific mRNA is detectable in brain and different smooth muscle tissues (Hullin et al. 1992; Ludwig et al. 1997). The ß$_2$ transcript is extensively spliced resulting in at least four different isoforms (Perez-Reyes et al. 1992; Hullin et al. 1992) Fig. 4). The rabbit cardiac ß$_{2a}$ (Hullin at al. 1992) and the rat brain ß$_{2a}$ (Perez-Reyes et al. 1992) are N-terminal splice variants of the same gene. The rat brain ß$_{2a}$ has two cysteines at position 3 and 4 which are palmitoylated in vivo (Chien et al. 1996; Qin et al. 1998a). The ß$_{2a}$ expressed in rabbit heart does not contain the amino terminal cysteines (Qin et al. 1998a) and is identical to the cloned cardiac ß$_{2a}$ (Hullin et al. 1992). Coexpressed with the α_{1E} subunit, the brain ß$_{2a}$ reduces the rate at which α_{1E} inactivates in response to depolarization and causes a rightward shift in the steady-state inactivation curve. The brain ß$_{2a}$ subunit does not support facilitation of the α_{1C} current (Olcese et al. 1994; Qin et al. 1998a). It prevents prepulse potentiation caused by G protein ßγ subunit interaction with neuronal α_1 subunits (Herlitze et al. 1996). Prevention of the palmitoylation of the brain ß$_{2a}$ by mutation of the two cysteines to serines changes its properties to that of the cardiac ß$_{2a}$, i.e. the mutated ß$_{2a}$ subunit shifts the steady-state inactivation curve to hyperpolarized potentials, supports facilitation of the α_{1C} current and interferes poorly with block of α_{1E} channel by carbachol (Qin et al. 1998a). The extent of palmitoylation is affected by mutation in other regions of the neuronal ß subunit, i.e. in a src homology 3 motif and in the ß subunit interaction domain (Chien et al. 1998) (see also under B.II.2).

A. II. 2.3 The γ Subunit

The γ_1 subunit is an integral membrane protein consisting of 222 amino acids with a predicted molecular mass of 25 kDa (Bosse et al. 1990; Jay et al. 1990), which is exclusively expressed in skeletal muscle (Eberst et al. 1997). Recently, a second γ_2 subunit has been identified in brain which has 25% identity with γ_1 and whose expression is highest in cerebellum, olfactory bulb, cerebral cortex, thalamus, CA3 and dentate gyrus of the hippocampus (Letts et al. 1998). Additional putative γ subunits γ_3 and γ_4 have been identified (Klugbauer et al. 1999c). The γ_3 clone is highly homologous to the γ_2 subunit and expressed mainly in various brain regions. The γ_4 clone has 25% identity with the γ_1 subunit and is present in muscular and other non-neuronal tissues. The human γ_1 and γ_2 subunits are encoded on chromosome 17q23 and 22q12–13, respectively (Powers et al. 1993; Letts et al. 1998). Hydrophobicity analysis reveals the existence of four putative transmembrane helices with intracellular amino- and carboxy-termini. The presence

of two extracellular potential N-glycosylation sites is consistent with the observed strong glycosylation of these subunits. Coexpression of each γ subunit together with α_1, $\alpha_2\delta$ and ß subunits in oocytes induces a shift in the steady-state inactivation curves in the hyperpolarizing direction (Singer et al. 1991; Letts et al. 1998). The γ_2 gene is mutated in stargazer mice leading to spike-wave seizures characteristic of absence epilepsy with accompanying defects in the cerebellum and inner ear (Letts et al. 1998).

B.I Functional Domains of the α_1 Subunit

B.I.1 The Pore and Ion Selectivity Filter

Part of the pore structure of the calcium channel is formed by the linker connecting the S5 and S6 transmembrane segments in repeats I to IV (Guy and Conti, 1990). This P region is thought to contribute to the outer vestibule of the channel pore and to span the outer half of the membrane. In analogy to the recently obtained crystal structure of the *Streptomyces lividans* potassium channel (Doyle et al. 1998), the calcium channel pore can be envisioned to have the structure of an inverted teepee with the vertex

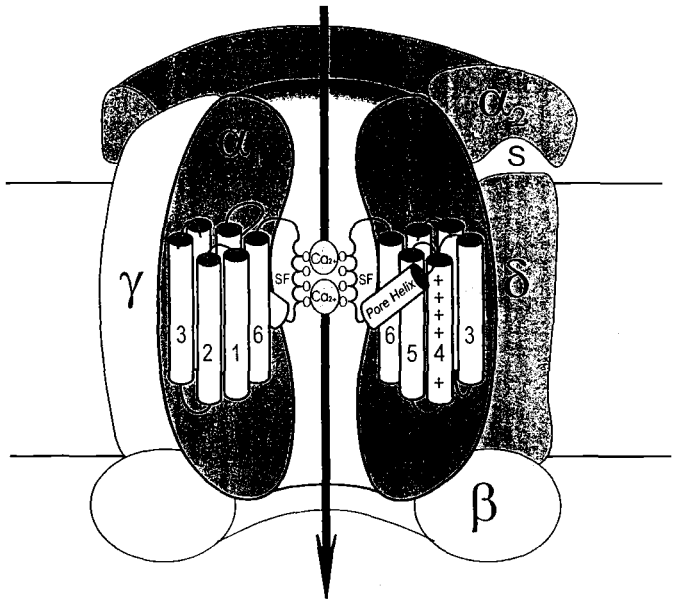

Fig. 5. Proposed structure of the calcium channel complex with transmembrane segments of two repeats. The model has been designed according to the structure of the *Streptomyces lividans* potassium channel

inside the cell. The helices of the four S6 segments would form the poles of this teepee, which are widely separated near the outer membrane surface and converge towards a narrow zone at the inner surface. This outer structure would stabilize an inner ring formed by the four P-regions, which control the speed of permeation and the ion selectivity (Fig. 5).

Mutational analysis of the α_{1C} (Tang et al. 1993; Yang et al. 1993) and α_{1A} (Kim et al. 1993) channel has shown that the four glutamic acid residues E413, E731, E1140 and E1441 (amino acid numbering is according to the $\alpha_{1C\text{-}b}$ sequence (Biel et al. 1990)) in the P regions of repeat I, II, III and IV are critical in determining the ion selectivity of the calcium channel. Equivalent glutamates are present in all HVA calcium channels. Mutation of these glutamates decreased dramatically the ability of Ca^{2+} or Cd^{2+} to block monovalent ion permeation (Yang et al. 1993; Kim et al. 1993; Yatani et al. 1994; Ellinor et al. 1995; Parent and Gopalakrishnan 1995). The studies showed that these glutamates form the high affinity Ca^{2+} binding site responsible for the Ca^{2+} selectivity within the pore. The glutamic acid residues of each repeat contribute differently to the Ca^{2+} affinity, selectivity and speed of permeation (Tang et al. 1993; Parent and Gopalakrishnan 1995; Ellinor et al. 1995). Mutation of E1140 in repeat III has a much greater effect on ion selectivity and permeation than comparable mutations in the other three repeats. LVA channels have aspartates instead of glutamates in the pore of repeat III and IV. This difference may be the cause of their distinct ion selectivity (Perez-Reyes et al. 1998a; Cribbs et al. 1998; Klugbauer et al. 1999b).

To explain rapid permeation of calcium ions, different models have been discussed with one or two – high and low affinity – site(s) for Ca^{2+} (Hess and Tsien, 1984; Tsien et al. 1987; Rosenberg and Chen, 1991; Armstrong and Neyton 1991; Kuo and Hess, 1993). In a recent study, Ellinor et al. (1995) demonstrated that these glutamates form a single high affinity Ca^{2+} site within the pore. This site may be accessed by two Ca^{2+} ions at the same time thereby allowing rapid permeation. The cloned smooth muscle $\alpha_{1C\text{-}b}$ channel allows rapid permeation of Ca^{2+} at physiological pH and voltages and has a high unitary conductance (Gollasch et al. 1996), whereas the unitary conductance of the skeletal muscle α_{1S} subunit is half that of the cardiac α_{1C} subunit (Dirksen et al. 1997). The unitary conductance was reduced from that of cardiac to that of skeletal muscle, when the skeletal muscle IS5-IS6 linker was introduced into the cardiac α_{1C} subunit (Dirksen et al. 1997). The net charges of the vestibule part of the cardiac and skeletal muscle IS5-IS6 linkers are −5 and −2, respectively. It is plausible that the more negatively charged vestibule of the cardiac channel compared to the skeletal muscle channel increases conduction by electrostatic attraction of Ca^{2+} ions into the channel pore.

Increased extracellular proton (H^+) concentrations which occur during episodes of intense neuronal activity or with ischemia in the heart strongly inhibit ion permeation through open calcium channels (Kuo and Hess 1993). A single H^+ binding site has been invoked. Analysis of the mutated α_{1C} subunit localized this site to the glutamates of the pore region. Controversial data have been published suggesting that H^+ binding requires either only E1140 in repeat III (Klöckner et al. 1996) or E413 and E1140 in repeats I and III (Chen and Tsien 1997). The two glutamate model may more readily explain the unusually high pKa (pH > 8) of the protonated site than the single glutamate model. The interpretation of these results is further complicated by the observation that removal of protons increases L-type current only when the α_{1C} subunit is expressed together with the cardiac $ß_{2a}$ subunit (Schuhmann et al. 1997).

B.I.2 Channel Activation

Mutational analysis in K^+ (Papazian et al. 1991; Liman et al. 1991) and Na^+ (Stühmer et al. 1989) channels suggested that the positive charges of the S4 segments in each repeat function as the voltage sensor. Mutation of individual S4 arginines in repeats I and III of a skeletal/cardiac α_1 chimera affected the midpoint and time constant of activation, whereas those of repeat II and IV were without effect (Garcia et al. 1997). Mutation of the leucine heptad motif present in the region of S4–S5 in repeat I and III yielded inconclusive results. The speed of calcium channel activation is a property of the α_1 subunit and is modulated by the $\alpha_2\delta$ (see under A.II.2.1) and ß (see under A.II.2.2) subunits. An over five-fold difference in the speed of activation was observed between the skeletal (slow) and cardiac (fast) α_1 subunits. Functional expression of chimeric calcium channels showed that repeat I determines the speed of activation (Fig. 2) (Tanabe et al. 1991). Initially, the S3 segment and the linker IS3–IS4 were shown to control slow and fast activation (Nakai et al. 1994). Analysis of several skeletal/cardiac chimeras suggests that although unitary conductance and speed of activation are encoded in different parts of repeat I, the linker IS5–IS6 affects not only the unitary conductance but also the speed of activation (Dirksen et al. 1997). In addition, the sequence between IIIS5 and IVS6 contributes to the speed of channel activation (Wang et al. 1995).

B.I.3 Channel Inactivation

HVA-calcium channels show two types of inactivation: slow and fast inactivation. Slow inactivation is voltage-dependent, whereas fast inactivation is

caused by the permeating calcium ion. The kinetics of slow/voltage-dependent inactivation, which is observed with all HVA calcium channels, differ considerably between the various types of calcium channels and are important in determining the amount of calcium entry during electrical activity. The IS6 segment and its flanking regions are critical for the inactivation properties of the channel (Zhang et al. 1994) as determined with chimeric α_1 subunits of channels with different inactivation rates, i.e. the α_1 subunits of the class C, class A and doe-1, an α_1 subunit cloned from the marine ray Discopyge ommata. Chimeras between the α_{1C} and α_{1S} calcium channels confirmed this conclusion (Parent et al. 1995). However, inactivation of the α_{1C} channel is also controlled by the intracellular carboxy terminal sequences (Wei et al. 1994). Removal of the carboxy terminus of the $\alpha_{1C\text{-}a}$ or $\alpha_{1C\text{-}b}$ subunit up to aa 1733 or 1728, respectively, increases the expressed current (Wei et al. 1994; Klöckner et al. 1995; Seisenberger et al. 1995) without increasing the charge moved or the density of DHP binding sites (Wei et al. 1994). Therefore, truncation of the channel up to aa 1733 does not increase the number of channels but removes an inhibitory action of the carboxy terminus. Similar results have been obtained *in vivo* by perfusion of cardiac myocytes with trypsin (Hescheler and Trautwein 1988). However, the trypsinized channel lost its calcium sensitivity, whereas the truncated channel still showed calcium-dependent inactivation.

Calcium-sensitive inactivation of α_{1C} channels is a biological negative feedback mechanism, by which the increase of intracellular calcium speeds up channel inactivation and prevents calcium overload of the cell. Using the L-type calcium current of guinea pig cardiac myocytes, Hescheler and Trautwein (1988) showed that intracellular application of trypsin or carboxypeptidase increased the amplitude of calcium or barium currents and decreased calcium dependent inactivation. The trypsin dependent increase in current amplitude was confirmed by others (Schmid et al. 1995; You et al. 1995), whereas the loss of calcium-dependent inhibition was seen by You (You et al. 1995) but not by Schmid (Schmid et al. 1995). These discrepancies were clarified by the use of the cloned α_1 subunits (Fig. 2). Fast/Ca^{2+}-dependent inactivation is especially prominent in the cardiac and the smooth muscle channels and requires only the α_{1C} subunit (Welling et al. 1993b; Neely et al. 1994; Zong and Hofmann 1996). Intracellular Ca^{2+} inactivates the calcium current by binding to a single site with an IC_{50} of 4 μM Ca^{2+} (Höfer et al. 1997) supporting the hypothesis of the presence of a single EF hand (Babitch 1990). Exchange of amino acids between residues 1572 and 1651 by exons only found so far in the α_{1C} gene increases the speed of inactivation and, depending on the substitution, removes calcium dependent inactivation (Soldatov et al. 1998; Zühlke and Reuter 1998). Exchange of the

same region of α_{1C} sequence for those of α_{1E}, a calcium insensitive channel, also results in a loss of calcium-dependent inhibition (de Leon et al. 1995; Zhou et al. 1997). However, no agreement exists on the importance of the EF hand binding motif, since its exchange or removal affected calcium sensitivity (Soldatov et al. 1998; Zühlke and Reuter 1998) or had no effect (Zhou et al. 1997). Further complication comes from the work of Adams and Tanabe (1997). An α_{1C}/α_{1S} chimera, in which the carboxy terminal α_{1C} sequence 1633 to 2166 was replaced by the skeletal muscle sequence 1510 to 1873, had lost calcium-dependent inactivation. However, the same chimera, in which the last 211 amino acids from the skeletal muscle (sequence used 1510 to 1662) were removed, regained Ca^{2+}-dependent inactivation. It is quite likely that these very different sequence modifications affected either the Ca^{2+} binding site or the conformation of the carboxy terminus that mediates channel inhibition or both. Agreement exists only in sofar as that Ca^{2+}-dependent inactivation requires only the α_{1C} subunit and binding of Ca^{2+} to the intracellular amino acid stretch between residues 1513 and approximately 1700.

B.II Sites for Interaction with Other Proteins

The α_1 subunit interacts with a number of proteins such as its auxiliary $\alpha_2\delta$, ß and γ subunits and proteins such as the G protein ßγ subunit, the ryanodine receptor and proteins necessary for fusion of neurosecretory vesicles with the presynaptic membrane. The potential interaction sites for the γ subunit and the $\alpha_2\delta$ are unknown or have been outlined above (see A.II.2.1).The major sites for interactions are the cytosolic loops between repeats I and II, II and III and the carboxy terminal tail sequence. Proteins which interact with the loop I-II and the C-terminal sequences usually modify channel kinetics, whereas loop II-III transduces the signal to intracellular partners.

B.II.1 Interaction of the α_1 Subunit with the Ryanodine Receptor

In cardiac muscle, e-c coupling does not require a direct contact between the calcium channel and the ryanodine receptor type 2 (RyR-2). Calcium release from the sarcoplasmic reticulum (SR) is triggered by the calcium flowing through the open L-type α_{1C} calcium channel into a restricted space between the plasma membrane and the SR (Sham et al. 1995). In contrast, in skeletal muscle e–c coupling requires direct coupling between the α_{1S} subunit and the ryanodine receptor type 1 (RyR-1). The cytoplasmic loop between repeats II and III of the α_{1S} subunit, but not that of the α_{1C} subunit, affects

ryanodine binding to the skeletal muscle RyR-1 and induces calcium release from the skeletal muscle SR (Tanabe et al. 1990). The α_{1S} subunit can be replaced by a peptide containing the skeletal sequence E666 to L791 (Lu et al. 1994). Later refinement of this peptide showed i) that phosphorylation of S687 (Röhrkasten et al. 1988) in the peptide E666–E726 prevents activation of calcium release from the SR (Lu et al. 1995), ii) that activation of RyR-1 requires only the sequence T671–L690 (El-Hayek et al. 1995) which contains the essential basic cluster RKRRK (El-Hayek and Ikemoto 1998), iii) that activation of the RyR-1 by the peptide T671–L690 is prevented by the peptide E724–P760 which is localized in the carboxy terminal part of the II–III loop of α_{1S} (El-Hayek et al. 1995). Using α_{1S}/α_{1C} chimeras expressed in dysgenic myotubes, Nakai and coworkers (1998b) have slightly revised the site which interacts with the RyR-1. Transfer of the skeletal muscle sequence between residues 711–765 to a cardiac α_{1C} subunit yields skeletal muscle type e-c coupling. The core region between residues 725–742 is necessary for e–c coupling but gives only a weak response (Nakai et al. 1998b).

Activation of the RyR-1 is not affected by truncation of the intracellular tail of the α_{1S} sequence at N1662 suggesting that this part of the tail is not necessary for normal e–c coupling in skeletal muscle (Beam et al. 1992). RyR-1 expression is not only necessary for normal e–c coupling, but also for a high density of the DHP receptor complex in skeletal muscle (Nakai et al. 1996) and neurons (Chavis et al. 1996). Work with chimeric RyR-1/RyR-2 showed that the sequence from aa 1635 to 2636 of the RyR-1 couples to the α_{1S} subunit of the DHP-receptor, increases the density of the DHP receptor complex and is necessary for calcium release from the SR (Nakai et al. 1998a). In addition, the carboxy terminal sequence aa 2659–3720 couples to the DHP-receptor complex as evidenced by an increase in calcium current, but does not allow calcium release from the SR (Nakai et al. 1998a) suggesting multiple contact sites between the skeletal muscle calcium channel complex and the cytosolic part of the RyR-1.

The in vivo interaction between the α_{1C} or α_{1S} subunit and RyR-2 or RyR-1 may depend on the presence of a 22 kDa protein, named sorcin. Sorcin immunoprecipitates with the cardiac α_{1C} and the skeletal muscle α_{1S} protein (Meyers et al. 1998). C-terminal peptides from the α_{1C} subunit suggest that sorcin binds to the sequence between residues 1622 and 1748. This sequence is N-terminal of the putative truncation site at residue 1870 and suggests that interaction with small peptides may be necessary to couple the calcium channel with the ryanodine receptor and to trigger calcium release from the sarcoplasmic reticulum in heart and skeletal muscle.

B.II.2 Interaction of Ca^{2+} Channels with Synaptic Vesicle Proteins

Neurotransmitter release is initiated by influx of Ca^{2+} through voltage-activated N- and P/Q-type calcium channels within 200 μs of the action potential arriving at the synaptic terminal, as the Ca^{2+} concentration increases from 100 nM to > 200 μM. Exocytosis requires a high Ca^{2+} concentration with a threshold of 20–50 μM and half-maximal activation at 190 μM. The synaptic membrane proteins syntaxin, synaptosome-associated protein of 25 kDa (SNAP25), and vesicle-associated membrane protein (VAMP)/synaptobrevin, are thought to form a synaptic core complex (SNARE), which mediates vesicle docking and membrane fusion. Synaptotagmin may be the low affinity Ca^{2+} sensor, but other Ca^{2+} sensors are involved as residual neurotransmission persists in synaptotagmin deficient mice (for details see Südhof 1995; Südhof and Rizo 1996). The close proximity of calcium channels and neurotransmitter containing vesicles in the presynaptic terminal is a prerequisite for synaptic transmission. The synaptic membrane proteins syntaxin and SNAP25 bind to the sequence 713–933 of the α_{1A} subunit, which is present in the intracellular loop between repeat II and III (Leveque et al. 1994; Sheng et al. 1994, 1996). The interaction is optimal at a Ca^{2+} concentration of 15 μM and falls off at lower and higher Ca^{2+} concentrations. Syntaxin 1A interacts with the α_{1B} loop II–III sequence with its membrane anchor between residues 181–288 (Sheng et al. 1994). The same site, identified as a "synprint" site, also interacts with the SNARE complex in vitro and in vivo. An identical site is present in the II–III loop of the α_{1A} subunit (residue 722–1036 of the rabbit BI clone (Mori et al. 1991)), which binds syntaxin and SNAP25 calcium-independently (Martin-Moutot et al. 1996; Rettig et al. 1996; Kim and Catterall 1997). Synaptotagmin I binds to the same site in both channels calcium independently with its calcium binding site C2B in vitro (Charvin et al. 1997; Sheng et al. 1997; Kim and Catterall 1997). This interaction may not occur in vivo, since syntaxin and SNAP25 bind with slightly higher affinity to the loop II–III sequence (40–50 nM versus 70 nM) (Charvin et al. 1997; Sheng et al. 1997). Phosphorylation of the synprint sequence of the N-type channel by protein kinase C and calmodulin kinase II up to a stoichiometry of 3–4 mole/per peptide prevents the binding of syntaxin 1A, SNAP25 and the SNARE complex (Yokoyama et al. 1997). The rat isoform of the α_{1A} channel (Starr et al. 1991) binds SNAP25 and synaptotagmin I, but not syntaxin (Rettig et al. 1996; Kim and Catterall 1997).

The functional significance of the interactions is not clear. Coexpression of the α_{1A} or α_{1B} calcium channel and syntaxin 1A in Xenopus oocytes decreased channel availability, slowed the recovery from inactivation and shifted the voltage for steady state inactivation by -20 mV (Bezprozvanny et

al. 1995). These responses would suggest that interaction of the proteins decreases presynaptic Ca^{2+} entry. Similar experiments were reported for the coexpression of the α_{1C} channel with syntaxin and SNAP25 (Wiser et al. 1996), although a physical interaction of an L-type loop II–III sequence with these proteins has not been reported. Injection of the synprint sequence of the α_{1B} subunit into embryonic spinal neurons indicated that the synprint peptide suppressed neurotransmitter release by 25% (Rettig et al. 1997). Recalculations of these results implied that 70% of the vesicles formerly linked to the calcium channel were uncoupled by the peptide. The peptide would interfere with the coupling of the SNARE complex with the N-type channel and sever thereby vesicle docking. It is possible that assembly and dissassembly of the membrane vesicle complex is modulated by cysteine string proteins, which are molecular chaperones and bind in vitro directly to the loop II-III sequence of the α_{1A} subunit (Leveque et al. 1998).

B.II.3 Interaction of the α_1 Subunit with the ß Subunit

Coexpression of a ß subunit with α_1 subunits alters the voltage-dependence, kinetics and magnitude of the calcium channel current. The differences in reported effects most likely depend on the particular combination of both subunits and splice variants. These modulatory effects are the consequence of conformational changes in the quarternary structure resulting from the specific interaction of subunit surfaces (Neely et al. 1993). To identify the ß subunit interaction site on the α_1 subunit, an epitope library of the α_{1S} subunit was screened with a labelled $ß_{1b}$ subunit probe (Pragnell et al. 1994). The ß subunit probe binds to the cytoplasmic linker between domain I and II of the α_1 subunit (Fig. 3). A detailed analysis of different α_1 subunits revealed that a highly conserved sequence motif, called AID for alpha subunit interaction domain, is reponsible for this specific interaction, i. e. 428QQ-E--L-GY--WI---E445 (amino acid numbering is according to the $\alpha_{1C\text{-}b}$ sequence (Biel et al. 1990)), positioned 24 amino acids from the IS6 transmembrane domain in each α_1 subunit. Further mutations showed that only the sequence -437Y--WI441- is essential for high affinity binding of the ß subunits (DeWaard et al. 1996b). Mutation of the tyrosine to a serine (-Y--WI- to -S--WI-) reduces the affinity of the AID for ß subunits dramatically (Witcher et al. 1995). This mutation abolishes the increase of peak currents and the changes in the inactivation kinetics and the voltage-dependence of activation by the ß subunit (DeWaard et al. 1996b). In a biochemical assay, De Waard and collegues (1995b) showed that the AID of the α_{1A} subunit binds ß4 with a K_D of 5 nM. The relative affinities for the various ß subunits to the AID_A were ß4>ß2a>ß1b>>ß3. A second low affinity binding site (K_D about

100 nM) for the $ß_4$ and $ß_3$ subunit has been detected in the carboxy terminal sequence of the α_{1A} subunit between residues 2090 and 2424 (Walker et al. 1998). The neuronal $ß_{2a}$ subunit binds with the homology domain D4 (residue 206–414) to the α_{1E} subunit between residues 2035 and 2068 (Tareilus et al. 1997; Qin et al. 1997).

Since all four ß subunits can modulate the kinetics and voltage dependence of the α_1 subunit and bind to the AID, it is likely that ß subunits contain a conserved motif, which binds to AIDs. To identify this structural domain, a series of truncated and mutated $ß_{1b}$ subunits was constructed and it was tested whether they interact with α_{1A} in vitro (De Waard et al. 1994). A 30 amino acid domain of the ß subunit (aa 215–245 of $ß_{1b}$) is sufficient to induce all the modulatory effects of this subunit. This sequence stretch is located at the amino terminus of the second region of high conservation among all four ß subunits. Modifications in this region changed or abolished the stimulation of calcium currents by the ß subunit and the binding to the α_1 subunit. This central core region of the ß subunit is also required to relocate the α_{1C} subunit from the ER to plasma membrane (Gao et al. 1999).

Deletion of the $ß_1$ subunit gene showed that a proper targeting of the α_{1S} subunit in skeletal muscle depends on the coexpression of the $ß_{1a}$ subunit (Gregg et al. 1996). Transient transfection of the $ß_1$ cDNA in deficient myotubes restored Ca^{2+} current, charge movement and Ca^{2+} transients (Beurg et al. 1997). Slightly different results were reported when the homozygous dysgenic (mdg/mdg) cell line GLT was used (Neuhuber et al. 1998a). This cell line does not express the α_{1S} subunit. Proper targeting of the $ß_{1a}$ subunit required coexpression of the α_{1S} subunit, in which the binding site for ß subunits in the I–II loop (AID) was not mutated at the essential tyrosine (Neuhuber et al. 1998a). Further experiments on the interaction and targeting of the α_{1S} subunit by the $ß_{1a}$ or neuronal $ß_{2a}$ in tsA201 cells yielded similar results (Neuhuber et al. 1998b). The biological significance of these findings is not clear since i) the $ß_{1a}$ subunit is expressed in the absence of the α_{1S} subunit in mdg/mdg myotubes, ii) the neuronal $ß_{2a}$ subunit is targeted by palmitoylation of the two amino terminal cysteines to the plasma membrane, iii) palmitoylation of the $ß_{2a}$ subunit is affected significantly by mutations in the BID and other domains (Chien et al. 1998) and iv) it is difficult to understand how the ß subunits affect barium currents without colocalizing with the α_{1S} subunit (Neuhuber et al. 1998a, b). However, clear results were published by Yamaguchi and collegues (1998), who expressed the α_{1C} subunit in Xenopus oocytes. Injection of the $ß_3$ subunit protein rapidly modulated the current kinetics and voltage dependence of activation, whereas a large augmentation of the peak current amplitude occurred over a longer time scale. Prevention of protein translocation by bafilomycin A_1 did

not affect the modulation of the current kinetics or voltage dependence, but completely abolished the increase in peak amplitude. These results provide clear evidence that, at least in Xenopus oocytes, the ß subunit has two functions: one is an allosteric modulation of the α_1 subunit function and the other is a chaperoning of the α_1 subunit to the plasma membrane. In support of this interpretation is the finding that the loopI-II of the α_1 subunit contains a ER retention signal, which is inactivated when the ß subunit binds to the AID site (Bichet et al. 1999).

B.II.4 Interaction of the α_1 Subunit with G Proteins

The rapid release of neurotransmitters and secretion of hormones is initiated by an increased calcium influx through presynaptic N- and P/Q-type calcium channels. The activities of these channels are modulated by G protein-dependent pathways. Previous studies in neurons have identified five G protein-dependent pathways for N-type calcium channel inhibition (Hille 1994). One pathway is membrane-delimited and may involve only calcium channels, heterotrimeric G proteins and neurotransmitter receptors. Calcium channels inhibited via this pathway exhibit positive shifts in the voltage dependence of activation, slowed activation kinetics, and reduced current amplitude. Such channels are described as "reluctant" to open and can be transiently reconverted into "willing" channels by a strong depolarizing prepulse (Bean 1989). This reconversion is known as facilitation. Initially it was thought that inhibition is mediated by specific G-protein α subunits (Hescheler and Schultz 1993), until Ikeda (1996) and Herlitze and coworkers (1996) showed that the G protein ßγ subunit confers the reluctant "phenotype" to N- and P/Q-type calcium currents. In both studies, expression of Gα did not mediate the voltage-dependent inhibition of the channels. Overexpression of the G-protein $ß_1\gamma_2$ and $ß_2\gamma_3$ subunits inhibited the N- and P/Q-type current, respectively (Ikeda 1996; Herlitze et al. 1996). Sequence analysis, site directed mutagenesis and various chimeras of the α_{1A} (P/Q-type current), α_{1B} (N-type current) and α_{1E} (R-type current) channels showed that high affinity binding of the Gßγ subunit requires the sequence QXXER which is present in the intracellular loop between repeat I and II (Herlitze et al. 1997; DeWaard et al. 1997; Zamponi et al. 1997; Qin et al. 1997). The QXXER motif is not present in L-type calcium channels, which have the sequence QXXEE at the same position. In agreement, L-type channels are not regulated/ inhibited by G protein ßγ subunits. Mutation of the QXXER motif to QXXEE in the α_{1A} or α_{1B} subunit prevented inhibition by the Gßγ subunit (Herlitze et al. 1997; DeWaard et al. 1997; Zhang et al. 1996), whereas mutation of the motif QXXEE to QXXER in the α_{1C} subunit allowed regulation by

the Gßγ subunits (Herlitze et al. 1997). Identical conclusions were obtained by Furukawa and collegues (1998a, b). These authors also showed that current inhibition of the α_{1A} and α_{1B} channel could be induced by overexpression of the G_{i3} α subunit and required the intracellular C-terminal sequence of the α_1 subunits (Furukawa et al. 1998a). They provided evidence, that the Gßγ subunit binds directly to loop I–II and the $G_{i3}\alpha_1$ subunit directly to a C-terminal sequence (Furukawa et al. 1998b).

Although these results appeared to be quite clear, later work indicated that the functional effects of the binding of the G protein ßγ subunit also required a domain in the cytosolic loop I–II. Zhang and coworkers (1996) indicated that the reluctant phenotype of the N-type current required part of the N-terminal-repeat I and the C-terminal sequence of the α_{1B} subunit, whereas Meza and Adams (1998) suggested that the loop I-II, but not the C-terminal sequence of the α_{1B} subunit is necessary for the functional effects of the Gßγ subunit. Slightly different results were reported by Qin and coworkers (1997) who used the α_{1E} subunit. The Gßγ subunit bound to the I–II loop and to the C-terminal sequence between residues 2035–2068. Inhibition of the current was mediated by the C-terminal sequence. This sequence also bound the neuronal ß subunit of the calcium channel. Overexpression of the $ß_{2a}$ subunit inhibited the functional effects of the Gßγ subunit. A competition between the inhibitory effect of the G protein and the calcium channel ß subunit had been reported earlier (Campbell et al. 1995). This group also suggested that the N-terminal-repeat I-loop I–II sequence and part of the C-terminal sequence of α_{1B} or α_{1Elong} is involved in the inhibition of calcium current (Stephens et al. 1998; Page et al. 1998). From these results, it may be concluded that the functional binding pocket of the Gßγ subunit includes residues of the intracellular N and C-terminal sequence and part of repeat I and the loop I–II sequence of the α_{1A}, α_{1B} and α_{1E} subunit. The differences reported probably reflect the use of different splice variants of the α_1 subunits and of the ß subunits.

B.III Binding Sites for L-Type Calcium Channel Agonists and Antagonists

B.III.1 The Dihydropyridine Binding Site

The L-type calcium channel ligands represent a clinically and experimentally important set of blockers and agonists. The major classes of these drugs are the dihydropyridines (DHP), phenylalkylamines (PAA) and benzothiazepines. Different techniques have been used to localize potential binding sites of these drugs on the calcium channel complex. Earlier experimental

observations from photoaffinity labelling and peptide mapping studies on the skeletal muscle channel revealed that all three classes bind to the transmembrane region of repeat IV of the α_1 subunit (Regulla et al. 1991; Catterall & Striessnig 1992; Kuniyasu et al. 1998) with additional sites on repeat III (Catterall and Striessnig 1992; Kalasz et al. 1993) and repeat I (Kalasz et al. 1993) for the DHPs. These localizations were refined by the use of chimeric α_{1C}/α_{1A} and α_{1C}/α_{1E} channels and site-directed mutagenesis of single amino acids in the α_{1S} or α_{1C} subunit (Fig. 6 top). High affinity block of α_{1C}-mediated barium current (I_{Ba}) by the DHP blocker isradipine or (–)R-202-791 is prevented by mutation of the L-type specific amino acids (amino acid numbering is according to the $\alpha_{1C\text{-}b}$ sequence (Biel et al. 1990)) Thr1061 and Gln1065 in IIIS5 (Ito et al. 1997; He et al. 1997), Ile1175, Ile1178, Met1183 and the conserved Tyr1174 of IIIS6 (Bodi et al. 1997; Peterson et al. 1997) and Tyr1485, Met1486, Ile1493 and the conserved Asn1494 in IVS6 (Schuster et al. 1996; Peterson et al. 1997) (Fig. 3). The largest effects were observed with mutation of Thr1061 to Tyr, which lowered the affinity for isradipine more than 1000-fold (He et al. 1997; Ito et al. 1997). The removal of stimulation of I_{Ba} by the DHP agonists Bay K 8644 or (+)S-202-791 required mutation of fewer amino acids: Thr1061 in IIIS5 (Ito et al. 1997),

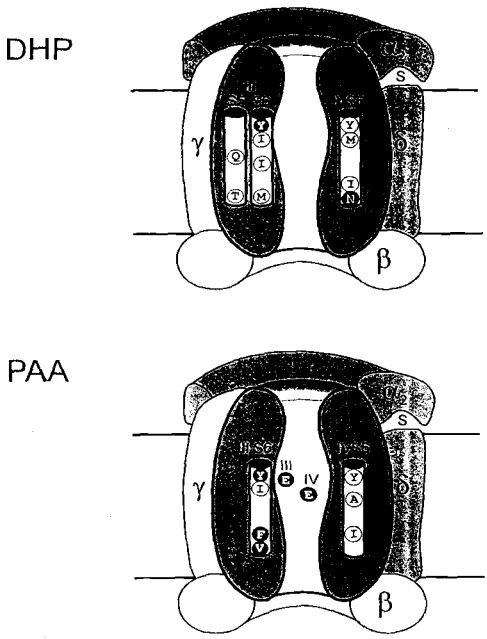

Fig. 6. (**Top**) Localization of interaction sites for DHP calcium channel antagonists and agonists on the IIIS5, IIIS6 and IVS6 segments. (**Bottom**) Localization of interaction sites for PAA calcium channel antagonist on the IIIS6 and IVS6 segments and the pore region. Letters on white background indicate residues that are different between DHP-sensitive and -insensitive channels. Letters on black background are residues that are conserved in all calcium channels and participate in the interaction with different ligands

Tyr1174 in IIIS6 (Bodi et al. 1997) and Tyr1485, Met1486 in IVS6 (Schuster et al. 1996). In contrast to these mutations, the replacement of the L-type specific Phe1484 in IVS6 by Ala decreased the IC_{50} for the DHP blocker isradipine from 6.8 nM to 0.014 nM (Peterson et al. 1997). More or less identical results were obtained when the binding affinity of the mutated α_{1C} or α_{1S} subunit for isradipine was determined (Peterson et al. 1996; He et al. 1997). High affinity binding of DHPs requires Ca^{2+} ions (Schneider et al. 1991), which are coordinated by the glutamates in the pore region I, II, III, IV (Mitterdorfer et al. 1995). Mutation of the respective Glu to Gln in the α_{1S} pore region III and IV decreased the affinity for isradipine 10- to 40-fold (Peterson and Catterall 1995). Although not completely excluded, it is unlikely that the high affinity binding of DHPs involves direct binding to the pore region glutamates. Most likely, the coordination of Ca^{2+} is required to maintain the optimal conformation for high affinity binding. In contrast, isradipine binds with low affinity (IC_{50} about 1 μM) to the open state of an α_{1C} subunit as revealed by the use of a channel in which Tyr1485, Met1486, Ile1493 of IVS6 were mutated (Lacinova and Hofmann 1998). Possibly, binding to the pore region is involved in this low affinity block.

The transfer of parts of the α_{1C} sequence to the DHP-insensitive neuronal α_{1A} subunit (Grabner et al. 1996) confirmed the above concept. Detailed analysis using the α_{1A} subunit (Sinnegger et al. 1997; Hockerman et al. 1997b) or the α_{1E} subunit (Ito et al. 1997) showed that the L-type specific and the non-conserved amino acids (see above) had to be present to allow high affinity block and stimulation of these channels by the DHP antagonist isradipine and agonist Bay K 8644. The IC_{50} values for block of the chimeric channels was in the range of 10 to 100 nM. A similar range is obtained with the wild type α_{1C} channel at a holding potential of –80 mV suggesting that these amino acids transfer the affinity for a "resting block". The high affinity block by DHPs requires inactivation of the L-type Ca^{2+} channel. Inactivation of the channel results in IC_{50} values of 0.1 nM or less. At present, it is not clear if this high affinity state requires the transfer of additional amino acids or cannot be obtained with the α_{1A} and α_{1E} subunit, because these channels inactivate at different membrane potentials leading to a different conformation of the binding site. Testing of the different mutations of the α_{1C} channel with charged and noncharged DHPs (Bangalore et al. 1994) indicated that inactivation of the mutated channel affected the channel block differently. The noncharged DHP behaved like the commonly used isradipine (Lacinova et al. 1997). In contrast, the charged DHP blocked wild type and mutated α_{1C} channels with similar affinities indicating that charged DHPs may bind to a different conformation of the channel and interact with different amino acids than the neutral DHPs. The involvement of additional amino acids in

the high affinity block of the L-type channel by DHPs is supported by the results with a recently cloned L-type calcium channel α_1 subunit from jellyfish (Jeziorski et al. 1998). The expressed jellyfish α_1 subunit contains all amino acids identified in IIIS5, IIIS6 and IVS6 which are necessary for high affinity block of the mamalian α_{1C} channel. However, this channel is neither blocked by isradipine at submicromolar concentrations nor stimulated by (-)Bay K 8644.

The work of several groups suggested that the coexpression of a ß and $\alpha_2\delta$ subunit is required for high affinity binding of DHPs (Mitterdorfer et al. 1994; Wei et al. 1995; Suh-Kim et al. 1996; but see Lacinová et al. 1995). However, at present it cannot be decided, whether these subunits help to localize the α_1 subunit in the membrane, to obtain a correctly folded α_1 subunit or directly influence the binding site. It was reported that high affinity binding of DHPs is already observed when the α_{1C} subunit was expressed alone (Welling et al. 1993a). Investigation of several splice variants of the α_{1C} subunit showed that additional sequences affect the DHP sensitivity (Welling et al. 1993b). In-depth analysis of the α_{1C-a} (cardiac) and α_{1C-b} (smooth muscle) sequences showed that the alternative exon 8a or 8b, which codes for the IS6 segment, affects the affinity for neutral DHPs (Welling et al. 1997). The α_{1C-a} channel, which contains the segment IS6a and is expressed in cardiac muscle, is blocked at higher concentrations of nisoldipine than the α_{1C-b} channel, which is expressed in vascular smooth muscle (Welling et al. 1997). IC_{50} values for isradipine were 32 and 8 nM at a holding potential of -80 mV and 10 and 1.3 nM at a holding potential of -50 mV for the α_{1C-a} and α_{1C-b}, respectively (Lacinova et al. 1998). Similar results were reported by Zühlke and coworkers (1998) and Morel and coworkers (1998) demonstrating that the IS6 segment significantly affects DHP block. It was possible that the change in affinity was caused by different inactivation kinetics of the two splice variants, since the IS6 segment strongly affects the inactivation kinetics of the channel (Zhang et al. 1994). However, the inactivation kinetics of the two channels are either identical or opposite to expectation, i.e. the cardiac α_{1C-a} channel inactivated at more negative membrane potentials than the smooth muscle α_{1C-b} channel (Hu and Marban 1998). Together with the earlier photoaffinity results (Kalasz et al. 1993), it is obvious that the increased affinity of the smooth muscle L-type calcium channel for DHPs is caused by structural differences in the IS6 segment, which contribute directly to the DHP binding pocket and not to the inactivation kinetics. Additional splice variations at the IIIS2 segment and in the intracellular carboxy terminal sequences could also contribute to an altered DHP affinity (Zühlke et al. 1998).

B.III.2 The Phenylalkylamine and Benzothiazepine Binding Site

Phenylalkylamines (PAA) such as verapamil, gallopamil or devapamil block L-type calcium currents in a use-dependent manner from the intracellular side of the membrane (Hescheler et al. 1982) and affect the binding of DHPs by allosteric interaction (Striessnig et al. 1993). In addition, benzothiazepines (BTZ) such as diltiazem interact allosterically with the binding of DHPs (Striessnig et al. 1993). In contrast to PAAs, benzothiazepines label extracellular sites in the linker sequence between IVS5 and IVS6 in the α_{1S} subunit (Watanabe et al. 1993), in agreement with a recent report that the quaternary 1,5 BTZ DTZ417 blocks the cardiac L-type channel only when applied from the extracellular side (Kurokawa et al. 1997). More recently, it was shown that like the PAA devapamil (Catterall and Striessnig 1992), the 1,4-BTZ semotiadil labels a short sequence of the IVS6 segment (Kuniyasu et al. 1998). The PAA verapamil blocks the L-type α_{1C} Ca^{2+} channel and the non-L-type α_{1A} and α_{1E} Ca^{2+} channels at similar concentrations in a state-dependent manner (Cai et al. 1997), whereas diltiazem blocks all three channels at similar concentrations, but only the α_{1C} Ca^{2+} channel in a state-dependent manner (Fig. 6 bottom).

Molecular analysis of the α_{1C} subunit (Schuster et al. 1996; Hockerman et al. 1995, 1997a) showed that the L-type channel-specific Ile1175 and the conserved Tyr1174, Phe1186 and Val1187 in IIIS6 and the L-type-specific Tyr1485, Ala1489 and Ile1492 in IVS6 are necessary to form a high affinity PAA site (Fig. 3). In addition, the two glutamates (Glu1140 and Glu1441) in the pore region of repeat III and IV are necessary (amino acid numbering is according to the $\alpha_{1C\text{-}b}$ sequence (Biel et al. 1990)) (Hockerman et al. 1997a). The effect of the mutation of the conserved Tyr1174 depends on the replacing amino acid. Substitution by phenylalanine decreased the affinity for devapamil 18-fold, whereas substitution by an alanine increased the affinity 7-fold (Hockerman et al. 1997a). The increased affinity of the Y1174A mutant is most likely caused by a shift of the steady state inactivation curve by -11 mV. Transfer of the three IVS6 amino acids Tyr1485, Ala1489 and Ile1492 from the α_{1C} to the α_{1A} subunit introduced PAA and BTZ sensitivity, when measured in a use-dependent protocol (Hering et al. 1996). Furthermore, it was shown that the triple mutation Y1485A, A1489S and I1492A in IVS6 of the α_{1C} channel reduced use-dependent block of the three PAAs , devapamil, verapamil and gallopamil, reduced the resting and depolarized block by devapamil, but poorly affected the resting and depolarized block by verapamil and gallopamil (Johnson et al. 1996).

Together, these results show that the IVS6 segment interacts with various PAAs and BTZ. State-dependent block of the L-type channel is mediated by

the same three amino acid residues in IVS6 for diltiazem and devapamil. However, different amino acids are required to allow high affinity interaction at the resting state for diltiazem, verapamil and gallopamil. A further problem arises from the finding that DHPs, PAAs and BTZs interact with the same (Tyr1485) or with the adjacent (Ile1492and Ile1493) amino acid side chain. It is difficult to reconcile this close location of interacting site chains with the allosteric modulation of DHP binding by diltiazem or phenylalkylamines decribed previously (Striessnig et al. 1993).

C.I Modulation of Expressed Calcium Channels by Protein Kinases

C.I. 1 Modulation by cAMP-Dependent Phosphorylation

In the heart, the positive inotropic action of catecholamines is mainly caused by an increased calcium influx through L-type calcium channels. cAMP-dependent phosphorylation of the α_1 subunit or a closely associated protein increases the current 3- to 7-fold (Osterrieder et al. 1982; Kameyama et al. 1985; Hartzell and Fischmeister 1992). Phosphorylation increases the probability of channel opening upon depolarization by modulation of channel gating. Cardiac calcium channel currents also show facilitation during high frequency stimulation (Lee 1987) or after strong depolarization (Pietrobon and Hess 1990). Depolarization-induced facilitation was supposed to require voltage-dependent phosphorylation of the channel by cAMP kinase (Artalejo et al. 1992). However, these results of Artalejo and collegues were probably caused by the removal of secreted substances from the external solution and not by channel phosphorylation (Garcia and Carbone 1996). The adult skeletal muscle calcium channel is apparently not regulated by phosphorylation to a large extent. In contrast, the calcium channel of embryonic rat skeletal muscle myoballs shows voltage- and cAMP kinase-dependent facilitation (Sculptoreanu et al. 1993b). Facilitation depending on a strong depolarizing prepulse requires membrane localization of cAMP kinase (Johnson et al. 1994) by a 15 kDa cAMP kinase anchoring protein (Gray et al. 1998).

In adult skeletal muscle, two forms of the α_{1S} subunit are present, a large 212 kDa form, containing the complete sequence of the cloned α_{1S} cDNA, and a small 190 kDa form, which is truncated between amino acid 1685 and 1699 (De Jongh et al. 1991). About 5% of the α_{1S} subunits are the large 212 kDa form and over 90% are processed to the small 190 kDa form (De Jongh et al. 1991). In intact rabbit skeletal muscle myotubes, cAMP kinase rapidly phosphorylates Ser1757 and Ser1854 in the large 212 kDa form and slowly phosphorylates Ser687 in the small 190 kDa form, which does not contain

the cAMP kinase sites at Ser1757 and Ser1854 (Rotman et al. 1995). Expression of an α_{1S} cDNA, which is truncated at Asn1662 and encodes the small form, fully restored both e-c coupling and calcium current in dysgenic myotubes, consistent with the idea that the small form of the α_{1S} subunit performs both functions in adult muscle without cAMP-dependent phosphorylation (Beam et al. 1992). These results are in line with the conclusion that the long form of the skeletal muscle α_{1S} channel is modulated by cAMP kinase in myoballs, but that this modulation is attenuated or not present in adult skeletal muscle, in which the short form prevails.

In contrast to the skeletal muscle L-type calcium channel, the precise mechanism of phosphorylation of the cardiac α_{1C} calcium channel is less clear. The fact that cAMP kinase-dependent phosphorylation significantly affects the function of the channel *in vivo* is undisputed. However, the mechanism causing the channel modulation is controversial. Rabbit heart sarcolemma contains a large 240 kDa and a small 210 kDa form of the α_{1C} subunit (De Jongh et al. 1996). The small 210 kDa form is truncated at residue 1870 in the carboxy terminal sequence. The 240 kDa form is phosphorylated by cAMP kinase at Ser1928 (De Jongh et al. 1996). The expressed full length 250 kDa $\alpha_{1C\text{-}a}$ subunit is phosphorylated *in vivo* in CHO cells (Yoshida et al. 1992) and HEK 293 cells (Gao et al. 1997). Phosphorylation of the α_{1C} subunit is prevented by the mutation S1928A (Gao et al. 1997). The mutation S1928A also prevents a decrease in barium current induced by the cAMP kinase inhibitor H-89 in Xenopus oocytes (Perets et al. 1996). However, a direct effect of cAMP kinase on current amplitude was not observed in Xenopus oocytes (Singer-Lahat et al. 1994; Bouron et al. 1995; Perets et al. 1996). In contrast to studies in oocytes, a cAMP-dependent increase in current amplitude was reported by several groups, who used either CHO or HEK cells as the expression system (Haase et al. 1993; Perez-Reyes et al. 1994). The peak barium inward current showed facilitation following a prepulse to positive membrane potentials in CHO cells dialysed with active cAMP kinase (Sculptoreanu et al. 1993a). cAMP kinase-dependent facilitation was also reported by Bourinet and coworkers (1994), who used the neuronal $\alpha_{1C\text{-}c}$ splice variant and the oocyte expression system. In a recent report, these authors observed facilitation of barium currents in the absence of cAMP-dependent phosphorylation and showed that facilitation was observed only in the presence of the $ß_1$, $ß_3$ and $ß_4$ subunits and was not supported by the neuronal $ß_{2a}$ subunit (Cens et al. 1998). Identical results were reported by Qin and coworkers (1998a), who used an N-terminally truncated $\alpha_{1C\text{-}a}$ (expressed residues 60–2171) subunit. These recent results are in agreeement with the earlier reports by Kleppisch and coworkers (1994) and Bouron and coworkers (1995) that facilitation of the α_{1C} current is independ-

ent of cAMP kinase-dependent phosphorylation. In a careful study, which used the α_{1C-a} and α_{1C-b} splice variants stably expressed in CHO and HEK 293 cells and transient expression of α_{1C-b}, cardiac $ß_{2a}$ and $\alpha_2\delta$-1, Zong and coworkers (1995) showed that the current amplitude in these cells was not affected significantly by internal dialysis with cAMP kinase inhibitor peptide, catalytic subunit of the cAMP kinase or a combination of cAMP kinase and okadaic acid. Similar results were obtained by the coexpression of all subunits of the calcium channel complex, whereas the calcium current of cardiac myocytes was increased 3-fold during internal dialysis with active cAMP kinase or external superfusion with isoproterenol. Furthermore, dialysis of cardiac myocytes with the phosphatase inhibitor microcystin stimulated the calcium inward current more than 2-fold, whereas the current of the expressed calcium channel was not affected. These conflicting results were apparently solved when Gao and coworkers (1997) reported that cAMP kinase-dependent stimulation of barium current required the coexpression of the cAMP kinase anchoring protein AKAP 79, α_{1C-a} and the neuronal $ß_{2a}$ subunit in HEK 293 cells. AKAP 79 anchors the kinase at the plasma membrane. These authors reported that phosphorylation of Ser1928 was required for cAMP-dependent stimulation of barium currents. However, a careful reexamination of these results using overexpression of AKAP79 – cloned from HEK 293 cells and identical to that used by Gao and coworkers (1997) – failed to reproduce a cAMP kinase-dependent increase in current amplitude or facilitation of the current by strong depolarization (Dai et al. 1998). In contrast, cAMP-independent facilitation was observed when α_{1C-a} and cardiac $ß_{2a}$, or α_{1C-a} truncated at residue 1733 were used. Prepulse facilitation was prevented by expressing the α_{1C-a} and cardiac $ß_{2a}$ subunits together with the $\alpha_2\delta$-1 or $\alpha_2\delta$-3 subunit, in line with the known effect of the $\alpha_2\delta$ subunit on the gating of the channel. These results demonstrate clearly that facilitation of the cardiac L-type current can be observed with channels which do not contain the established cAMP kinase phosphorylation site at Ser1928.

C.I.2 Modulation by Protein Kinase C-Dependent Phosphorylation

L-type calcium channels are tightly regulated by hormonal and neuronal signals. Protein kinase C (PKC) is one such regulator, which enhances cardiac, smooth muscle and neuronal L-type currents (Lacerda et al. 1988; Yang and Tsien 1993; Schuhmann and Groschner 1994) by an increase in the open probability of the channel (Yang and Tsien 1993). The response to PKC activators is usually biphasic, with an increase followed by a later decrease

Table 1. Genetic Diseases associated with HVA-Calcium Channels GD, genetic disease; KO, gene disrupted by targeting in knockout mice

Gene	Species	Location	GD/KO	Function	Reference
L-Type Calcium Channels					
α_{1S}	mouse	skeletal muscle	GD	lethal	a
$ß_1$	mouse	skeletal muscle	KO	lethal	b
γ_1	mouse	skeletal muscle	KO	no apparent phenotype	c
α_{1S}	human	skeletal muscle	GD	Hypokalemic periodic paralysis	d, e
α_{1C} like	C. elegans	muscle	GD	Myotonic; several phenotypes	f
α_{1F}	human	retina	GD	X-linked congenital stationary night blindnee	g,h
α_{1C}	mouse	heart, smooth muscle	KO	lethal	i
Non-L-Type Calcium Channels					
α_{1A}	human	brain	GD	Familial hemiplegic migraine and episodic ataxia type 2	k
α_{1A}	human	brain	GD	Cerebellar ataxia	l
α_{1A}	mouse	brain	GD	Tottering mouse/Absence epilepsy	m
$ß_3$	mouse	brain	KO	Reduction in N-type channels	n, p
$ß_4$	mouse	brain	GD	Lethargic mouse/Ataxia and seizures	q
γ_2	mouse	brain	GD	Stargazer mouse/Absence epilepsy	r

a) Chaudhari 1992; b) Gregg et al. 1996; c) Freise et al. 1998; d) Ptácek et al. 1994; e) Jurkat-Rott et al. 1994; f) Lee et al. 1997; g) Strom et al. 1998;h) Bech-Hansen et al. 1998; i) Seisenberger et al. 1998; k) Ophoff et al. 1996; l) Zhuchenko et al. 1997; m) Fletcher et al. 1996; n) Namkung et al. 1998; p) Cavalié et al. 1998; q) Burgess et al. 1997; r) Letts et al. 1998.

(Lacerda et al. 1988; Schuhmann and Groschner 1994). The biphasic response to PKC stimulators was fully reconstituted when the α_{1C-a} subunit was expressed in Xenopus oocytes (Singer-Lahat et al. 1992). Bouron and coworkers (1995), who used a human α_{1C} splice form, which has the same amino terminus as the α_{1C-b} subunit, observed only a decrease in current suggesting that PKC-dependent regulation may be controlled by the different amino termini of the two splice variants. This prediction was confirmed (Shistik et al. 1998). Deletion of amino acids 2–46 in the amino terminus of the α_{1C-a} subunit prevented the PKC-dependent current increase. The effects of PKC activation were larger in the presence of the α_{1C-a} and $\alpha_2\delta$-1 subunits and were decreased by the coexpression of the cardiac $ß_{2a}$ subunit. Upregulation of the current was not affected by truncation of the α_{1C-a} subunit at residue 1665, or mutation of the proposed PKC phosphorylation site Ser533 in the I–II linker. Upregulation depended on the splice variation of the amino terminus and was not observed with the amino terminus of the α_{1C-b} subunit. In agreement with Wei and coworkers (1996), these studies show that, depending on the splice variant, the amino terminus affects channel gating and mediates PKC-dependent upregulation.

D.I Genetic Mutations of Calcium Channels and Associated Phenotypes

Inheritable disorders of ion channels include a variety of different diseases such as cardiac arrythmias, epilepsy, migraine headache or episodic ataxias. These disorders are caused by mutations in sodium, calcium and chloride channels in skeletal muscle, in calcium and potassium channels in neurones or in neuronal nicotinic and glycine receptors. The molecular bases of channelopathies have recently been reviewed (Ptacek 1997; Ophoff et al. 1998; Terwindt et al. 1998) (Table 1).

D.I.1 Linkage of α_{1S} Calcium Channel Mutations to Hypokalaemic Periodic Paralysis

Hypokalaemic periodic paralysis is an autosomal dominant skeletal muscle disorder in which episodic weakness is associated with low serum potassium levels. The defect cosegregates with the skeletal muscle α_{1S} gene. Mutations occur at one of two adjacent nucleotides within the same codon resulting in the substitution of a highly conserved Arg1239 in the IVS4 segment by a His or Gly (Ptacek et al. 1994; Fouad et al. 1997) and Arg528 in the voltage sensor of repeat II by His (Jurkatt-Rott et al. 1994). Initially, it was reported that in myotubes from affected patients, the mutation R1239H decreased current

amplitude, whereas mutation of R528H shifted the voltage-dependence of steady state inactivation by -40 mV (Sipos et al. 1995). Later analysis of the effect of the R528H mutation on channel kinetics, which used mutated α_{1C} channels (Lerche et al. 1996), mutated α_{1S} channels (Lapie et al. 1996), mutated α_{1S} channels and myotubes from affected patients (Jurkatt-Rott et al. 1998) or only myotubes from affected patients (Morrill et al. 1998), did not confirm the earlier findings. It is not clear in which way the mutation leads to the phenotype. Inactivation of the α_{1S} gene (Chaudhari 1992) or the β_1 subunit gene (Gregg et al. 1996) in mice is associated with a lethal phenotype, because e–c coupling is impaired in muscle preventing breathing after delivery. In contrast, deletion of the γ_1 subunit gene does not lead to gross physiological defects (Freise et al 1998). Taken together, these results do not establish the cause of the hypokalaemic periodic paralysis phenotype.

D.I.2 Linkage of α_{1A} Calcium Channel Mutations to Familial Hemiplegic Migraine and Cerebellar Ataxia

Three disorders are known that are caused by mutations in the neuronal P/Q type calcium channel α_{1A} subunit. Familial hemiplegic migraine (FHM) is a rare autosomal dominant disorder that is characterized by migraine attacks with a transient hemiparesis. To date, four different missense mutations have been identified in the α_{1A} subunit gene (Ophoff et al. 1996). A R192Q mutation in IS4, analogous to the mutation in the voltage sensor of α_{1S}, changes a basic amino acid residue into an uncharged residue. The T666M mutation is located in the pore loop structure of repeat II that determines the ion selectivity of the channel. Changes in this region are believed to affect the normal functioning of the channel. The other two point mutations implicated in this disease were identified in the S6 segments of repeat II (V714A) and IV (I1811L). Introduction of these mutations into the rabbit α_{1A} sequence showed no effect for the R192Q mutation (Kraus et al 1998). The other three mutations, which are located in the pore region of the channel, alter the inactivation kinetics and may provide the basis for the postulated neuronal instability in patients with FHM.

Truncating mutations of the α_{1A} subunit gene were found to be associated with episodic ataxia type 2 (EA2) (Ophoff et al. 1996). EA is a neurological disorder in which patients suffer from recurrent attacks of generalized ataxia and other symptoms of cerebellar dysfunction. The disorder is characterized by two clinically distinct syndromes. Episodic ataxia with myokymia (EA1) that includes brief episodes of ataxia and dysarthria is thought to be associated with missense mutations in a potassium channel gene. The other type of episodic ataxia without myokymia (EA2) is heterogenous and is charac-

terized by paroxysmal cerebellar ataxias, paroxysmal vestibulocerebellar ataxias and hereditary paroxysmal cerebellar ataxias. One of the errors associated with EA2 is a splice site mutation of an invariant G nucleotide of the intron consensus sequence that leads to aberrant splicing. This mutation causes a disruption of the reading frame in the transmembrane segment S2 of repeat III. The other known EA2 mutation is a single nucleotide deletion of C4073 which also causes a premature stop in IIIS1. The truncated proteins are thought to be unable to form a functional calcium channel and may also, as a dominant negative effect, influence proper P/Q-type calcium channel assembly in the membrane.

Numerous unstable expansions of trinucleotide repeats have been reported to be associated with neurodegenerative disorders that include muscular atrophy, different types of spinocerebellar ataxia and Huntington's disease. Normally, there are about 20 trinucleotide CAG repeats, whereas affected people either have 2- to 3-fold or even hundredfold increase in trinucleotide repeats. A multiplication of the CAG repeat was also identified in patients with chronic cerebellar ataxia SCA6 in the 3'-untranslated region of the α_{1A} calcium channel gene (Ophoff et al. 1996). Another study showed that there are at least six different transcripts of α_{1A}, of which three contain an insertion of five nucleotides which results in a shift of the open reading frame and translation of the CAG repeat as a polyglutamine stretch in the C-terminus (Zhuchenko et al. 1997). However, the pathogenic effect of the small expansion of the polyglutamine region in α_{1A} calcium channel subunits is still a matter of debate. First, the number of repeats (21–27) is considerably smaller than that of other neurodegenerative disorders (more than 100). Secondly, alternative splicing in the C-terminal part of the α_{1A} subunit RNA may result in transcripts in which the CAG repeat is either translated as glutamine or is part of the noncoding region. It is still not known how the different transcripts contribute to the onset of cerebellar ataxia.

Mutations of the α_{1A} calcium channel have also been described in the tottering (tg) and leaner mice (tg^{la}) (Fletcher et al. 1996). Mice with the recessive tottering mutation have been extensively studied as models for human epilepsy (Noebels and Sidmann, 1979). Similar to the FHM disorder, the mutation in the tottering mouse is also a missense mutation in the pore loop region of repeat II. The more severe phenotype of the leaner mouse is associated with a splice site mutation that results in an aberrant intracellular C-terminus (Fletcher et al. 1996). The location of these mutations suggests that the underlying molecular mechanisms in leaner mice lead to a loss of function and in tottering mice to a gain-of-function mutation.

D.I.3 Diseases Caused by Mutations of L-Type α_1 Calcium Channel Subunits

Mutations in calcium channel genes have led to the identification of the new, tissue specifically expressed α_{1F} subunit belonging to the family of L-type calcium channels. This novel retina-specific gene was characterized in families with incomplete X-linked congenital stationary night blindness and was found to encode an L-type calcium channel α_{1F} subunit (Strom et al. 1998; Bech-Hansen et al. 1998). Mutation analysis of this α_{1F} subunit revealed a variety of different types of changes in the primary structure, including missense and nonsense mutations and the deletion and insertion of nucleotides. The nonsense and insertion/deletion mutations cause premature protein truncations and are distributed across the α_{1F} subunit from the cytoplasmic loop between repeat II and III to the first portion of the cytoplasmic C-terminus. Missense mutations were also found in different regions of the channel. The significance of these mutations is supported by the high structural conservation of the affected residues between the α_1 subunits (Strom et al. 1998). The aberrations in the α_{1F} gene are thought to cause the decrease in neurotransmitter release from photoreceptor presynaptic terminals frequently seen in the incomplete form of X-linked congenital stationary night blindness.

In *Caenorhabditis elegans*, the *egl-19* gene plays a pivotal role in regulating muscle excitation and contraction (Lee et al. 1997). The product of the *egl-19* gene is a homologue of the α_{1C} calcium channel. Two myotonic mutations have been identified in the IS6 segment which is involved in voltage-dependent inactivation (Zhang et al. 1994). A third myotonic mutation implicates the IIIS4 segment, which is part of the voltage-sensor. In addition several lethal mutations of the *egl-19* gene have been identified. In agreement with these findings, deletion of the α_{1C} gene in the mouse is lethal (Seisenberger et al. 1998). Surprisingly, the embryos develop normally up to day 14 pc and have a beating heart. Cardiac beating is suppressed at low concentrations of nisoldipine, a dihydropyridine. The channels involved are unknown.

D.I.4 Diseases Caused by Mutations of Calcium Channel ß and γ Subunits

Not only the alteration of the main pore-forming α_1 subunit results in different heritable neurological disorders, but also the mutation of the auxiliary ß subunit has similar severe effects. Defects in the $ß_4$ subunit gene on chromosome 2 are associated with seizures and ataxia in recessive lethargic (lh) mice (Burgess et al. 1997). An insertion of four nucleotides into a splice donor site of the $ß_4$ gene results in exon skipping and in a translational

frameshift. The truncated protein thereby loses the binding site for the α_1 subunit and probably represents a loss of function mutation. The lethargic phenotype has many features in common with the tottering mouse including absence epilepsy and spontaneous focal motor seizures and ataxia. Previously it was shown that the α_{1A} and $ß_4$ subunits are colocalized and could be part of the P/Q-type calcium channel (Ludwig et al. 1997). It is therefore possible that mutation of the $ß_4$ subunit affects the cerebellar P/Q-type calcium current. Recently, it was demonstrated that neither a mutated nor a truncated $ß_4$ protein is present in the lethargic mouse brain (McEnery et al. 1998). The absence of the $ß_4$ protein is associated with with decreased expression of the N-type channel (α_{1B}) in forebrain and cerebellum and an increased expression of the $ß_{1b}$ subunit (McEnery et al. 1998). It was suggested that the persistence of an immature population of N-type channels is the basis for at least part of the lethargic phenotype.

In contrast to the inactivation of the $ß_1$ and $ß_4$ subunit, mice with a targeted disruption of the $ß_3$ gene are viable and indistinguishable from the wild type with no gross morphological or histological differences (Namkung et al. 1998; Cavalié et al. 1998). Minor effects could only be detected in sympathetic neurons in which L- and N-type currents were reduced. In addition, the voltage dependent activation of P/Q-type calcium channels was also altered. These extremely different phenotypes clearly indicate that the ß subunits are not redundant and are needed to "stabilize" specific calcium channels at specific locations.

Similar to the identification of the α_{1F} subunit, a new auxiliary calcium channel γ subunit was described by genetic analysis of epileptic seizures of stargazer mice (Letts et al. 1998). The γ_2 subunit is expressed only in the brain and was shown to shift steady-state inactivation of heterologously expressed α_{1A} calcium channels in the hyperpolarizing direction. The discovery of a novel γ subunit implicates that neuronal and other HVA calcium channel complexes may contain a γ subunit as a fifth protein.

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Aging of the Male Endocrine System

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Contents

1 Preface

The current phenomenon of population aging will be a major determinant for future health trends. Due to this demographic change affecting developed as well as third world countries, a major aim of geriatric medicine is in the maintenance of quality of life for the elderly and the containment of costs for care associated with age-related diseases. As most of the diseases that affect elderly people are in some way or another associated with the endocrine system, this aim can only be achieved by first elucidating the pathogenetic mechanisms that determine the most important endocrine diseases of later life, and secondly by clarifying the consequences of hormonal changes in the elderly. It is important to be clear that the goal is to improve the quality of life in old age rather than to extend the length of life, regardless of quality. Given the universal character of aging and the requirement to provide an array of medical and health-related services for the aged, world-wide attempts are on their way to understand mechanisms and consequences of age-related changes in the secretion of hormones, the major contributors in the orchestration of the processes necessary for orderly development and aging.

2 Theories of Aging

Aging-as a highly complex process-is still poorly understood despite of the rapid development of new biomolecular tools. A wide spectrum of approaches, ranging from descriptive up to reductionistic studies, in the examination of various age-related mechanisms at cellular or molecular levels, have led to several theories of aging (Carlson and Riley 1998). These can roughly be subcatagorized into genetic or epigenetic. A comprehensive description of age-related aspects like for example oxidative damage by free radicals, protein glycation, the failure of DNA repair, "gerontogenes", decreased or aberrant immune function, a decreased membrane fluidity or telomeric shortening would be beyond scope of this review (for information see Kirkwood 1996; Carlson and Riley 1998). As human beings undergo changes in all domains of their lives i.e. genetic, biochemical, metabolic, physiological, psychological and social, it is not expected that any single, overarching, predictive theory of senescence will ever be found. In addition the various theories of aging are not independent.

The "disposable soma theory", proposes that aging is due to the accumulation of unrepaired somatic defects and the primary genetic control of longevity operates through selection to raise or lower the investment in basic

cellular maintenance systems, in relation to the level of environmental hazards (Kirkwood 1992).

The genetic paradigm assumes that aging is a continuation of the developmental process in which a program of successive gene activations and inactivations leads to differentiation and maturation. The fact that rates of aging are variable among, but characteristic for, particular species, indicate the existence of genetic factors that influence the ageing process. The identification of the gene responsible for the Werner's syndrome (Yu et al. 1996), a rare genetic disease in which individuals age prematurely, and the "age-1" gene found in Caenorhabditis elegans (Johnson 1990), which, when mutated, increases mean life span by 65%, to name just two examples, favour the theory that genes are being related in some way or another to life span.

One of the oldest theories on aging states that longevity is inversely proportional to the metabolic rate (Pearl 1928). Although simplistic in nature, the observation of the inverse association between longevity and metabolic rate among many species is intriguing. However, its significance for the aging process *per se* is uncertain, as the prolongation of life span may also be a result of delayed maturation, hence leaving the rate of aging unchanged (Rose 1991).

A link between metabolism and aging is provided by the "free radical theory" (Harman 1956; Martin et al. 1996). According to this theory, random tissue damages caused by oxygen radicals, produced by normal aerobic metabolism, accumulate during life and lead to various breakdown events at cellular or molecular levels. Aging would then result from this gradual accumulation of cell and tissue damage caused by interactions with their milieu.

While an examination of the mechanisms of aging invariably seems to lead to the cellular and subcellular level (Rattan and Derventzi 1991), the meaning of aging may be lost in the absence of an organismal context (Carlson and Riley 1998). Comfort's aging model, the concept of a "hierarchy of clocks" suggests that aging is not caused by a single regulatory system but rather by the interaction between a number of processes, each with independent timing. The fastest of any of the clocks can lead to deterioration or death (Comfort 1979).

One candidate for such a pacemaker in humans is the endocrine system. The precise organisation of physiological, cellular and molecular events is governed by hormones and leads not only to processes like reproductive cyclicity in women but also to their cessation (Wise et al. 1996). The dramatic and fairly rapid endocrine changes brought about by menopause have biological, social and cultural implications that profoundly influence the second half of a woman's life. Endocrine changes are also observed in men,

although not as drastic and sudden as in women (Warner et al. 1985). Thus, age-related changes in hormone secretion appear to be phenomenons affecting both genders.

3 Endocrine Changes in Aging Men

Human senescence is accompanied by various physiological and morphological changes at molecular, cellular (Conover 1997) and organ levels. These range from alterations in receptor signal transduction and subsequent gene expression, to structural and morphological changes affecting – at least in part – endocrine functions. Although common, the pattern of changes can be highly variable between individuals and many aspects of the aging phenotype bear marked similarities to states of hormone deficiency or excess. Nevertheless, the once popular concept that aging is a consequence of a single or multiple states of hormone deficiencies certainly does not hold true and has to be converted into a modern concept appreciating the complexity and heterogeneity of the aging process. Whatever the underlying mechanisms, the aging process leads to a state in which there are reductions in the number and/or functional reserve capacities of various types of differentiated cells and the inability to repair defects or replace affected cells by proliferation and/or differentiation of stem cells. This state may well apply to endocrine organs and their regulatory centers. As a consequence, hormonal circadian or seasonal biorhythms, pulsatile frequency or amplitude as well as absolute hormonal levels may change with age.

A description of age-related endocrine dysfunctions must discriminate between intrinsic age-related changes and those of a variety of age-associated, potentially confounding, comorbid variables or even changes caused by extrinsic factors like nutritional status, physical activity or the use of medications by individuals. The establishment of more precise normative data on the effects of aging on the endocrine system will improve interpretation of diagnostic endocrine tests during illness. More importantly, it will help identifying endocrine changes that may contribute to the normal aging processes *per se* and ultimately help us to discriminate normal processes of aging, from those with pathological relevance that require treatment. The findings that hormonal treatment beneficially affects non elderly hormone-deficient adults and that secretion of certain hormones is reduced with aging raises hopes that hormonal administration in the elderly might reverse or prevent some of the physiological effects associated with old age (Villareal and Morley 1994).

Three male hormonal axes during aging are characterized by changes in concentrations of circulating hormones:

(I) The hypothalamic-pituitary-testicular axis (andropause/climacterium virile/PADAM): The magnitude of age-related alterations of the endocrine system is highly variable and sex dependent. In contrast to the clearly demarcated event of the menopause in women, aging of the endocrine system in men is a more gradual, less defined and highly variable process. A discussion concerning the menopause, being the most dramatic and rapidly occurring physiological change in women around the age of 50 (Wise et al. 1996) would be beyond scope of this review devoted to changes in the endocrine system of aging men.

The term "andropause" defined as "an indefinite syndrome composed of several constellations of physical, sexual, and emotional symptoms brought about by a complex interaction of hormonal, psychological, situational and physical factors" (Henker 1977) designated in analogy to the female menopause is a misnomer as in general there is no hormonal discontinuity in the reproductive lives of men and no upper age limit for male fertility. Besides the gradual decline of reproductive endocrine function and high variability among individuals in the degree of this reduction, not all men will become hypogonadal to a clinically significant degree. The complex of symptoms like nervousness, psychological depression, impaired memory, inability to concentrate, fatigue, insomnia, hot flushes, periodic sweating and loss of sexual vigor were first described by Werner in 1939 under the term "male climacteric" (Werner 1939; Werner 1946).

In addition to "andropause" and "climacterium virile" a third term "PADAM" (partial androgen deficiency of the aging male) was proposed to describe the symptom complex of the aging male. The contribution of age *per se* to the questionable physiologic and epidemiologic basis of a male climacteric syndrome has been heavily discussed. Since various factors like psychological stress, acute or chronic non endocrine illnesses, physical activity, obesity, malnutrition and drug or medication use have an influence on the hypothalamic-pituitary-testicular axis as well as on sexual behaviour, it is hard to discriminate between such effects and the effects of aging *per se* (McKinlay et al. 1989).

(II) The hypothalamic-pituitary-adrenal axis (adrenopause): The adrenopause in both sexes is characterized by the age-related gradually declining serum levels of the adrenal androgens dehydroepiandrosterone (DHEA) and its sulfo-conjugated derivative DHEA-sulfate (DHEAS). Although being the most abundant circulating steroids in the human organism, their biological role in human aging remains undefined. Their progressive decrease with age suggests a role for both steroid hormones in the aging process. The zonation of the adrenal gland in aging men shows changes like a reduced size of the zona reticularis and a relative increase of the outer cortical zones. A reduced

mass of the zona reticularis may be at least one cause for the diminished production of DHEA and DHEAS in aged men (Parker et al. 1997). In view of the easy restoration of serum DHEA levels to those of young men, it would be of great interest to clarify the cause-and-effect relationship between the decreasing levels of DHEAS with age, and the physiological and pathological manifestations of aging.

(III) The GH-IGF-I axis (somatopause): Within the somatopause, the growth hormone (GH)/insulin-like growth factor I (IGF-I) axis shows a gradual age-associated decline in hormone production. This is paralleled by clinical signs similar to those of GH-deficient adults (for reviews see Corpas et al. 1993; deBoer et al. 1995).

4 The Hypothalamic-Pituitary-Testicular Axis

4.1 Testosterone

Aging in men is accompanied by clinical signs, such as changes in bone mineral density (Riggs et al. 1981), a decrease in Leydig cells (Neaves et al. 1984), a decline in Sertoli cell function (Tenover et al. 1988), a decrease in muscle mass (Vandervoort and McComas 1986), and signs of decreased virility (Swerdloff and Wang 1993; summarized in: Vermeulen and Kaufman 1995). Whether or not aging is also associated with a decrease in plasma testosterone concentrations, has long been a matter of debate (reviewed in: Vermeulen 1991). A decline in androgen levels with aging was first reported in 1958 using bioassays (Hollander and Hollander 1958). Further studies using radioimmunoassays to measure testosterone (T) levels in the sera of elderly men yielded in part contradictory results. This was mainly due to differences in the characteristics of the men studied, the study-design (morning or afternoon blood sampling for androgen levels and the frequency of blood sampling in view of the episodic nature of T secretion) and the cross-sectional character of the studies. However, several recent well-designed studies, controlling these variables, have shown convincingly that T levels do indeed decrease with age (Deslypere and Vermeulen 1981; Nahoul and Roger 1990; Gray et al. 1991; Simon et al. 1992; Morley et al. 1997). The investigation with the largest sample of men comprised 415 healthy men and 1294 men with one or more ailments, aged between 39 and 70 years old (Gray et al. 1991). In this study, obesity was associated with lower T levels and there were no significant differences between healthy and less healthy men, although T levels being 10–15% lower in the latter. Total serum T levels decline by 0.4% per year, sex hormone binding globulin (SHBG) (the carrier protein that binds approx. 60% of circulating T) increases by 1.2%

per year, with the net effect that free T levels decline by 1.2% per year (Gray et al. 1991). The high-affinity binding of SHBG for T, and to a lesser extent for estradiol, influences the circulating levels of these sex steroid hormones and their biodisposal to target cells as well as their mutual balance.

Whether plasma dihydrotestosterone (DHT) levels change with age is a matter of controversy since some investigators have found decreased serum DHT levels with aging (Pazzagli et al. 1975). Others have found no change (Pirke and Doerr 1975) and Bremner et al. (1983) even describe age-related increases in DHT serum levels of elderly men. The reduction products of DHT, androstanediol and androstanediol glucuronide, both considered to be biochemical markers of androgen action in target organs, are decreased in elderly men (Gray et al. 1991; Simon et al. 1992).

4.2 Mechanisms of Decreased Reproductive Endocrine Function

There is no consensus about the causes of decreased reproductive endocrine function with age in men. Changes at all three levels of the hypothalamic-pituitary-testicular axis, predominantly at the testicular level, seem to be associated with the modest decline of T serum levels in aging men. The lower basal serum T levels may originate from primary testicular changes as suggested by a decreased number of Leydig cells (Neaves et al. 1984), an impaired testicular perfusion (Suoranta 1971) and a reduced release of T upon stimulation by hCG (Rubens et al. 1974; Harman and Tsitouras 1980). In addition to the prevailing view of the primarily testicular origin as the cause of a decrease in bioavailable T in aging men, the relative contribution of the hypothalamic-pituitary-testicular axis to this decrease has been debated. This alternative perspective is supported by the fact that the amplitude of the nycthemeral variations in plasma T concentrations are significantly reduced in elderly men, probably a consequence of a decreased LH rhythm (Deslypere et al. 1987; Bremner et al. 1983), and/or the higher sensitivity of the gonadostat to the feed-back effect of androgens and/or estrogens in elderly men (Winters et al. 1982; Deslypere et al. 1987). Notably, the elevated basal serum levels of LH in response to the decline of T levels with aging (Deslypere and Vermeulen 1981) are lower than those observed in younger men with similarly decreased T levels (Korenman et al. 1990). The decline of bioactive LH levels with aging, which is not appreciated when LH levels are determined by immunoassays (Mitchell et al. 1995) and the reductions in the episodic frequency and amplitude of LH with aging result in a less effective stimulation of T secretion.

The reduced number of spontaneous high amplitude LH pulses in elderly men does not seem to be a consequence of a decreased sensitivity of the

gonadotrophs to LHRH. Instead, they may be the consequence of the release of smaller amounts of LHRH at each pulse (Kaufman et al. 1991).

4.3 Osteoporosis

Osteoporosis is widely viewed as a major public health concern. After some point in early middle age, bone loss ensues and is a universal phenomenon related to aging (Riggs et al. 1981). In men as in women, a higher incidence of osteoporosis is related to the decline in gonad function with increasing age. Although far greater attention has been given to osteoporosis and bone fractures in aging women, the incidence of osteoporotic fractures also increases in aging men (Santavirtas et al. 1992). Due to the slow and progressive decline of gonadal function in men compared to women and the higher peak bone mass at skeletal maturity, men are affected later and to a lesser degree than women (Crilly et al. 1981). The estimated frequency of non traumatic osteoporotic vertebral fractures in men is one sixth to that in women (Riggs and Melton 1983). In men and in women, hypogonadism is a well recognized cause for overt osteoporosis as well as asymptomatic osteopenia (Francis et al. 1986; Seeman et al. 1983; Greenspan et al. 1986). Whether the physiological reduction of gonadal function in aging men (Baker et al. 1976; Foresta et al. 1984) leads to the age-related bone loss is still a matter of debate (Crilly et al. 1981; Meier et al. 1987). Besides being a slow and progressive process, the decline of male gonadal function can occur at different ages, even when very late in life it can result in primary hypogonadism with low plasma levels of T and its metabolites, such as androstendione, and elevated gonadotropin plasma levels (Baker et al. 1976; Pirke et al. 1977; Madersbacher et al. 1993).

Mastrogiacomo and co-workers observed a positive linear correlation between the bone mineral content (using the relief of the percent cortical area at the level of the second phalanx of the left-hand index finger by Garn's method) and T, androstendione and estrone plasma levels in 30 male subjects aged between 60 and 90 years (Foresta et al. 1984). In addition to increasing age, hypogonadism is also associated with osteoporosis in men (Odell and Swerdloff 1976; Swerdloff and Wang 1993). Bone mineralization, total osteoid surface, relative osteoid volume and bone density can be improved by T therapy in androgen deficient men (Finkelstein et al. 1989). In hypogonadal men, bone mineral density can be normalized and maintained in the normal range by continuous, long term T substitution (Behre et al. 1997). The underlying mechanism by which androgens influence bone metabolism are still not understood (for review see Orwoll 1996). Several mechanisms are conceivable:

(I) Osteoporosis of male hypogonadism may be a secondary estrogen deficiency rather than a primary T deficiency (Crilly et al. 1981). Although not direct, the positive effects of estrogens on bone metabolism in women and the positive effects of a hormonal estrogen therapy are obvious. In addition to the stimulation of calcitonin secretion, estrogen treatment of postmenopausal women also increases calcium resorption by raising the serum levels of $1,25(OH)_2D$ (Gallagher et al. 1980). Estrone, the main postmenopausal estrogen, which is largely derived from androstendione by peripheral conversion, is significantly reduced in postmenopausal osteoporotic women (Marshall et al. 1977). Whether the peripheral conversion of androgens into estrogens accounts for some of the osteoporotic effects seen in males, remains to be elucidated. Taking into consideration the elevated estrogen/androgen ratio in elderly males, other mechanisms leading to osteoporosis in aging male must still be considered:

(II) T may act directly on the bone. Human bone contains the enzyme 5α-reductase, which can convert T into DHT (Schweikert et al. 1980).

To conclude, as in women, the decline of gonadic function correlates with increased bone resorption in men. Due to the later and more gradual onset of the partial sex-steroid deficiency, elderly men are less affected by this process than elderly women (Foresta et al. 1984).

4.4 Gonadotropins: The SENIEUR Protocol

Solid studies of the aging process of the endocrine system are fundamentally based on two prerequisites, i.e. reliable assay systems and well characterized study populations. The Human Immunology Group of European Community's Concerted Action Programme on Aging (EURAGE) designed admission criteria for gerontological studies, the SENIEUR protocol (Ligthart et al. 1990). This protocol primarily aims at defining "healthy" populations for immuno-gerontological studies. It should replace the current widely spread practice using "apparently healthy" or "subjects without overt disease" as reference populations for age related investigations. These rather vague definitions of reference populations do not exclude underlying disease or medication and therefore might bias results and conclusions. In contrast, the SENIEUR protocol excludes, as much as is possible, endogenous and exogenous influences on the immune system and provides a standardized study population suitable for joint international gerontological studies. It is worth noting that any medication known to affect the immune and endocrine system, such as anti-inflammatory drugs, hormones, and analgetics lead to exclusion of probands on the basis of the SENIEUR-protocol.

In addition to the "classical" pituitary-derived gonadotropins luteinizing hormone (LH) and follicle stimulationg hormone (FSH), chorion gonadotropin (CG), which was thought to be present only during pregnancy and in the course of certain malignancies has been identified in sera of healthy non-pregnant women and even in apparently healthy men (Madersbacher et al. 1992; Odell and Griffin 1987; Stenman et al. 1987). This was achieved by ultrasensitive MCA-based time-resolved immunofluorometric assays which have advantages over other detection systems due to their wide assay ranges, high sensitivities and the non-radioactivity of the europium chelate (Madersbacher et al. 1993).

The origins of hCG in non-pregnant individuals are most likely the pituitary gland and the testis (Odell et al. 1990; Dirnhofer et al. 1996; Berger et al. 1994). It is therefore not surprising, that the serum levels of CG as those of LH and FSH increase with in both sexes age (Stenman et al. 1987; Madersbacher et al. 1992); but it was only in the female population that the rise of hCG serum levels were statistically significant.

As hCG was also present in SENIEUR-compatible individuals, it is obvious that a physiological production of hCG, other than in pregnancy and malignancy, does exist. The biological function of extratrophoblastic hCG as well as the mechanism leading to an age-related increase are unknown. In parallel to the identification of hCG in the serum of elderly males, the free α-subunit common to all four GPHs has been shown recently (Madersbacher et al. 1992). Free α-subunit production can also be demonstrated under malignant conditions *in vitro* and *in vivo* and therefore has the potential of a tumor marker (Madersbacher et al. 1992). A biological function in males of the free α-subunit *in vivo* is presently unknown. In parallel to GPH, we were able to demonstrate an age dependent increase of the free α-subunit secretion in SENIEUR protocol compatible individuals of both genders (Table 1 A, B).

Recently, the impact of aging and SENIEUR status on numerous non-endocrine parameters, such as immune variables (including functional tests), serum lipids, lipoproteins and neopterin has been intensively investigated. Reibnegger et al. observed a clear impact of age on the respective analytes, however no statistically significant difference between SENIEUR and NON-SENIEUR individuals was seen (Reibnegger et al. 1988). The same holds true for the endocrine parameters hLH, hFSH, hCG and free α none of which were significantly different between SENIEUR and NON-SENIEUR, despite clear age dependencies. This key observation led to the following conclusions: The rise of serum gonadotropins and of the free α subunit in old age is caused by the aging process per se, as this rise can be demonstrated in "healthy" SENIEUR-individuals, and it is not modulated or due to

Table 1 A. Means, S.D.s and two-way ANOVA analysis of serum hFSH, hLH, hCG and free α in males. * n=20, age: 27 ± 4a; ** n=18, age: 72 ± 3a

Parameter	Males		Levels of significance	
(ng/L)	Young*	Aged**	Age	SENIEUR
hFSH	619 ± 437	1948 ± 1749	0.0002	0.0473
hLH	54 ± 137	487 ± 296	0.015	0.1325
hCG	4 ± 3	7 ± 7	0.191	0.4687
free α	142 ± 54	279 ± 142	0.005	0.7132

Table 1 B. Means, S.D.s and two-way ANOVA analysis of serum hFSH, hLH, hCG and free α in females. * n=21, age: 24 ± 2a; ** n=16, age: 72.5 ± 4a

Parameter	Females		Levels of significance	
(ng/L)	Young*	Aged**	Age	SENIEUR
hFSH	665 ± 442	15312 ± 5995	0.0001	0.6771
hLH	466 ± 613	2041 ± 1186	0.0001	0.9428
hCG	6 ± 5	71 ± 47	0.0001	0.3582
free α	186 ± 163	431 ± 206	0.0001	0.8472

According to reference Madersbacher et al. 1993.

underlying disease or medication. As SENIEUR and NON-SENIEUR individuals exhibited comparable hormone values, a randomly chosen, "apparently healthy" population may be sufficient for reliable endocrinological studies of serum GPH.

Lastly, the endocrinological changes in SENIEUR individuals underline the need for age adjusted hormone reference intervals. The presence of hCG and its free subunits in SENIEUR-individuals and the definition of age- and sex-adjusted reference values may have significant impact on the application of these molecules in the field of oncology as they have been shown to be secreted by a variety of tumors (Marcillac et al. 1992; Dirnhofer et al. 1998; Madersbacher et al. 1992; Madersbacher et al. 1994). Human CG and/or free α are well recognized as tumor markers particularly for testicular cancer, bladder cancer, molar pregnancy and choriocarcinoma. As these malignancies are present in both sexes and in young as well as aged patients such definitions are the basis for their reliable clinical application (Madersbacher et al. 1992).

5 The adrenopause

5.1 Dehydroepiandrosterone Levels

Dehydroepiandrosterone and its sulfo-conjugated derivative DHEAS, synthesized in the zona reticularis of the adrenal glands, are, quantitatively, the most abundant circulating steroids in humans, with 30–50% higher levels in men than in women. Despite their abundance in the circulation their biological function, besides the partial transformation into sex steroids (Ebeling and Kovisto 1994), is unknown. During aging, the circadian rhythm of DHEAS secretion is lost (Del Ponte et al. 1990) and the levels in old men are only one-quarter to one-third of those observed in young adults (Orentreich et al. 1984) suggesting a possible relevance of this steroid hormone to the aetiology and management of a number of age-related clinical disorders. It is consistently observed that the response of DHEA secretion to adrenocorticotrophic hormone stimulation decreases markedly with age (Vermeulen et al. 1982) as do the DHEAS levels in serum (Orentreich et al. 1992). Moreover the wide variability seen in DHEAS serum levels among individuals within normal populations still remains unexplained.

A possible role for DHEA or DHEAS in atherosclerosis, as suspected earlier, was not confirmed in a study with which the DHEAS serum levels of a cohort of men, initially free of clinically detectable coronary heart disease, stroke and cancer, were compared between 238 cases who had definite coronary heart disease during the subsequent 18 years and 476 age-matched controls who survived the follow up period and remained free of clinically detectable coronary heart disease (LaCroix et al. 1992). The findings do not support a role of DHEAS in the development of nonfatal myocardial infarction or the progression of atherosclerosis.

A potential effect of substitution with DHEA or DHEAS could be in reversing the decline in immune functions of elderly people (Daynes et al. 1993) but extended studies are required to verify the potential therapeutic benefits of DHEA. Being a potent antiglucocorticoid, DHEA may also be important for cerebral activity. The significant age-related decrements in both blood and cerebrospinal fluid of DHEA and DHEAS and the direct action of DHEAS on membrane-bound transmitter events such as gamma-aminobutyric acid (GABA(A)) receptors point to a role of DHEA and DHEAS in age-related alterations of brain function (Guazzo et al. 1996). This deserves further investigation since the concurrent influence of the neuroactive steroid DHEAS on transmitter-gated ion channels and gene expression regulating neuronal function, extends the concept of "cross-talk" be-

tween membrane and nuclear hormone effects and provides a new role for the therapeutic applications of this steroid (Rupprecht 1997).

Concerning the physiological and pathological significance of the conversion of DHEA into the potent sex steroid T, Roberts and coworkers discussed that the fall in total T with age probably reflects an age-related decrease in enzymatic capacity by forming DHEA or DHEAS, as precursors of T (Morley et al. 1997). Their work revealed that it was especially the 17,20 lyase activity of the cytochrome $P450_{c17}$ enzyme that was affected. Activity of this enzyme which catalyzes the side chain cleavage in 17α-OH pregnenolone and 17α-OH pregnenolone sulfate to form DHEA or DHEAS (Miller et al. 1997) decreases with age, restricting the conversion into T.

Another potential function of DHEAS may reside in the facilitation of T binding to albumin, thereby influencing the transport of T to its receptor. The molar concentration of albumin (≈ 6.3 x 10E-4 M) is independent of age in healthy individuals. DHEAS and T can both bind to albumin, DHEAS, due to its anionic nature, with considerably higher affinity than T. DHEAS, at concentrations of 10 E-5 M or less, forms a 1:1 complex with albumin that has a greater affinity for T than unbound albumin. This is due to the high flexibility of the albumin molecule which can exist in different configurations when bound to various ligands. A molecule of T may bind to the DHEAS-albumin complex forming a ternary 1:1:1 complex, with T and DHEAS binding to separate sites on the same albumin molecule, thereby DHEAS may serve not only as a reservoir or precursor for T but also influence the targeting of T to specific receptors for rapid actions at the cellular level (Morley et al. 1997).

6 The Somatopause

6.1 Growth Hormone

Growth hormone and IGF-I serum levels decrease with age and appear to contribute to the decline of body functions that is associated with normal aging (Rudman 1985; Corpas et al. 1993; Rosen and Conover 1997). Nevertheless more information is required before the conclusion of a direct relationship between changes in the GH/IGF axis and the functional alterations of aging can be drawn (for review see Corpas et al. 1993; Rosen and Conover 1997). While in boys GH secretion can be as high as 1.0–1.5 mg/day, healthy elderly men can produce as little as 50 μg/day (Veldhuis et al. 1995). Since GH secretion can be influenced by an array of age-related factors like lower sex steroid hormone serum-levels, a poor nutritional status, reduced physical activity, altered body composition, aberrant sleep patterns and adiposity,

GH-serum levels can be highly variable between individuals (Veldhuis et al. 1995). Interestingly, the strong positive correlation of the daily GH secretion with T in lean individuals cannot be observed in obese men. Latter show reduced GH secretion rates across all T concentrations (Iranmanesh et al. 1991). A special characteristic of GH-deficient men is the increase in the amount of intra-abdominal (visceral) fat. Similarly, with increasing age, there is an increase in visceral fat and a correlation between the periodic GH release and visceral fat in aged men (Iranmanesh et al. 1991). Serious metabolic consequences including insulin resistance and an increased cardiovascular risk may result thereof (Thorner et al. 1997).

6.2 Mechanisms Underlying the Decline of Growth Hormone Function

The mechanisms underlying the hypoactivity of the GH/IGF-I axis in elderly men are still unclear, since there is little evidence of a change in sensitivity to IGF-I feedback or a diminution of the somatotroph cell mass with aging. A potential mechanism for the age-related decline in GH secretion may either reside in the decreased release of GH-releasing hormone (GHRH) or in an increase of somatostatin. As shown in rats, it is due to both, an increased somatostatin tone and an impaired activation of the GHRH-R in the somatotrophs. The latter is likely to be caused by a primary GHRH deficiency, as implied by the diminished GHRH expression in and release from the hypothalamus of old rats (Sonntag and Meites 1988). Due to the differences in neuroendocrine regulatory mechanisms between man and rodents, it is not clear whether and to what extent the above mechanisms contribute to decreased GH secretion in elderly men. As shown by administration of a synthetic growth hormone-releasing factor (hpGRF-44) to young and old men, the somatotroph cells become less sensitive to GHRF with aging (Shibasaki et al. 1984). The finding that repetitive administration of GHRH can restore the attenuated response also suggests that somatotroph cells become less sensitive to GHRH with normal aging (Iovino et al. 1989). Nevertheless, differently from the GHRH-induced GH release, the somatotroph response to combined administration of arginine (a GH secretagogue, probably acting via inhibition of hypothalamic somatostatin release) and GHRH, does not vary with age, pointing to an increased somatostatinergic activity underlying the reduced GH secretion in normal aging (Ghigo et al. 1990). The function of GHRH-producing neurons in man can be tested by a withdrawal of SRIH infusion, promoting a rebound GH response. Measuring the baseline IGF-I levels before and after withdrawal of SRIH infusion, Uberti et al were able to show significantly lower levels of IGF-1 in elderly compared to young men. These findings are compatible with the view that an age-

related decrease in endogeneous GHRH function may at least in part contribute to the defective GH secretion in elderly men (Uberti et al. 1997).

Circulating IGF-I and -II in their majority are bound to specific IGF-binding proteins (IGFBP). In healthy aging adults the molar ratio of IGF-1/IGFBP-3 decreases, leading to lower levels of free (biologically active) IGF-I serum levels (Juul et al. 1994). The generalized malnutrition and protein depletion (common conditions in the elderly) can be associated with marked changes in IGF-I serum levels and altered circulating IGFBPs, independent to the GH status (Clemmons and Underwood 1991), making the interpretation of a single IGF-I measurement difficult. The function of the IGFBPs still requires a better understanding, especially concerning age-related changes in serum IGF-I. Interestingly conditioned medium levels of senescent human fibroblasts have significantly increased levels of IGFBP-3, and IGFBP-3 was found to be among the overexpressed genes in a senescent cell cDNA library. Besides influencing the IGF bioeffectiveness, IGFBPs may also possess intrinsic biological activities (Conover 1997).

7 Pulsatile Patterns of Hormone Secretion and Aging

The dynamic regulation is a characteristic of the endocrine system. In addition to varying hormone levels, hormonal information can also be encoded by distinct pulses with changing frequencies. Thus, with the development of sensitive methods for hormone measurements and high frequency blood sampling, the relevance of the pulsatile patterns of secretion for regulation of endocrine axes and its implications on diagnosis as well as on endocrine diseases was investigated in a series of studies (for review see Brabant et al. 1992).

Pulsatile patterns of hormone secretion are essential for target-cell regulation. Changes of mean serum concentrations of LH, FSH, TSH, prolactin, GH, ß-endorphin and adrenocorticotrophic hormone (ACTH) are generated by a modulation of the frequency and/or amplitude of hormone pulses (Veldhuis et al. 1990). Pulsatile, but not continuously applied, gonadotropin-releasing hormone (GnRH) is able to stimulate the secretion of bioactive LH and FSH capable of fully inducing gonadal steroid secretion, follicular growth, or ovulation in women with hypothalamic amenorrhea (Southworth et al. 1991). LH and FSH synthesis and secretion are differentially regulated by the frequency of GnRH pulses. A low frequency of GnRH stimulation preferentially increases FSH, whereas LH is maximally stimulated by higher GnRH pulse frequencies (Knobil 1980; Shupnik and Fallest 1994). Elderly men have similar basal LH pulse frequencies compared to younger men, but the frequency of high LH pulses amplitude (greater than 2IU/L), mean and

maximal LH pulse amplitude, and pulse area are lower (Vermeulen et al. 1989).

The reproducibility of mean 24-h hormone levels and the responsiveness of LH or GH to exercise or fasting by an alteration of both pulse amplitude and frequency (Weltman et al. 1990; Ho et al. 1988; Cameron et al. 1991) indicate a physiological and functional significance of the pulsatile hormone patterns. Increased secretory irregularities with advancing age, as a widespread hormonal phenomenon, can also be demonstrated in older males since they secrete LH and T more irregularly, and jointly more asynchronously, than younger males. In this context the need for quantifications such as the model-independent statistic approximate entropy (ApEn) and cross-ApEn have to be emphasized. Mean hormone concentrations between two age groups may show no differences, giving no information concerning the irregularity of hormonal secretion in one of the two cohorts (Pincus et al. 1996).

8 Hormonal Replacement in Elderly Men

8.1 Testosterone

Despite the moderate fall in T levels, aging men show features compatible with androgen deficiency. The clinical significance of the age-related decline of T levels in men should be questioned. Should elderly men suffering from androgen deficiency related clinical signs like asthenia, impotence, a decrease in muscle and bone mass or a decrease in libido be substituted with androgens (for review see Gooren 1996)? In absolute terms, T levels in aging men are nearly always within the normal reference values, although they may have fallen significantly in an individual's lifetime (Gray et al. 1991). Despite the recognition of decreasing T levels in aging men, indications for T replacement therapy in elderly men remain to be defined. This is especially difficult since the symptoms of T deficiency may be subtle or unrecognised. A number of clinical problems prevalent in older men may be related to androgen deficiency, including muscle weakness and wasting, changes in body composition, osteopenia, increased prevalence of hip and vertebral fractures, sexual dysfunction, decreased hematopoiesis, and memory loss. Although all of these disorders are multifactorial, it has been speculated that age-related T deficiency or insensitivity plays a role in their pathophysiology, and that T replacement may help prevent or reverse these disorders.

The most common indication for androgen therapy in men is hypogonadism which has numerous similarities with the aging process. These in-

clude: decreased musculoskeletal mass, increased adipose tissue and reduced hematocrit and all can be in part adversed by T therapy in hypogonadal men (Greenspan et al. 1986; Jackson et al. 1987; Bhasin et al. 1997). Several T delivery systems have been approved, and many more are under development (Bagatell and Bremner 1996; Bhasin and Bremner 1997). Preliminary T replacement studies (Table 2 A) in old hypogonadal men (bioavailable testosterone less than 70 ng/dl) aged 69 to 89 years have shown beneficial effects on libido, density lipoprotein levels and a significant increase in bone density and lean body mass (Katznelson et al. 1996; Finkelstein et al. 1989). Adverse effects of T treatment included an increased hematocrit and higher prostate specific antigen (PSA) levels (Morley et al. 1993). In a study conducted by Tenover (1992) 13 healthy men, aged 57 to 76 with low or borderline serum testosterone levels received intramuscular T enanthate (100 mg per week) for 3 months. Following treatment, lean body mass was significantly increased and urinary hydroxyproline excretion reduced. In addition, an increase in hematocrit was also documented as a negative side effect of the therapy. Furthermore, a decrease in cholesterol and a sustained elevation in serum PSA levels appeared in the T treated group (Tenover 1992). Although there appear to be some positive effects such as improved fitness and general well being after androgen treatment in aging males (Tenover 1994), the question about the long term side effects still persists. The sparse data regarding the effects of hypogonadism on age-associated physical and cognitive declines has led to a study in which the year-long effects of T administration were observed. Fifteen hypogonadal men (T levels of less than 60 ng/dL, mean age 68 ± 6 yr) were randomly assigned to receive placebo, and 17 hypogonadal men (mean age 65 ± 7 yr) to receive T (200 mg testosterone cypionate, biweekly for 12 months). The main outcome of the study was an improved strength, increased haemoglobin, and lower leptin levels in those probands treated with T. There were no significant changes between the two cohorts in memory or levels of PSA. Three probands receiving testosterone had to withdraw from the study due to an abnormal elevation in hematocrit (Sih et al. 1997).

Although supraphysiologic doses of T, especially when combined with strength training, increase fat-free mass, muscle size and strength in normal men (Bhasin et al. 1996), the long term substitutions of supraphysiologic doses of T in frail elderly men are not recommended. Testosterone may have a role in treatment of frailty in males with hypogonadism; however, older men receiving T must be carefully monitored because of its potential risks. Future experiments will have to address this question, especially concerning a possible stimulation of malign prostatic growth after androgen treatment.

Table 2 A. Effects of androgen administration in elderly men

Author	Subjects	Androgen regimen	Effects	Side-Effects
Morales et al. 1994	13 men, (40–70 yrs)	DHEA, 50 mg, nightly, oral, for 6 months	increase in IGF-I, decrease in IGFBP-I, increased well-being	None reported
Morley et al. 1993	14 hypogonadal men (75–80 yrs)	Testosterone enanthate (n =8) (200 mg/ml), im injections every 2 weeks for 3 months controls (n = 6)	increased hematocrit, right hand muscle strength, osteocalcin; decrease in cholesterol	None reported
Sih et al. 1997	32 hypogonadal men (62–74 yrs)	Testosterone cypionate 200 mg, im injections biweekly for 12 months (n = 17); placebo (n = 15)	increased bilateral grip strength and hemoglobin; no significant changes in PSA or memory	abnormal elevation in hematocrit
Tenover 1992	13 men (57-76 yrs) (T ≤ 13.9 nmol/L)	Testosterone enanthate im injections 100 mg weekly	significant increase of lean body mass, decrease of hydroxyproline excretion, decline in total cholesterol and low density lipoprotein	significant increase in hematocrit, increase in serum PSA levels

The undesired effects of androgens depend on both the type and dose administered (for review see Bagatell and Bremner 1996). Since only low-dose T supplements are used for elderly men, side-effects like those reported in athletes using high doses of alkylated androgens are not likely to be a problem of androgen-therapy. Due to the physiological androgenic and estrogenic actions of androgens accounting for the main side effects of androgen replacement therapy, the cardiovascular system and the prostate deserve higher monitoring in androgen therapy. Despite the elementary importance of androgens for prostatic growth and the fact that BPH and prostate cancer do not develop without the exposure to T during puberty there is presently no evidence that replacement of androgens in hyopogonadal men will lead to BPH or even prostate cancer (Behre et al. 1994). Until now only moderate increases in prostate size and PSA levels have been documented in elderly men who were supplemented with androgens (Morley et al. 1993; Tenover 1992; Holmäng et al. 1993). Although androgens stimulate the growth of clinical prostate cancers, there are no data available showing that androgen administration enhances the progression from preclinical to clinical cancer. Nevertheless, at the second International Androgen Workshop in Long Beach, California in February 1995, it was recommended that androgen administration is not started if PSA levels are above normal, and should be discontinued if there is an increase of 2.0 ng/ml at any time or an increase of 0.75 ng/ml per year over a 2-year period (Gooren 1996).

Until now a hormonal supplementation is only reasonable given the clinical signs of a latent or manifested T deficiency i.e. a bioavailable T level of less than 70 ng/dl (2.43 nmol/L) on at least two occasions (Villareal and Morley 1994). The so far beneficial effects reported in studies on androgen-supplement therapy in aging men (see Table 2 A) will have to be verified by larger and long-term studies ruling out placebo effects, before a definitive conclusion concerning the prescription of androgens to elderly men can be drawn.

8.2 Dehydroepiandrosterone

In a randomized placebo-controlled cross-over trial of nightly oral DHEA administration (50 mg) of 6-month duration (13 men) the DHEA and DHEAS serum levels were restored to those found in young adults within 2 weeks of DHEA replacement. The levels were sustained throughout the 3 months of the study. Restoring the levels to those of young men resulted in an improvement of physical and psychological well-being, an increase in the bioavailability of IGF-1 and a small rise in androstendione serum levels. The

Table 2 B. Effects of GH administration in elderly men

Author	Subjects	GH regimen	Effects	Side-Effects
Rudman et al. 1990	21 healthy men (61–81yrs) with low IGF-I	rhGH, 0.3 mg/kg (n = 12) or placebo (n = 9) sc injections 3 x weekly for 6 months	Increased LBM and spinal bone density. Decreased fat mass	Increased blood pressure and glucose (normal range) carpal tunnel syndrome*)
Marcus et al. 1990	16 men and women (> 60 yrs)	rhGH, 0.03, 0.06 or 0.12 mg/kg im injections daily for 7 days	rise in circulating somato medin C and PTH, decrease in urinary nitrogen, calcium and sodium excretion,	impairment of oral glucose tolerance and reduced insulin sensitivity
Kaiser et al. 1991	10 malnourished men (64–99 yrs)	rhGH, 0.01 mg/kg (n = 5) or placebo (n = 5) im injections daily for 21 days	Increased nitrogen retention weight and mid-arm circum ference	None reported
Suchner et al. 1990	6 severely malnourished men on parenteral nutrition (54–73 yrs)	rhGH, 30 µg/kg/day for 4 days, then 60 µg/kg/ day for 4 days	Improved nitrogen balance, increase in energy expenditure and fat oxidation	None reported
Taaffe et al. 1994	18 healthy men (65–82 yrs)	rhGH, 0.02 mg/kg/day or placebo for 14 weeks (with excercise)	Increase in body lean mass, decrease in body fat mass, no effects on muscle strength	None reported
Papadakis et al. 1996	52 healthy men (older than 69 yrs)	rhGH, 0.03 mg/kg or placebo, 3 x weekly for 6 months	Increase in body lean mass, decrease in body fat mass, no effects on knee or hand grip strength	26 men had 48 incidents of side effects.

beneficial effects of DHEA replacement and the absence of side effects support the need for long term studies of DHEA supplementation in older men. All studies that have been conducted in humans thus far (for review see Bellino et al. 1995), showed beneficial effects, and essentially no toxicity of DHEA treatment at doses that restore serum levels. This is very promising, but needs to be confirmed and warrants further well controlled long termed studies. Moreover, it needs to be clarified whether there is a cause-and-effect relationship between the decreasing levels of DHEAS with age and the physiological and pathological manifestations of aging. The easy restoration of DHEA serum levels by DHEA administration (Morales et al. 1994), its long biological half life (approx. 8–10 hrs), the limited physiological diurnal variations and the lack of noticeable changes of metabolism in aging people make DHEA an ideal candidate for an exogeneously admininstered hormone, for restoring the young *in vivo* situation.

8.3 Growth Hormone

The availability of recombinant hGH (rhGH), the new hexapeptide agonists of the growth hormone releasing peptide (GHRP) family and the nonpeptide analogues of such agonists, has led to a renewal of scientific and clinical interest in investigating wider indications for GH administration. The pharmacological and clinical aspects of hGH replacement therapy have been extensively studied (reviewed in: Jorgensen 1991). Reversing or attenuating the effects of GH deficiency by rhGH therapy (Salomon et al. 1989) has led to the question of whether some effects of normal aging may be overcome by treating aged people with rhGH. Because GH deficiency (Jorgensen 1991) and normal aging (Rudman 1990) are both associated with decreases in protein synthesis, percent of lean body and bone mass, and with increases in percent of body fat, it is possible that reduced GH secretion and IGF-I levels may account, at least in part, for one or more of the above effects of aging. Thus, some elderly people might benefit from treatment with rhGH in a way GH deficient adults do when treated with GH (Carrol et al. 1989; Jorgensen et al. 1989). Several studies (Table 2 B) have shown the beneficial effect of short-term treatment to older post-surgical patients (Ponting et al. 1988; Ziegler et al. 1992) or older malnourished people (Binnerts et al. 1988; Suchner et al. 1990; Kaiser et al. 1991). Treatment of 18 healthy men and women aged 60 years and older for 1 week with three different doses of rhGH, revealed a dose-dependent increase in plasma IGF-I accompanied by reductions in daily urinary excretion of nitrogen, phosphate, and sodium (Marcus et al. 1990). In a study conducted by Rudman 12 healthy men, aged 61–81 yr, who had basal plasma IGF-1 levels less than 350 U/liter, were treated with sc

injections of rhGH three times weekly for 6 months, with the dose adjusted monthly based on IGF-I responses. Administration of rhGH produced an 8.8% increase in lean body mass, a 14.4% decrease in adipose tissue mass, and a significant increase in skin thickness (Rudman et al. 1990). Whether the stimulation of bone turnover as a result of GH treatment, as shown in hypogonadal men (Bravenboer et al. 1997), can also be achieved in elderly men, remains to be shown. As shown by Taaffe et al. (1994) supplementation of healthy elderly men (65–82 yrs) with rhGH does not augment the response to strength training.

Since the pituitary GH releasable pool is still present in the aged, the GH axis may be stimulated by administration of either GHRH, GH-releasing peptide (GHRP) or GHRP mimetics like MK-677 (for review see Thorner et al. 1997). As shown by Corpas et al. (1992) the decreased GH and IGF-I levels in old men can be reversed by the administration of GHRH 1–29. Single nightly doses of GHRH are less effective than multiple daily doses of GHRH in eliciting GH- and/or IGF-I mediated effects (Vittone et al. 1997).

The hexapeptide agonists of the GHRP family and the nonpeptide analogs work by facilitating GHRH action, leading to an enhanced pulsatile GH release, restoring IGF-I concentrations in GH deficient adults (Chapman et al. 1997) as well as in elderly men (Chapman et al. 1996). In contrast to the exogenous administration of GH, the feedback regulation at the pituitary level and the physiological pulsatile pattern of GH release are still preserved by these new agents.

Although a positive regulation of body composition in older adults by the restored GH serum levels favor a therapeutic role of secretagogues for aged men, long term clinical trials are still necessary before such conclusions concerning efficacy and benefits may be drawn. It is still questionable whether GH serum levels in old men resembling GH serum levels in young man can be considered physiological. Finally, the results of a recent study showing that higher plasma levels of IFG-I are associated with higher rates of malignancy in the prostate gland, raise the concern that administration of GH or IGF-I over long periods may increase the risk of prostate cancer (Chan et al. 1998).

8.4 Melatonin

Melatonin (N-Acetyl-5-methoxytryptamine) is mainly biosynthesized from tryptophan by the pineal gland. Its secretion and release depend upon a number of exo- and endogenous factors e.g. light, drugs, sex and age. There is little or no melatonin secreted before the age of 3 months; Then melatonin production commences, becomes circadian, and reaches highest nocturnal

levels at the age of 1–3 years. During childhood nocturnal peak levels drop progressively by 80% until adult levels are reached. This alteration appears to be the consequence of increasing body size in face of constant melatonin production during childhood (Waldhauser et al. 1988). The biological significance of this decrease in melatonin secretion is presently unknown. Besides its well documented effects on the regulation of circadian rhythms (e.g. body temperature) and its sleep inducing properties, melatonin has also been shown to be a powerful antioxidant (reviewed in: Reppert and Weaver 1995). Due to its lipophilic and hydrophilic nature it potentially affects not only every cell but also every subcellular compartment. Intracellular actions of melatonin, some of which are receptor-independent, have become the focus of current investigations. Melatonin is capable of scavenging free radicals produced during catecholamine autooxidation (Miller et al. 1996), which have been implicated in the loss of dopaminergic neurones in the nigrostriatal region of the brain. This occurs during normal aging and in Parkinson's disease, thus the antioxidant effect of melatonin might have implications for the brain's antioxidant defences and may protect against subsequent dopaminergic neurodegeneration. In view of the pharmacological doses of melatonin required in order to see such scavenging effects, it is unlikely that it has such effects in vivo (Reiter et al. 1995).

In addition, melatonin has been shown to possess immunoenhancing properties, which may be relevant when counteracting immunodepression following acute stress, drug treatment, viral diseases or aging. Physiological as well as pharmacological concentrations of melatonin induce interleukin 4 production in T-helper cell type 2 lymphocytes, which in turn activates bone marrow stromal cells to release hematopoietic growth factors (Maestroni 1995).

Although the potential effects of melatonin in neuroimmunoendocrine interactions is presently being explored, there are too few clinical studies to establish a meaningful estimation of melatonin's involvement in human neuroimmunoendocrine interactions (Reppert and Weaver 1995).

The claim that melatonin can reverse aging is based on a study performed in mice where the administration of melatonin in drinking water to aging mice prolonged their survival from 23.8 to 28.1 months. These data seem to indicate a place for melatonin in the physiological regulation of aging (Pierpaoli and Regelson 1994). The major drawback of this study is that these mice have a well-described genetic defect in pineal melatonin biosynthesis and do not produce melatonin, so the assertion that melatonin can increase longevity in mice in general can not be made.

Presently, the only therapeutic application of melatonin in humans (jet lags and some circadian-based sleep disorders) takes advantage of its ca-

pability of modulating the circadian rhythms (Reppert and Weaver 1995). An interesting issue which merits further evaluation is its ability to induce sleep in humans (Dollins et al. 1994), especially in the light of the GH-secretion patterns which are known to be most reproducible shortly after the onset of sleep (Van Cauter and Plat 1996). Sleep loss in young subjects results in endocrine disturbances which mimic those observed in the elderly (Van Cauter et al. 1998). It is therefore conceivable that the decrease in sleep quality which characterizes aging and seems to contribute to age-related alterations in hormonal function might be counteracted via the administration of melatonin.

9 Conclusion

A better understanding of the aging process will improve our geriatric care. In regard to this, the potential role of hormonal changes in the development of senescence is of highest priority, since the signs and symptoms of endocrine gland failure are often similar to some of the classical age-related changes that result in a deterioration of quality of life and/or the development of frailty. The structural and functional changes during aging may at least in part be a consequence of the hormonal alterations seen in aged individuals. The improved methodology to measure hormones and the understanding that it is unlikely that a single hormone deficiency will explain the aging process *per se* have led to new interest in the putative role of declining hormonal levels in relation to frailty syndromes seen in association with male aging.

Aging in men produces a variety of changes at the hypothalamic, pituitary, and testicular level, thereby possibly contributing to aging "syndromes" such as frailty with its concomitant impact on morbidity and mortality.

The potential usefulness of hormonal replacement in elderly people deserves further investigation due to the beneficial effects in individuals having subnormal hormone levels, thereby suffering from symptoms similar to those of aged people.

Long term studies using novel methods of T administration such as enhanced transdermal systems, transscrotal patches, or subcutaneous implants will show whether the benefits outweigh the long term risks and T can truly prevent or reverse frailty. Besides producing a more physiologic replacement of T than depot injections these new application systems also offer an easier, more acceptable mode of therapy in older individuals (for review see Kaiser and Morley 1994).

Despite its decrease (Labrie et al. 1997a) the clinical significance of DHEAS for the aging process is uncertain. The local or intracrine biosyn-

thesis and action of androgens synthesized by steroidogenic enzymes from the precursors DHEA and DHEAS minimizes the risks of undesirable masculinizing or other androgen-related side effects of DHEA (Labrie et al. 1997b). In addition, the activity of the steroid-converting enzymes present in peripheral tissues do not seem to decrease during aging (Belanger et al. 1994). Interestingly, in rhesus monkeys, restriction of caloric intake, which extends life span and retards aging in laboratory rodents, slows down the postmaturational decline of serum DHEAS levels (Lane et al. 1997).

Due to the complexity of the endocrine system it is unlikely that hormonal replacement of one or a few hormones will reverse or stop aging *per se*. Nevertheless a hormonal therapy may alleviate some of the symptoms of aging, helping us not to extend life but to improve the health such that it increasingly fits the life span to which we all now aspire.

The potentially unlimited supply of biosynthetic hGH renewed the interest in the actions of GH, going far beyond promotion of longitudinal growth in children or GH replacement therapy in GH-deficient adults (Cuneo et al. 1998). Normal aging is associated with declining GH serum-concentrations, a reduction in lean body mass and an increase in fat mass, in this way resembling adult GH deficiency. Although the administration of GH in the elderly has shown increments in lean body mass and reductions in fat mass, potential side effects like mitogenic actions of GH on potentially malignant cells, the risk of heart failure via fluid retention and increased resting heart rate, or the insulin antagonist actions of GH in a population with a high prevalence of non-insulin-dependent diabetes, to name just a few, should, especially in view of long-term administrations, not be underestimated. Another important point would be to define the gender differences observed in response to GHRH analog administration. Nightly administation of a GHRH analogue for 4 months in age-advanced men and women induced anabolic effects favoring men more than women (Khorram et al. 1997a). Interestingly no sex differences in the immune response were detected with the same GHRH analog ([norleucine27]GHRH (1–29)-NH2). Administration of that substance to aging men and women had profound immune-enhancing effects, pointing to a potential therapeutic benefit in states of comprised immune function in elderly people (Khorram et al. 1997b).

As already stated in the Vienna International Plan of Action on Aging (1982), "research and practical experience have demonstrated that health maintenance in the elderly is possible and that diseases do not need to be essential components of aging". Recognizing some of the missing links and increasing our understanding of the endocrine changes affecting elderly men will hopefully enable us to ameliorate age-related symptoms and improve quality of life.

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Effect of Age on Thymic Development, T Cell Immunity, and Helper T Cell Function

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Contents

1 Introduction

Adaptive immunity, measured by the level of protective antibodies or cytotoxic T cells, wanes with age (Schwab et al. 1989). As a consequence, the elderly suffer greater morbidity and mortality from infections, autoimmune diseases such as pernicious anemia, and cancer. Aging of the immune system, immune senescence, leads to the most common but not the most severe, immunodeficiency state. Unlike severe acquired immune deficiency states, aging is not associated with a decrease in the concentration of serum immunoglobulins or in the number of lymphocytes. Immune senescence also leads to a state of immune dysregulation characterized by polyclonal B cell activation, autoantibody formation, and clonal lymphocyte expansions (LeMaoult et al. 1997).

The most striking anatomical change in the immune system with age is the involution of the thymus gland. Thymic involution begins early in life and by mid-life the cellular mass of the thymus has fallen by 75 to 85% (Boyd 1932). The principal consequence of thymic involution is the decreased capacity of the thymus to export a diverse repertoire of naive T cells (Scollay et al. 1980). The elderly, despite a decreased output of naive T cells from the involuted thymus, maintain a normal peripheral T lymphocyte count as self-renewal, combined with the small number of T lymphocytes produced by the thymus, is capable of making up for the normal loss of peripheral T lymphocytes (Rocha et al. 1989). However, when peripheral T cells are depleted, for example following intensive chemotherapy, the output of the involuted thymus gland does not permit the rapid regeneration of a normal T lymphocyte count observed in young individuals (Mackall and Gress 1997). It would be expected that the elderly could not maintain or regenerate their peripheral T lymphocyte population as well as young individuals when the rate of peripheral T cell destruction increased following, for example, radiation therapy or HIV infection.

2 Thymic Development and Aging

The thymus gland first appears as a collection of epithelial cells which is colonized by thymocyte precursors and macrophages from the bone marrow midway through the fetal development (Miller 1992). The thymic epithelial cells form the microenvironment in which bone marrow-derived thymocyte precursors develop. Thymocyte development is rigorously censored so that less than 5% of thymocytes leave the thymus as mature T lymphocytes to populate the secondary lymphoid compartments (Scollay et al. 1980).

Anatomists had identified the lobulated thymus gland located in the upper thoracic cavity and recognized its age-associated involution before its immunological function was established (Miller 1992). Each lobule of the thymus gland is divided into four zones (Stevens and Lowe 1997). The outermost zone, the subcapsular region, is the point of entry of bone marrow-derived thymocyte precursors. The outerzone of the cortex is just below the subcapsular region and contains actively dividing thymocytes. The inner zone of the cortex contains small, largely nondividing cells which then pass into the medulla. The medulla contains the most mature thymocytes prior to their immigration to the secondary lymphoid compartments.

The stages of thymocyte maturation are defined by their location within the thymus gland as well as by their expression of cell surface molecules and the state of arrangement of the T cell antigen receptor (TCR) genes (Aspinall 1997; von Boehmer and Fehling 1997). The earliest T cell precursors in mice are CD3-, CD4low, CD8-. The next stage of thymocyte maturation is identified by the loss of surface CD4 expression. The CD3-, CD4-, CD8- thymocytes have the genes coding for the β chain of the TCR in their germline configuration and express CD44 but not CD25 on their surface. Thymocytes which have rearranged their β chain TCR genes express cell surface CD25 becoming CD3-, CD4-, CD8-, CD25+, CD44+. The product of the rearranged TCR β chain genes is expressed on the cell surface in association with the TCR α chain equivalent (von Boehmer and Fehling 1997).

The next stage of thymocyte development is identified by the expression of both CD4 and CD8 on the cell surface (von Boehmer and Fehling 1997). These "double positive" thymocytes make up the largest population of cells within the thymus. During this stage of development, thymocytes rearrange their α chain genes and express the mature TCR consisting of both the α and β chains of the TCR. Double positive thymocytes are activated when their mature TCR interact with MHC-peptide complexes expressed on the surface of thymic epithelial and hematopoietic-derived cells.

Double positive thymocytes whose TCR bind with high affinity to self-MHC class I or class II-peptide complexes enter a pathway of activation-induced cell death, termed apoptosis. This process in the thymus is called negative selection (Miller 1992). Negative selection contributes importantly to the establishment of self-tolerance by preventing the release of autoreactive T cells from the thymus. In contrast, thymocytes that interact with either self-MHC class I or self- MHC class II molecules with moderate affinity are activated to proliferate, a process called positive selection (Miller 1992). Those thymocytes that are not activated die of "neglect". Double positive thymocytes which interact with self-MHC class I-peptide complexes lose their cell surface CD4 while double positive thymocytes which interact with

self-MHC class II-peptide complexes lose their CD8 molecules. The "single positive" thymocytes move from the thymic cortex into the medulla prior to their immigration to the secondary lymphoid compartments.

The age-associated involution of the thymus gland results predominantly from the decreasing mass of the thymic cortex (Boyd 1932). This suggests that age-associated defects in the early stages of thymocyte development are largely responsible for thymic involution. There is also some evidence that under competitive conditions the capacity of bone marrow thymocyte precursors from old mice to reconstitute a young thymus gland is less than that of precursors from young mice (Sharp et al. 1996). It is not known whether the impaired capacity of bone marrow-derived thymocyte precursors to colonize the thymus reflects a lower number of circulating thymocyte precursors released from the bone marrow of old mice or whether the thymocyte precursors in old mice are impaired in their capacity to enter the thymus gland.

Aging is associated with a declining number of all thymocytes after the CD3-, CD4-, CD8-, CD25+, CD44- stage of thymocyte development (Aspinall 1997). A similar age-associated change in thymocyte development has been seen following irradiation of old mice (Thoman 1997). The crucial transition from CD3-,CD4-,CD8-,CD25+,CD44- to CD3-,CD4-,CD8-,CD25+, CD44+ thymocytes depends upon the rearrangement of the TCR β chain genes. Thus, it is possible that an impaired capacity of thymocytes in old mice to rearrange their TCR β genes underlies the decline in thymocyte number and thymic involution. This hypothesis predicts that old mice with rearranged TCR β and α chain transgenes, which do not need rearrangement prior to their expression, would not suffer thymic involution. Results consistent with this prediction have recently been published (Aspinall 1997). Thus, TCR β and α chain transgenic mice were reported to maintain thymocyte number during aging and not to undergo thymic involution. How completely thymus mass and thymocyte number are maintained appears to vary in different mice and with different transgenic TCR genes (P-J Linton et al. 1997).

The precise mechanism underlying the age-associated impairment in the rearrangement of TCR β chain genes in old mice has not been defined. However, a role for IL-7 in thymic involution must be considered. First of all, the addition of IL-7 to cultures of thymocytes increases their viability and proliferation associated with an increase in RAG gene expression by the cultured thymocytes (Muegge et al. 1993). Furthermore, the failure of thymus gland development in athymic, nude, mice also appears to be related to defects in the production of IL-7. Thus, nude mice that express an IL-7 transgene under the control of an immunoglobulin promoter were reported

to develop thymocytes and functional peripheral T cells (Rich and Leder 1995).

While decreased rearrangement of TCR genes appears to be a dominant factor underlying age-associated thymic involution other intrathymic age-associated factors may also influence the development and function of T cells in old mice. Thus, decreased expression of MHC antigens on bone marrow-derived stromal cells and macrophages may impair positive or negative selection of thymocytes (Farr and Sidman 1984). The decreased expression of MHC molecules would be expected not only to decrease thymocyte proliferation but also to decrease the stringency of self-MHC restricted T cell responses. Elderly humans and old mice have been shown to have less stringent self-MHC-restricted T cell responses which may reflect impaired positive selection of thymocytes that could result from the decreased expression of intrathymic MHC molecules (Schwab et al. 1992; Russo et al. 1993).

The decreased output of T cells from the involuted thymus does not lead to a decrease in the number of peripheral T cells during aging. Although self-renewal of peripheral T lymphocytes is thought to maintain the T lymphocyte count despite thymic involution, it is also possible that extra-thymic pathways of T cell production. There is little to suggest that T cell formation in the gut maintains the number or function of α/β TCR-expressing peripheral T cells during aging. In contrast, the capacity of oncostatin M (OM) to stimulate the generation of functional T cells within lymph nodes of thymectomized or nude mice suggests a potential mechanism contributing to T cell development after thymic involution (Clegg et al. 1996).

OM is a member of the IL-6 subfamily of cytokines that is expressed in hematopoetic tissues. Mice that express the OM gene, under the control of a p56lck promoter, develop large numbers of CD4-CD8- and CD4+CD8+ T cell precursors in their lymph nodes. Furthermore, the T cells that are stimulated to develop by the OM transgene are immunocompetent as assessed by their capacity to control the growth of an allogenic melanoma line. The thymic independence of this developmental pathway was demonstrated by showing that these early T cell precursors developed in thymectomized mice which were given transgenic but not control bone marrow cells. OM protein can also stimulate the development of T cells in nude mice. Thus, sublethally irradiated nude mice treated with OM showed an increase in CD4+CD8+ T cells in their mesenteric lymph nodes compared to unirradiated controls. These results suggest that OM stimulates an extrathymic pathway of peripheral T cell development although the origin of the T cell precursors that accumulate within the lymph nodes remains to be defined. The fact that the immature T cells stimulated by OM express the CD8 α/β molecule and not the CD8 α/α molecule, typical of gut-derived T cells, sug-

gests a pathway that is independent of both the thymus and gut. Additional studies are necessary to show whether OM would be useful in reversing the age-associated defect in the generation of a diverse population of naive T cells by the thymus gland.

Once it was realized that the thymus gland was the primary lymphoid organ responsible for the development of peripheral T cells and that this gland involuted with age, investigators began to study the effect of age on T cell function in vivo and in vitro (Price and Makinodan 1972; Roberts-Thomson et al. 1974; Weksler et al. 1974). It was shown that T-dependent antibody responses, T cell mediated delayed-type hypersensitivity reactions and the proliferation of T cells in culture were impaired in old compared to young subjects. Another prediction, following from the decreased export of a diverse repertoire of naive T cells from the thymus, was an age-associated decrease in the diversity of the T cell repertoire. The age-associated appearance of clonal T cell populations observed in both old mice and elderly humans is consistent with this prediction (Callahan et al. 1993; Posnett et al. 1994; Schwab et al. 1997). As the number of lymphocytes does not increase with age, clonal expansions must occur at the expense of other clones. Some clones may be completely lost creating "holes" in the lymphocyte repertoire.

Although the involution of the thymus gland is the most striking anatomical change in the immune system during aging, it is not "unique within the immune system." as previously thought (George and Ritter 1996). Thus, we and others have shown that the generation of mature B cells from bone marrow B cell precursors is also decreased with age (Zharhary 1988; Stephan et al. 1996; LeMaoult et al. 1997). The defect in B lymphocyte development reflects the impaired transition of Pro-B cells into Pre-B cells (Szabo et al. in press). Pre-B cells appear to be an important check point for B cell development. To pass through this stage of B cell development, the immunoglobulin (Ig) heavy chain genes must be rearranged and their product expressed on the cell surface in association with the surrogate light chain (Li et al. 1993). Ig heavy chain genes are rearranging in Pro-B cells and we have preliminary evidence that evidence that RAG activity-mediated DNA breaks are lower in Pro-B cells from old compared to young mice. These observations may be the functional consequences of the age-associated decrease in RAG gene expression observed in bone marrow B cell precursors (Ben-Yehuda et al. 1994; LeMaoult et al. 1997). Further support for the age-associated defect in passing the Pre-B cell checkpoint in B cell development comes from the increased rate of apoptosis in Pre-B cells from old compared to young mice Kirman et al. 1998). In summary, there is evidence that age-associated "functional involution" occurs in both the thymus and bone marrow, the primary sites of B and T cells generation, respectively. At both sites the

mechanism underlying the "functional involution" appears to be impaired RAG-mediated rearrangement of antigen receptor genes.

3 Effect of Age on the Structure and Function of T Cells

As thymic involution is the most striking change in the immune system with age, it is not surprising that most investigators of immune senescence have studied age-associated changes in the distribution and function of thymic-derived, T lymphocytes. Because T lymphocytes are found in lymphoid compartments throughout the body, the effect of age on the structure of the lymphoid tissue and lymphocyte function has usually involved comparisons of the same compartment in young and old individuals. Human studies have usually involved blood lymphocytes obtained from young and old donors. In mice, the spleen, lymph node or bone marrow cells from young and old animals have been compared.

In addition to thymic involution, there are other important age-associated changes in the structure of the lymphoid tissues. There is a marked increase in the number of mature lymphocytes and plasma cells in the bone marrow and a marked reduction in the number of germinal centers in the lymph nodes and spleen with age (Benner et al. 1981; Gonzalez-Fernandez et al. 1994). Although it is clear that bone marrow becomes a dominant site of antibody production in old mice, the mechanism and significance of this age-associated change remains unclear. In contrast, the age-associated decline in T lymphocyte function explains the decrease in the number of germinal centers. Furthermore, as somatic mutation and isotype switching, the mechanisms underlying the generation of high affinity and IgG antibody, take place within germinal centers, the decline in the number of germinal centers with age explains the age-associated impairment in the most protective high affinity and IgG antibodies (Goidl et al. 1976; Zheng et al. 1997).

Despite the age-associated involution of the thymus gland and the consequent decreased generation of naive T cells from the thymus, most studies report no change in the number of T cells although some studies report a decrease in T cells in elderly compared to young individuals (Sansoni et al. 1997). The consensus is that the number of peripheral T cells change modestly, if at all, with age. Similarly, the expression of the α/β TCR or CD3 complex by resting T cells does not change with age, although it has been reported that the expression of both TCR α/β and CD3 are reduced on mitogen-activated T cell from old compared to young T mice (Wakikawa et al. 1997). Clonal T lymphocyte expansions become more frequent with age and

lead to a skewing of the T cell repertoire with respect to BV usage (Schwab et al. 1997).

There is no consistent shift in the ratio of CD4+ to CD8+ T cells with age although reports exist that the ratio of CD4+ to CD8+ T cells both increases or decreases with age (Miller 1996). It is possible that the observed differences in the ratio of CD4+ to CD8+ T cells reflect a difference in the frequency and size of the age-associated CD8+ and CD4+ T cell clonal expansion (Callahan et al. 1993, Schwab et al. 1997).

There is general agreement that there are significant age-associated changes in the distribution of the T lymphocytes expressing CD45R, CD28, and CD40L cell surface molecules (DePaoli et al. 1988; Miller 1996; Wakikawa et al. 1997). Aging is associated with a 20 to 50% increase in the ratio of CD45RO+, memory T cells to CD45RA+, naive T cells in both humans and mice. This shift from naïve to memory T cells with age is observed in both the CD4+ and CD8 peripheral T cell populations. The age-associated increase in the ratio of memory to naive T cells reflects decreasing generation of naive T cells by the thymus gland, greater immunological experience, and possibly, an increased apoptosis among naive T cells and a decreased apoptosis among memory T cells (Mountz et al. 1997; Schwab et al. 1997).

Memory T cells are more impaired with respect to age-associated defects such as decreased IL-2 secretion and T cell proliferation than naive T lymphocytes (Miller 1996). However, increased immunological experience and the shift from a naive to memory phenotype may not be a requirement for the expression of these defects (Linton et al. 1997). Thus, T cells from TCR transgenic mice maintain their naive phenotype during aging although they become impaired in their capacity to proliferate and secrete IL-2. These results suggest that interaction with the nominal antigen and the shift from the naive to memory phenotype are not essential for transgenic T cells to develop characteristics observed in the heterogeneous population of T cells during aging.

In addition to CD45R, there are other T cell surface markers which change during aging. The percentage of resting T cells expressing CD28 decreases with donor age (Pawelec 1995). CD28 expression by T cells also declines during the in vitro passage of T cell clones. Thus, expression of CD28 may be not only a biomarker of immunosenescence but also a possible explanation for the impaired T cell response to foreign antigens. The expression of CD40L by activated T cells has also been reported to decrease with increasing age (Li et al. 1995). Fewer T cells from old compared to young mice cultured with anti-CD3 antibody express CD40L. This age-associated change in cell surface phenotypic may also have functional con-

sequences with respect to the age-associated changes in humoral immunity and T cell activation.

The changes in the structure of the lymphoid tissues and in the phenotype of T lymphocytes associated with aging are associated with important defects in T cell function. Although the generation of a protective T cell-mediated immune response in vivo involves a multitude of processes including antigen presentation, lymphocyte or monocyte chemotaxis, lymphokine production, T cell proliferation, and the vascular responses to inflammatory mediators, it has become clear that T cells and not their environment are the major determinants of protective immunity. The most convincing experiments in this regard are the experiments which compare protection from tuberculosis and mortality in mice of different ages infected with tuberculosis (Orme 1987). Old mice suffer a much higher mortality from M. tuberculosis than young mice and this fact is attributable to changes in the function of T cells and not the T cell environment. Thus, when mice of different ages are given T cells from young donors, all recipients, regardless of age, develop the same level of protective immunity. In contrast, when young mice were reconstituted with T cells from young or old mice higher protective immunity was observed in the recipients of T cells from young than old mice.

The effect of age on the morbidity and mortality from three viral diseases, influenza, H. zoster, and HIV, has been studied. Both elderly humans and old mice suffer excess morbidity and mortality from influenza even when immunized prior to challenge (Ben-Yehuda et al. 1993). The age-associated increase in susceptibility to influenza is linked to the impaired generation of both protective cytotoxic T cells and antibodies. Approximately one half elderly humans and old mice fail to develop protective anti-influenza immunity after immunization with the conventional influenza vaccine.

The reactivation of the latent Varicella-Zoster (VZ) virus leads to "shingles", a vesicular skin lesion that follows the distribution of the nerve supply to the skin. The VZ virus is acquired during childhood and lies dormant in the dorsal root ganglia of the peripheral nervous system. The latent virus reactivates when the level of anti-VZ viral T cell immunity declines. The incidence of shingles, and even more disturbing to the patient post-herpetic neuralgia, increases dramatically in persons over 45 years of age. It has been clearly demonstrated that the frequency of VZ specific T cells declines with age leading to the reactivation of the latent viral infection (Levin et al. 1992). It has been demonstrated that immunization of elderly humans with the attenuated, live Oka VZ virus vaccine boosts the number of VZ-specific T cells. Clinical studies are being undertaken to determine whether

immunized elderly individuals will be less susceptible to shingles and post-herpetic neuralgia.

HIV is a viral disease which destroys peripheral CD4 T lymphocytes. For this reason it was speculated that the elderly, who are less able to compensate for peripheral T cell depletion would be more severely affected by HIV. This prediction was documented in hemophilic patients in whom the date of infection could be documented (Darby et al. 1996). There was an inverse correlation between age at infection and length of survival. Furthermore, the oldest cohort had a 4 fold greater death rate than the youngest cohort.

Because T cell immunity plays such an important role in immune senescence, the activity of T lymphocytes in culture has been examined in considerable detail. Many studies have focused on the effect of age on the in vitro proliferative responses of T lymphocytes cultured with mitogens, such as PHA, Con A, monoclonal antibodies to the CD3ε chain, or to antigens to which the lymphocyte donor had been sensitized (Miller 1996). There is a broad consensus that the proliferative response of T cells in vitro decreases with age. As the T cell receptors for these ligands are not altered with age, it is widely believed that the defect in the response of T lymphocytes from elderly person reflects impaired signal transduction. This concept is supported by a number of studies that have shown that mobilization of calcium and generation of phosphorylated intracellular proteins differ in activated T cells from old as compared to young subjects.

In addition to the early steps of T cell activation, there is considerable evidence that cytokine generation by activated T cells is also altered with age (Kirman et al. 1996). More than 20 years ago, we demonstrated that T cells from old humans are impaired in their production of and response to IL-2 (Weksler and Hutteroth 1974). There now is a general consensus that there is a reduced level of IL-2 gene transcription and IL-2 secretion by in vitro activated T cells from elderly humans and old mice compared to from young controls. However, not all cytokines produced by activated T cells decrease with age (Shearer 1997). For example, activated T cells from older donors secrete more IL-4 and IL-6 than do activated T cells from young donors. Considering the profile of cytokines produced by T cells from old and young subjects, there is an age-associated shift from a profile typical of Th1 to Th2 cells.

Since thymic involution seems to be the pacemaker of immune senescence, it was of interest to test the capacity of transplanting thymus into adult animals (Hirokawa et al. 1982; Kurashima et al. 1995). Thymus grafts from neonatal animals caused the most rapid reconstitution of the naive T lymphocyte population and the most complete recovery of responsiveness to cell mitogens and to T-cell dependent antigens. When thymus grafts were

taken from older animals, the pace of recovery was delayed and in many cases thymic-dependent immune function never reached that seen in intact animals or in animals reconstituted with neonatal thymus glands. Thus, the capacity of the thymus to affect the maturation of thymocyte precursors into mature T cells decreased with age.

4 T Cell Helper Function and Age

T lymphocytes not only mediate cellular immunity but also regulate humoral immunity. T cells modulate not only the quantity and quality of the antibody secreted by B lymphocytes but also the development of B lymphocytes from precursors within the bone marrow. The age-associated defect in the antibody response to most protein antigens and vaccines is a consequence of thymic involution and the resulting impaired T lymphocyte helper function. These conclusions are derived from studies in mice using antigens that have been shown to induce T lymphocyte dependent (TD) or thymic independent (TI) antibody responses and that stimulate antibody secretion by B1 or B2 lymphocytes (Zhao et al. 1995). It was shown that the antibody responses to TI antigens and to antigens that stimulate B1 lymphocytes do not diminish with age. In contrast, the response of old mice to TD antigens and to antigens that stimulate B2 lymphocyte responses are markedly diminished compared to the response of young mice. The quantitative impairment in humoral immunity is reflected in terms of the concentration of serum antibody or of the number of plaque forming cells specific for the nominal antigen. The qualitative impairment is reflected in the limited heterogeneity of antibody produced by old mice in terms of antibody affinity for the nominal antigen, antibody isotype, and antibody idiotype (Goidl et al. 1976; Goidl et al. 1980).

In parallel with the impaired antibody response of old mice to the nominal foreign antigen, there is a significant increase in the autoantibody response (Goidl et al. 1980). One class of autoantibodies, auto-anti-idiotypic antibodies react with the antigen combining sites of the B cell antigen receptor and, thereby, inhibit antibody secretion by the B lymphocyte specific for the nominal antigen. Thus, some autoantibodies not only reflect the dysregulated immune response associated with aging but contribute to the immune deficiency associated with aging.

Despite the decreased antibody response to most foreign antigens with age, there is a marked and generalized increase in the secretion of Ig by old mice manifested by the increased concentration of serum Ig and the increased number of Ig-secreting lymphocytes in old compared to young mice (Zhao et al. 1995; Gueret et al. 1996). It is likely, but not yet proven that the

loss of T cell-mediated regulation of B lymphocyte activity leads to polyclonal activation of B cells in old mice.

The antibody responses to TD antigens that are severely compromised with aging, depend upon cooperation between T and B lymphocytes within the secondary lymphoid tissue. In addition to the primary interaction between the nominal antigen and the B cell antigen receptor, there are critical cognate interactions between the B-7 and CD40 molecules on the surface of B cells with the CD28 and CD40L molecules on the surface of T lymphocytes as well as B cell exposure to cytokines such as IL-4 and IL-6 secreted by the T cell (Abbas and Singer 1996). These critical interactions between B and T lymphocytes take place within the germinal centers of the secondary lymphoid compartments. In the germinal centers, further diversification of B cells occurs, mediated by somatic mutation of the immunoglobulin genes, as well as isotype switching from IgM to IgG, IgA and IgE antibodies (Liu and Arpin 1997). The number and function of germinal centers are severely compromised with age as a consequence of thymic involution and the resulting defects in T lymphocyte function. The failure of normal germinal center development leads to the impaired production of the most protective high affinity and IgG antibodies is impaired.

While the influence of aging and thymic involution on the antigen-dependent phase of the B cell function, it is clear, aging and thymic involution is now being recognized to influence the antigen-independent phase of B cell development. Specifically, we have shown that T lymphocytes and the soluble factors they secrete regulate the development of B cell from their bone marrow precursors (Szabo et al. 1998). The development of B lymphocytes within the bone marrow, like the development of thymocytes within the thymus, can be separated into several stages. The early stages of B lymphocyte development up to and including the Pro-B lymphocyte stage changes little with age. In contrast, the number of Pre-B cells, is much reduced in old compared to young mice.

The transition of Pro-B to Pre-B cells depends upon the rearrangement of the Ig heavy chain genes and the expression of the Ig heavy chain on the cell surface in association with the surrogate light chains, lambda V and VpreB (Li et al. 1993). This series of molecular and cellular events is similar to the rearrangement of the heavy chain genes of the TCR genes and the expression of the TCR heavy chain in association with the surrogate light chain on thymocytes. The rearrangement of the BCR and TCR genes requires the activity of the RAG gene products. We have shown that RAG gene expression by bone marrow B cell precursors decreases with age (Ben-Yehuda et al. 1994). In preliminary studies, we have shown that RAG-mediated DNA breaks are decreased in Pro-B cells from old compared to young mice. It

appears reasonable to suggest that the age-associated defect in B lymphocyte development in old mice is due to the impaired capacity of B cell precursors to rearrange their Ig heavy chain genes. Impaired expression of RAG genes by B cell precursors is observed not only in old mice but also in athymic, nude mice (Szabo et al. 1998). Injection of IL-16 into old or athymic mice increases the expression of RAG genes by bone marrow B cell precursors and the number of bone marrow Pre-B cells. The impaired capacity of T lymphocytes from old mice to secrete IL-16 compared to young mice offers a mechanism linking age-associated T lymphocyte dysfunction with decreased expression of RAG genes by bone marrow B cell precursors and decreased numbers of Pre-B cells in thymic deprived mice. In summary, thymic involution not only compromises T lymphocyte development and cell mediated immunity but also compromises B lymphocyte development and humoral immunity as T cells and/or the factors they secreted are required for the normal development and function of B lymphocytes.

5 Clinical Significance of Immune Senescence

There is much evidence that immune senescence contributes to the increased morbidity and mortality that accompanies aging (Roberts-Thomsen et al. 1974; Doria et al. 1997). The first link between immune senescence and mortality was the observation that elderly humans with reduced delayed cutaneous hypersensitivity reactivity were at increased risk of death. Subsequently, studies showed that decreased delayed cutaneous hypersensitivity reactivity in humans over 60 years of age was associated with an increased mortality from pneumonia and cancer. In vitro immune parameters have also linked immune senescence to increased mortality. Thus, healthy men with a low lymphocyte counts were found to be at increased risk of death. Similarly, individuals between 86 and 92 years of age, with low proliferative responses to T cell mitogens, combined with low CD4, high CD8 T cell and low B cell counts were at increased risk of death. Some of the same factors have been studied in mice. Thus, mice with increased CD8 T cells and memory T cells had decreased survival.

There is every reason to believe that interventions that enhance adaptive immunity in the elderly will reduce their morbidity and mortality. Specifically, increasing the capacity of the immune system in the elderly to produce the most protective high affinity, IgG antibodies as well as protective cytotoxic lymphocytes should reduce the severity of infectious disease in the elderly. The regulation of autoantibody formation may well decrease the incidence of pernicious anemia and vascular diseases affecting the brain, heart, and kidney that are accelerated by the production of autoantibodies.

Finally, reversing the age-associated defects in immune surveillance that permit growth of neoplastic cells and clonal lymphocyte expansions, likely precursors of lymphomas should reduce the morbidity and mortality from cancer. For these reasons, it is reasonable to believe that preventing or limiting immune senescence will improve the health of elderly adults.

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Aging and Chromosomal Instability

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Contents

1 Introduction

"Aging is a progressive, generalized impairment of function resulting in a loss of adaptive response to stress and in a growing risk of age-related disease. The overall effect of these changes is summed up in the increase in the probability of dying, or age-specific death rate, in the population ..." (Kirkwood 1995).

During the last decades, life expectancy has significantly improved and the number of centenarians is expected to increase dramatically in the next century. Concurrently, the financial support for the elderly population will become an issue for public concern, especially with regard to the expenditure for health care for such age-related diseases as atherosclerosis, diabetes, neoplasias etc.

As a consequence of these problems, research on the mechanisms of aging has gained broad interest. Fundamental knowledge of the molecular processes underlying aging and senescence may enable medicine to alleviate or postpone age-related biological mechanisms. Thereby, the incidence of age associated diseases may be reduced permitting a vast majority of elderly people to enjoy life in good health and physical strength.

This review will discuss data that supports evidence for DNA instability (caused by DNA damage) playing a central role in cellular senescence and, thereby, organismal aging.

1.1 Theories of Aging

1.1.1 "Programmed Aging" or Aging Under Genetic Control?

The phenomenon of aging and mortality had fascinated scientists for centuries in their hunt for a formula for eternal youth. While many theories of aging have been proposed, two, each opposing, opinions have emerged: aging as a genetically programmed versus a stochastic process. Here, no comment on these theories will be made. (for review, see: Bernstein and Bernstein 1991). But with regard to the genetic character of this review, the term "programmed" needs to be specified. As long as programmed aging is understood as a carefully regulated sequence of genetically established physiological events leading to aging as a developmentally programmed adaptation (Finch 1972) is this theory assailable. Kirkwood (1984) and Hayflick (1987) argued that the existence of an inborn "clock" that limits life span in a deterministic way would not withstand the demands of selection and evolution. There is no rational explanation why programmed aging should render a species fitter (in terms of reproductiveness) than a longer

life. This statement, however, is not necessarily relevant for processes on the cellular level such as the programmed cell death (apoptosis) that, indeed, may be executed according to a special program. (for review, see: Evan and Littlewood 1998). But this kind of cell death is not a necessary consequence of aging. Thus, in the nematode Caenorhabditis elegans, mutations that block apoptosis do not extend life span (Driscoll 1995). They, rather, offer cells with irreparable damaged DNA the possibility to commit suicide and by that escape cancerogenesis.

This reservation towards the existence of "programmed aging"" does not disprove the fact that senescence-associated cellular events are genetically regulated. It is widely accepted that genes are involved in aging. Many (probably several hundreds) of these genes termed "gerontogenes" (Rattan 1985) are anticipated. Some have been identified, especially in lower eukaryotes but also in humans (Jazwinski 1995; Guarente et al. 1998). Thus, aging stands under genetic control.

1.1.2 Aging, a Stochastic Process?

If there is no deterministic program for aging provided, what then makes us age?

Aging could result from a sum of stochastic events. Orgel (1963) blaimed the protein-synthesis apparatus for becoming inaccurate over the years and proposed the famous "Error Catastrophe Theory of Aging". Many experiments were performed along that line which finally proved this hypothesis to be invalid (Harley et al. 1980; Rothstein 1987). Following the idea of stochastic events, other theories were suggesting that decay of one or the other organic system or macromolecules brought about the decay of the whole organism. (The neuroendocrine theory: Finch 1972; Herrmann and Berger, this volume. The immunological theory: Walford 1969; Shen et al., this volume. The Wear-and Tear Theory: Bernstein and Bernstein 1991. The Somatic Mutation Theory: Szilard 1959; the Disposable Soma Theory: Kirkwood 1977; the Free Radical Theory: Harmann 1956). All these theories are reduceable to a common denominator: Aging may evolve from the inefficency of the organism to maintain its somatic integrity. Since DNA contains information for all cellular requirements, this integrity can be maintained as long as error-free transcription is supplied from undamaged DNA. Thus, DNA damage and impairment of genetic information substantiated by chromosomal instability may be considered to be the key event in aging. The goal of this review will be to document the validity of this statement and to gather arguments for the linkage of genetic regulation and stochastic accumulation of damage as the initiator of aging.

1.2 "Quick-Motion" to Study Aging in Man

1.2.1 Fibroblasts in Culture Perform Replicative Senescence

The longevity of man along with the complexity of the human organism raise serious problems for the experimental gerontologist. Many short-living model systems have been established of unicellular as well as of multicellular organisms (yeast, nematodes, flies, rodents and others).

Even though key mechanisms of aging are expected to be universal, the comparability of these systems may be limited: adaptation to different environmental demands may have stimulated divergent age-controlling mechanisms impeding the direct application of experimental results to humans.

Since the introduction of cytology in the beginning of the last century by Mathias Jakob Schleiden and Theodor Schwann it became obvious, that all organisms and all tissues are organized from cells, the smallest living entity. Cells taken from an individual and propagated in tissue culture turned out to replicate by dividing as cells do in vivo, but it also became obvious that this proliferation capacity is limited. This finding was first described by Hayflick (1965) for normal human fibroblasts. The ability to proliferate is gradually lost and normal human fibroblasts subcultivated (passaged) continously enter proliferative senescence (Hayflick and Moorhead 1961). Thus, human diploid fibroblasts in culture promised to be a model system for human aging on condition that further properties in common could be found between cells in vitro and in vivo. Many experiments along this line proved, that a) the phenomenon of replicative senescence is not an artifact of cells in culture, b) cell senescence can be delayed but not reversed and reflects a process, which also occurs during organismal aging and c) senescence has a genetic background. At least four genes (involving mainly chromosomes 1, 4 and 7) can be expected according to complementation studies to control senescence in a dominant way (Smith and Pereira-Smith 1996). Campisi (1996) described a neutral β-galactosidase activity specific for senescent human cells in culture that is also expressed in vivo in human skin upon aging. Allsopp et al. (1992) reported telomer shortening in aging human fibroblasts and in lymphocytes not only in cell culture but also in vivo correlating with donor age. It could be shown that aging in cell culture is directly related to aging in vivo since the proliferative capacity of diploid fibroblasts decreases with increasing age and is directly related to the maximum lifespan of the species from which they are derived (Martin et al. 1970; Rohme 1981; Weirich-Schwaiger et al. 1994). A reevaluation of these results was published very recently (Christofalo et al. 1998). The authors analysed skin fibroblast cell lines from 42 healthy donors with biopsies taken under

standard conditions. They did not find a relationship between donor age and the replicative lifespan in human cells in culture. Indeed, results obtained in cell culture have to be interpreted carefully. In particular, experience in handling of primary human cells must be guaranteed so that the utmost lifespan of each cell line will be reached (Cristofalo reported a regression line for in vitro maximum replicative lifespan (PDL) of all cell lines of all donor ages of between 20 and 30 PDLs, which appears to be rather low). Whatever the interpretation of these and the earlier results may be, it remains that the in vitro life span of human diploid fibroblasts derived from young and healthy donors of 60-70 passages according to culture conditions renders this cellular model system suitable to observe parameters of human senescence similar to the image created by rapid motion cinematography.

1.2.2 Premature Aging Syndromes Exhibit Features of Accelerated Aging

The understanding of many biological processes has been promoted by the study and comparison of wildtype and mutant individuals. In the field of aging human individuals exist who suffer from inherited diseases that are accompanied by symptoms of premature aging. Since many though not all features of aging seem to be accelerated, these diseases are called segmental progeroid syndromes (Martin 1985). The autosomal recessive inheritance of most of these congenital disorders is evidence for their monogenic origin. Since only one gene should be involved in each of these one may hope to learn about aging by elucidating the molecular basis of these diseases. Clinical features of some of these diseases, including Hutchinson-Gilford Progeria, (Progeria of Childhood) and Werner Syndrome, (Progeria of the Adult) the two most prominent progeroid syndromes that appear, are summerized in Bernstein and Bernstein chapter 7 (1991). One of the main features, a shortened life expectancy, is also reflected in the replication potentials of cultured fibroblasts of these patients (Weirich-Schwaiger et al. 1994; Brown et al 1984; Kipling and Faragher 1997). Thus, analysis of cells in culture of young and old individuals and of patients with progeroid syndromes seems to be a legitimate and potent tool to study molecular events responsible for human senescence.

2 Chromosomal Instability as a Central Parameter of Aging

2.1 Chromosomal Instability: a Link Between Syndromes of "Chromosomal Instability" and "Premature Aging"

Aside from morphological changes and a reduced number of population doublings one striking feature of progeroid syndrome fibroblasts in culture is an increased chromosomal instability either spontaneous or induced by exogenous agents (Epstein et al. 1966; Salk 1985; Miozzo et al. 1998). In the past, an increased chromosomal instability had been attributed to the so-called "chromosomal instability syndromes", a group of congenital diseases like Xeroderma pigmentosum, Cockayne syndrome, Ataxia telangiectasia, Down`s syndrome, Bloomis syndrome, Nijmegen breakage syndrome, that were characterized by sensitivity to radiation and/or DNA damaging agents, chromosomal breakage and, most importantly, susceptibility to cancer. Defects in DNA repair mechanisms were suspected to be the molecular basis of these "reparatoses". Indeed some of these defects have been elucidated by cloning the mutated genes and the deduced proteins were shown to be involved in DNA repair processes. (Chu and Mayne 1996, review; Rotman and Shiloh 1998, review; Ellis et al. 1995; Schwaiger et al. 1989; Varon et al. 1998; Carney et al. 1998). Monitoring the symptoms of these "chromosomal instability syndromes", it became evident, that some of them (especially Cockayne syndrome, Ataxia telangiectasia, Down`s syndrome) exhibited features of premature aging and that there was an overlap between premature aging and chromosomal instability syndromes (Hirsch-Kauffmann et al. 1990).

2.2 Chromosomal Instability Results From Damaged or Structurally Altered DNA

Genetic instability is to be expected in all DNA containing compartments of the cell (for review, see: Gaubatz 1990). Reorganization of the mitochondrial genome has gained high significance especially with regard to aging. (For the interested reader, see: Osiewacz 1995; Richter 1995; Wallace 1995; Shigenaga et al. 1994). This review will be restricted to nuclear DNA.

2.2.1 Chromosomal Instability May be Reflected in Microscopic or Submicroscopic Alterations of Chromatin

Gross chromosomal instability of the nuclear genome may manifest in cultured cells as rearrangements of metaphase chromosomes, chromosomal loss, chromosomal breaks or as micronuclei (Heddle et al. 1978). The resultant instability may arise from either the injury itself (for example, strand-breaks following irradiation) or from incomplete attempts to repair injured DNA. Repair processes are initiated in order to free DNA from structural damage like crosslinks and bulky adducts, nucleotide or base alterations (for review, see: Sancar 1994; Lindahl et al. 1997). Such injuries may be brought about by endogenous processes such as hydrolysis of the DNA at 37 °C or oxidation by oxygen radicals arising from normal metabolic processes, or by exogenous noxes like DNA-damaging agents or radiation. Those defects amount to several thausend alterations per day per cell (Lindahl 1993) and if unrepaired would accumulate within the cell. Mechanisms to keep DNA stability and DNA flexibility in balance are numerous: antioxidants and genes controlling DNA stability and integrity (guardians of the genome) are involved in such processes. However, depending on the individual genotype and the individual environmental burden, repair capacity will be worn out sooner or later or repair genes themselves might become mutated, both leading to an insufficently repaired DNA and an accumulation of chromosomal aberrations.

At the submicroscopic level, DNA instability may be caused by structural alteration and relaxation of DNA domains such regions of methylation, heterochromatin, repetitive sequences and the telomeres. Such destabilized regions may become preferential targets for DNA damage.

2.3 Chromosomal Instability in Aging Cells

In order to verify whether chromosomal instability and waning DNA repair capacity mentioned above actually correlate with aging and actually play an important role in human senescence, signs of DNA destabilization have to be evident both in aging cells in vivo and in vitro and in cells from patients with premature aging syndromes.

One main problem in reviewing the experimental results on this issue is the inconsistency of the findings. As discussed by Norwood and Gray (1996), great effort has been made in the 1980s to solve this problem. Now, almost 20 years later, methods and knowledge in cell biology have significantly improved and more recent studies to evaluate chromosomal instability have to be cited.

2.3.1 Numerical Chromosomal Aberrations

As reviewed by Schneider (1985), aneuploidy increases in aging cells. The sex chromosomes in particular seem to be affected (Guttenbach et al. 1994). There, a higher level of aneuploid cells was found in fibroblasts of human progeroid syndromes (Mukherjee and Costello 1998). When one considers that the only viable human aneuploidy is Turner syndrome (XO) it could be surmised, that other aneuploid cells would have less chance to survive.

2.3.2 Structural Chromosomal Aberrations

Diverse structural aberrations result from chromosomal breakage events. In addition to chromatid and chromosome breaks, different kinds of translocations, deletions, inversions and dicentric chromosomes can be observed in metaphase spreads. The increase in dicentric chromosomes in senescent cells (Benn 1976; Sherwood et al. 1989) especially derive from telomeric association (TAS), that are stimulated by telomere shortening (see 2.3.3). The presence of dicentric chromosomes in turn initiates breakage-fusion-bridge cycles that further increase chromosomal instability (Toledo et al. 1992; Mondello et al. 1997).

Chromosomal fragments, if acentromeric, may become enveloped by nuclear membrane material and appear as micronuclei in interphase cells. The contradictory results, formerly obtained when age-dependency of the amount of chromosomal and chromatid breaks was examined, are summerized by Rattan (1989). In the main, cytogenetic studies were performed with cultured human lymphocytes (Marlhens et al. 1986) or with primary human fibroblasts (DellÒrco and Whittle 1981). In the latter a positive relation was found. Or, they were performed with lymphocytes from peripheral blood (Bender et al. 1989) or with fibroblasts in culture (Mayer et al. 1986). In the latter this effect could not be confirmed. Similar attempts with rodent cells in culture attained positive results (Martin et al. 1985; Sato et al. 1995). More recently, however, Weirich-Schwaiger et al. (1994) reported a significant increase in spontaneous chromosomal instability (chromosomal breaks and micronuclei) according to passage level in cultures of diploid human fibroblasts derived from young and old donors and from patients with premature aging syndromes (Down's-, Werner- Cockayne syndrome and Ataxia telangiectasia). Under constant laboratory conditions, a direct correlation between prolongation of population doubling time on the one hand, and accumulation of chromosomal breaks and micronuclei on the other could be observed in the course of passages. That an increase in breakage events can be noticed in high passaged cultures of young and of old individuals as well

as in those of patients with progeroid syndromes, endorses a causal relationship between senescence and DNA damage and once again justifies both the study of cultured cells and of premature aging syndromes as valid model systems in aging research.

In Werner syndrome do not only chromosome breaks accumulate extensively in fibroblasts during the shortened lifespan, but translocations, deletions and inversions are also reported (Salk et al. 1985; Fukuchi et al. 1989; Scappaticci 1990). That this instability does not reflect a cultural artifact but rather an outlet of the inborn error of this disorder is proven by the presence of the instability trait seen also in fibroblasts of the heterozygotic siblings, albeit to a lesser extent than with the homozygotes. (Weirich et al. 1996). A very recent publication confirmed these results: immortalized B lymphocytes of Werner syndrome patients and heterozygotic carriers are hypersensitive to 4-nitro-quinoline-1-oxide (4NQO) (Ogburn et al. 1997). This substance has a strong DNA-damaging, mutagenic and carcinogenic effect. Previously, Gebhart et al. (1988) had shown increased chromatid breaks and chromosomal interchanges after application of 4NQO to T-lymphocytes of Werner syndrome patients.

2.3.3 Telomere Shortening

The integrity of single chromosomes in eukaryotes is guarded by a nucleoprotein structure at both ends of the chromosomes, the telomeres (Blackburn 1991). The telomeric DNA consists of an array of short G/C rich tandem repeats (TTAGGG in man), with a length of 6–10 kb at birth (de Lange 1994) that is progressively shortened with increasing donor age (skin and blood telomeres by 15–40 bp per year) as shown, for example, in lymphocytes by Lindsey et al. (1991) and with increasing passage number in cultured fibroblasts (30–200 bp per population doubling. Observed telomere lengths of 18–25 kb in young and 8–20 kb in senescent human fibroblasts.) (Harley et al. 1990; Allsopp et al. 1992; Vaziri et al. 1994). Telomere shortening was first predicted by Olovnikov as early as 1973 based on the inability of the DNA replication enzymes to replicate the utmost 3`end of the parental DNA strand, thus leaving a gap after excision of the RNA primer at the 5`end of the lagging daughter strand (Olovnikov 1973). This process has been shown to be directly coupled to cell division in somatic cells (Allsopp et al. 1995). Shortening of human telomeres only takes place in somatic cells and was widely regarded as the "mitotic clock", whereby cell proliferation is blocked when a telomere length of about 4 kb is reached (von Zglincki et al. 1995). The awareness of a wide variety of telomere lengths present in an individual cell leads one to believe that a single chromosome, reaching a

critical telomere length, may be enough to induce senescence. Such a shortened chromosome may mimic a double strand break: A single double strand break, however, may stop the cell cycle in yeast (Sandell and Zakian 1993) and in human cells (di Leonardo et al. 1994) via induction of proteins like p53 and Sdi1 (synonyms: p21/Cip1/Waf1/Cap20), the latter being known to inhibit cyclin-dependent kinases and, by this means, the cell cycle (Sherr and Roberts 1995); Vaziri and Benchimol 1996). In germline cells (de Lange et al. 1990) and in most immortal cells (Counter et al. 1992; Kim et al. 1994) an enzymatic activity (telomerase) has been identified that aids in replicating telomeric DNA (Morin 1990; Kim et al. 1994; review: Greider 1996). The block of replication (termed M1) in somatic cells at a critical telomere length (the Hayflick limit) can be overriden by transforming agents. If this occurs, telomere shortening proceeds until "crisis" when cell death occurs in M2. At that point, a few cells may regain telomerase activity and become immortal (Harley et al. 1992; Guarente 1996).

Recently, it was suggested that the "end-replication problem" may not be the only reason for telomere shortening. An enzymatic activity was noticed that degrades the C bases containing strand of the telomeres. The discovery of up to 200 G-bases long single strands on both ends of the chromosomes has confirmed these findings (Wellinger et al. 1996; Makarov et al. 1997). Moreover, von Zglinicki et al. (1995) propose the accumulation of single-strand breaks, gaps or terminal overhangs, measured experimentally as sensitivity to S1 nuclease degradation, in telomeres induced by mild hyperoxia. This implies, that telomeres might be sensitive to oxidative DNA damage. Accumulation of S1 sensitive sites in telomeres is also evident in fibroblasts cultured for extended periods at confluency. Since this effect is less prominent under conditions of serum deprivation, the metabolic state of a cell seems to contribute to telomere shortening and, thereby, to cellular senescence (Sitte et al. 1998). Under various experimental conditions leading to oxidative stress, S1 sensitve sites were found to be significantly more frequent in telomeres than in the overall genome. Furthermore, the repair of single stranded regions induced by H_2O_2 is almost twentyfold slower in telomeres than in non-telomeric DNA (Petersen et al. 1998). This repair insufficency seems to be unique for oxidative damage. UV-light induced pyrimidine dimers are reported to be excised slower from telomeres than from actively transcribed genes but faster than from heterochromatin. At any rate, repair capacity declines with age (Kruk et al. 1995). Accumulated single strand regions, however, may accelerate shortening by nucleolytic degradation or by breakage events.

2.3.4 Telomere Shortening in Premature Aging Syndromes

2.3.4.1 Down's Syndrome

Vaziri et al. (1993) demonstrated enhanced telomere shortening by determination of telomeric restriction fragments (TRF) in Down's syndrome patients compared to age-matched controls. In cytogenetic studies, however, Kormann-Bortolotto et al. (1996) did not find any preferential damage of distal regions in cultured lymphocytes of young and old individuals and in patients with Alzheimer`s disease and Down's syndrome. These results do not exclude accelerated telomere loss but indicate the existence of a mechanism that protects chromosome ends from gross destruction.

2.3.4.2 Ataxia Telangiectasia

In Ataxia telangiectasia lymphoblastoid cell lines (Pendita et al. 1995)) and in peripheral blood lymphocytes (Metcalfe et al. 1996) an accelerated telomere shortening was observed. A higher frequency of chromosome end-to-end associations may in part contribute to telomeric associations (TAS).

2.3.4.3 Werner Syndrome and Progeria

The kinetics of telomeric repeat loss in four serially passaged fibroblast-like cell lines from Werner syndrome patients showed a faster decline of TRF lengths than those of three age-related control cell strains (Schulz et al. 1996). This result confirms a study by Kruk et al. (1995) where one Werner syndrome strain was compared to five control fibroblast cultures from donors of different ages.

Allsopp et al. (1992) investigated the mean TRF length in five fibroblast strains from donors with Hutchinson-Gilford syndrome compaired to five control strains and found the telomeres to be significantly shorter.

The results on telomeres presented so far collectively emphasize the important role telomeric instability plays in the course of cellular senescence even though it may not be the only relevant mechanism, since generalization of this phenomenon to all cell types is problematic. Mice, for example, have long telomeres and an active telomerase in their somatic cells while yeast telomeres do not shorten at all. However, senescence is a necessary mean for mammalian cells to escape immortalization and, thereby, cancer development (for review, see: Sedivy 1998).

2.3.5 Instability of Other DNA Regions

2.3.5.1 Ribosomal DNA (rDNA)

Gaubatz (1990) reviewed the loss of reiterated sequences during aging of human cells in vitro and in vivo. An age-related loss of DNA coding for ribosomal RNA (rDNA) also in humans has been reported for different tissues. Also, fragmentation of the nucleolus, a structure consisting of rDNA, rRNA and proteins, was observed in very old yeast mother cells (Sinclair et al. 1997; Guarente 1997). This fragmentation, with resultant aging, was enhanced in yeast mutated in the helicase SGS1. This protein, localized to the nucleolus, belongs to the RecQ subfamily of helicases and is associated to top2p and top3p (topoisomerases) (Gangloff et al. 1994; Watt 1995). Topoisomerases are involved in the faithful segregation of daughter chromosomes in transcription and recombination processes. Their function may be coupled to helicases and mutants in either transcription or recombination involved helicase may result in hyperrecombination, especially within the tandem repeats of rDNA copies, with subsequent loss of sequences. Transcription of rDNA may also be impaired and indeed Werner helicase, the protein affected in Werner syndrome (Yu et al. 1996) also belongs to the RecQ helicases and is localized to transcriptionally active nucleoli. Thus, SGS1p and WRNp may protect rDNA from hyperrecombination and damage and prevent precocious fragmentation of the nucleolus. (Sinclair and Guarente 1997).

The essential role of the integrity of rDNA for longevity (ribosomal proteins are of central importance for protein synthesis) may be deduced from the fact that proteins otherwise linked to telomeres such as the SIR-complex, become relocalized to the nucleolus in aging cells. The result is beneficial to cell longevity (see yeast 2.4.3.3) (for review, see, Guarente 1997).

2.3.5.2 Extrachromosomal Circular Genetic Elements

Circular DNA molecules with homology to chromosomal DNA found in many species as a consequence of aging are hypothezised to be recombination products (Kunisada et al. 1985). Flores et al. (1988) found such elements to hybridize to repetitive interspersed mammalian nucleotide sequences.

An increase in small polydisperse circular DNA (spcDNA) was reported in cultured fibroblasts in Fanconi anemia, one of the well established chromosome instability syndromes (Motejlek et al. 1993).

Extrachromosomal circular ribosomal DNA was shown to accumulate in old yeast cells promoting the fragmentation of the nucleolus. The amount of

such elements increased in yeast mutated for SGS1 followed by premature aging and life span reduction (Sinclair and Guarente 1997). It remains to be elucidated if mutations in the Werner syndrome gene, a homologue of yeast SGS1, have similar effects.

2.3.5.3 Chromatin Alterations that Effect DNA Condensation

One of the functions of chromatin organisation in eukaryotes serves regulation of gene expression. Genes located in dense heterochromatin domains are silenced due to an impaired accessibility of the transcription machinery to the DNA. This heterochromatin structure, however, is facultative since decondensation of the DNA may allow transcription of otherwise repressed genes according to the physiological state of a cell. Thus, during aging, heterochromatin stability may be altered in a cell cycle-coupled fashion (Howard 1996). After each round of replication, heterochromatic domains have to be accurately reassembled. Damage to the DNA or acetylation of the core histone H4, normally selectively underacetylated in heterochromatin (Csordas 1990) may result in irreversible loss of repressive chromatin domains. Studies on the chromatin structure in cultured cells by differential scanning calorimetry revealed a reduction in the melting temperature of DNA with age, indicating an increase of single-stranded DNA in chromatin of aging cells (Almagor and Cole 1989). Such structural changes were attributed to the accumulation of DNA damages that would lower the supercoiling potential of the DNA. Furthermore, with a highly sensitive assay, that provides for detection of one frameshift mutation in a background of 10^6 wild-type sequences, Jackson et al (1998) demonstrated oxidative damage to preferentially increase the instability of the repetitive sequences of microsatellite DNA.

The failure to maintain the silencing effect of heterochromatin may easily explain the dominant phenotype of senescence: dominant, antiproliferative genes may be activated that enhance senescence. Such a gene is Sdi1 (Noda et al. 1994), an inhibitor of the cyclin E/Cyclin dependent kinase2 complex, that is involved in the progression of cells from the G1-to the S-phase (Peter and Herskowitz 1994; Pines 1994). Along this line several model systems have been proposed explaining the phenomenon of aging. (Villeponteau 1997; Imai and Kitano 1998). Indeed, there is a report (Wareham et al. 1987) indicating a loss of heterochromatic silencing for the X-chromosome located ornithine carbamoyl transferase gene in liver tissue of old mice.

Heterochromatin loss also may be induced following telomere shortening as proposed in a model by Wright and Shay (1992), and documented in yeast (Gottschling et al. 1990; Guarente 1996).

2.3.5.4 DNA Methylation as a Source of DNA Stability

The 5`methylCpG content of genomic mammalian DNA has been reported to decrease with age (Wilson and Jones 1983; Singhal 1987). Actually, methyl-modification of bases may be considered to play a role in the stabilization of heterochromatin domains, since demethylation of human fibroblasts with 5-azacytidine shortens their in vivo lifespan (Fairweather et al. 1987; Holliday 1986). Gray et al (1991), however, could not induce cell cycle arrest by a transient demethylation of DNA in fibroblasts.

DNA hypomethylation leads to genomic instability as in, for example, ICF syndrome (immunodeficiency, centromeric instability, facial abnormalities). This syndrome is characterized by undercondensation of the variable heterochromatin regions of chromosomes 1, 9 and 16 and an increase in spontaneously formed micronuclei (Stacey et al. 1995). Genomic instability was also shown in murine embryonic stem cells nullizygous for major DNA methyltransferase (Dnmt 1). Elevated mutation rates were observed at the hypoxanthin-phosphoribosyl-transferase locus as well as at the thymidine kinase locus mainly due to deletions (Chen et al. 1998).

2.4 Deficiency in DNA Repair Capacity Links Chromosomal Instability to Aging

2.4.1 Oxidatively Damaged DNA Accumulates in Senescence

To a great extent, damage to DNA is caused endogeneously. Spontaneous DNA hydrolysis represents one source, reactive oxygen intermediates (ROI) caused by metabolism the other. With the aid of modern chromatography methods, a broad spectrum of oxidative base modifications can be distinguished (Dizdaroglu 1992). Poulsen et al. (1996) found a 33% increased rate of oxidatively modified DNA in 20 men subjected to extensive excercise up to 10 hours per day over a period of one month. They measured 8-oxo-7,8-dihydro-2`-deoxyguanosine excreted in urine as metabolic parameter indicating the excision-repair of this oxidized base, thereby documenting the risk to damaged DNA by excessive oxygen consumption. In healthy young organisms DNA repair (review: Demple and Harrison 1994) and antioxidative cellular defense mechanisms counteract the deleterious effects of ROI. However, an inverse relation was found between oxygen consumption (metabolism that generates radicals) and longevity of various species as published by Sohal and Orr (1994), indicating a correlation between oxidative damage and aging. Perez-Campo et al. (1998) reviewed the relationship of oxidative stress with the maximum lifespan in different vertebrate spe-

cies. They concluded the main characteristics of a long-lived species to be a high rate of DNA repair and a low production of free radicals in close proximity to DNA. Consequently, the most long-lived animals appeared to exhibit the lowest level of antioxidants. They deduced, that the low rate of free radical production may contribute to the low aging rate. A correlation between oxidative damage repair and aging has also been found in human senescence in culture. Homma et al (1994) examined the level of 8-hydroxydeoxyguanosine in the DNA of human diploid fibroblasts. They found a significant increase during cellular aging (50 population doublings) and a concomittant decline of the repair capacity for this oxidative lesion. Similar results were published by Chen et al. (1995). These authors found the level of 8-oxo-2`-desoxyguanosine in DNA to be about 35% higher in senescent human fibroblast cells than in young cells whereas old cells excised four times more 8-oxoguanine from the DNA than young-passage cells did. Cultivation of fibroblasts in 3% O_2 instead of 20% resulted in 50% more population doublings. The spin trapping agent α-phenyl-t-butyl nitrone, acting as an antioxidant, also delayed senescence. These findings could be confirmed in cultured lymphocytes from healthy donors of different ages. (Barnett and King 1995; King et al. 1997). With an ELISA, single strand breaks were recorded in peripheral blood lymphocytes after damaging DNA by H_2O_2 treatment. At the same time, the antioxidant status of the cells was determined by measuring the level of several antioxidative enzymes like SOD, glutathione peroxidase, catalase and others. The authors concluded that the age-related increase in mutation frequency is due to a decrease in the repair efficiency of oxidative DNA damages and not to a decrease in the efficacy of the antioxidant defense system.

Ames et al. (1993) emphazised that the radical production generated from normal metabolism with subsequent damage to DNA (and also to proteins and lipids) does not only contribute to aging but also to age-related degenerative diseases like cardiovascular diseases, immune-system decline, brain dysfunction , cataracts and cancer.

2.4.1.1 Lesson from a Premature Aging Syndrome: Antioxidants Must be in Balance to Defend the Cell From Oxygen Radical Injury

Work on Down's syndrome revealed the importance of a balanced antioxidant status to prevent premature aging. Schwaiger et al. (1988) showed that rat cells overexpressing the gene for the human Cu/Zn-superoxide dismutase (hSOD1) became X-irradiation sensitive to a similar extent as are cells from Down's syndrome patients. In Down's syndrome patients the additional chromosome 21 (or part of it) supplies an overdose of SOD, thereby

increasing the amount of deleterious H_2O_2 which, in turn, oxidatively damages DNA. Supporting results came from de Haan et al. (1996). They found higher levels of H_2O_2 and signs of premature senescence in SOD1 transfected cell lines that had an elevation in the ratio of SOD1 to GPX1 (glutathione peroxidase). They also found a perturbation in the ratio of these enzymes in Down's syndrome fibroblasts and signs of senescence in H_2O_2 treated cells. In all cells mRNA levels for Sdi1 were found to be elevated, the mediator, probably, for the slow proliferation potential.

Recently, Raji and Rao (1998) analysed DNA repair parameters like "unscheduled DNA synthesis", DNA polymerase activities and endodeoxyribonuclease activities in lymphocytes from Down's syndrome patients of different age and found a lowered repair efficiency and a declining repair capacity with age.

2.4.1.2 Strenghtening of the Antioxidant System Increases Lifespan in Drosophila Melanogaster

The most convincing proof to date for the involvement of oxygen radicals mediated DNA instability in aging comes from experiments with Drosophila melanogaster. Despite discordant results of earlier attempts to supplement the diet of a variety of species with antioxidants like α-tocopherol, β-carotene etc., Brack et al. (1997) increased the flies` median and maximum life span by feeding them the antioxidant N-acetylcysteine and found a dose-dependent increase: 10mg/ml food increased the life span by 26.6%. Spectacular experiments were performed with transgenic animals, introducing extragenes for SOD1 and catalase into the germline, thereby achieving a lengthening of the median and maximum lifespan by one-third. These experiments directly indicated that cellular oxidative status and aging are interrelated (Orr and Sohal 1994; review: Sohal and Orr 1995). Parkes et al. (1998) overexpressed SOD1 specifically in the motorneurons of transgenic Drosophila and thereby extended the life span of these cells by 40%. Thus, the oxygen burden and the antioxidative capacity of specific cells may be decisive for the whole organism.

2.4.2 Accumulation of DNA Damage Outwears DNA Repair Capacity and Stimulates Cell Senescence. – DNA Repair Capacity Decreases with Age

DNA repair mechanisms counteracting the endogeneous and exogeneous injuries to the integrity of the DNA are numerous. This repair capacity compensates DNA damages in young and healthy individuals but is outworn in healthy but elder people by an excess of noxious agents as has been shown,

for example, for endogeneous processes (Weirich-Schwaiger et al. 1994), for X-irradiation (Harris et al. 1986; Mayer et al. 1989), for UV-irradiation (Roth et al. 1989; Kruk et al. 1994; Moriwaki et al. 1996) and for cross-linking agents (Rudd et al. 1995).

2.4.2.1 The Molecular Defects of Progeroid- and DNA Instability-Syndromes are Associated with DNA Repair

As mentioned above (2.1), the molecular basis of some progeroid syndromes has been elucidated and the functions of the putative proteins have been associated with DNA repair processes. These findings indicate that unrepaired DNA lesions caused by DNA repair deficiency are of central importance in the aging process. A detailed discussion is not possible in this review. However, a few results should be summerized in order to illustrate that DNA repair appears to be multi-faceted, involving not only excision of damaged or modified bases (base excision repair, BER) or of nucleotides (nucleotide excision repair, NER) and reconstitution of the original DNA sequence (reviewed by Lindahl et al. 1997), but also RNA transcription, replication, recombination and cell-cycle checkpoints. Prevention of DNA destabilization (see 2.4.3.1) and prevention of excessive oxygen radical production (see 2.4.1.1) may also be considered as "repair processes".

⇒ *Xeroderma Pigmentosum and Cockayne Syndrome Display Deficiency in NER and in Transcription-Coupled Repair*

As reviewed by Chu and Mayne (1996), some proteins responsible for the seven complementation groups of Xeroderma pigmentosum (XP), all involved in NER, are helicases. This inherited disease is characterized by extreme sun-sensitivity, cancerproneness and premature neuronal death. The two complementation groups of Cockayne syndrome (CSA and CSB), an inherited disease with features of UV-hypersensitivity and premature aging, are defective in proteins, that probably play a role in transcription by interacting with transcription factors thus coupling transcription to DNA repair. The CSA and the CSB genes contain a typical helicase sequence. The CSB protein exhibits DNA dependent ATPase activity (Citterio et al. 1998). Transcription-coupled repair becomes essential when polymerase II is arrested at the site of DNA damage.

⇒ *ATM, the Protein Mutated in Ataxia Telangiectasia, is a Key Regulator in DNA Damage Signaling Cascades*

Ataxia telangiectasia (AT) is a disorder of autosomal recessive inheritance characterized by X-ray sensitivity, immunodeficiency, cancerproneness, cerebellar degeneration and premature aging of the skin. ATM is the gene-product found to be mutated in this disease (Savitsky et al. 1995). The ATM protein belongs to a family of proteins that share similarities to the catalytic domain of phosphatidylinositol 3-kinases at their C-terminus. ATM is a regulator of several cascades signaling DNA strand breaks and thereby activating cell cycle checkpoints, DNA repair and apoptosis (Rotman and Shiloh 1998). Yeast ATM homologues have been helpful in the elucidation of the protein functions. Thus, the homologue MEC1 acts as a phosphokinase, a highly conserved single-stranded DNA-binding protein, that phosphorylates the replication protein A (REP A), cell-cycle dependent and in response to radiation. Because AT cells are delayed in phosphorylation of REP A, the function of ATM may be similar to that of MEC1 (Brush et al. 1998). The ATM protein family has further been reported to be involved in telomere length regulation. In AT cells, however, this function could not be demonstrated unequivocally. Recently, yeast mutants could be cloned that were defective in two ATM homologues. All chromosomes in these cells were circular and lacked telomeric sequences, indicating the involvement of ATM in telomere stability (Naito et al.1998).

⇒ *Werner Syndrome Patients have mutations in a Protein that Belongs to the RecQ Helicase Subfamily*

Werner syndrome has been reviewed in numerous reports (for example: Epstein et al. 1966; Herd et al. 1993). It is the most prominent premature aging syndrome and the elaboration of the underlying defect has been in progress since the Werner gene was cloned in 1996 by Yu et al.

The cDNA encodes a protein (WRN) 1432 amino acids in length, with a significant similarity to DNA helicases belonging to the RecQ subfamily. Besides WRNp, Escherichia coli RecQ, human RecQL, Saccharomyces cerevisiae SGS1p, Schizosaccharomyces pombe Rqh1p and the Bloom`s syndrome protein BLM all exhibit a high homology in the seven helicase domains of these proteins. (see: Lombard and Guarente 1996; Oshima et al. 1996; Yu et al. 1997). Many mutations have been found in patients with Werner syndrome, not only in the central helicase region but distributed all over the gene. (Yu et al. 1996; Oshima et a.l. 1996; Yu et al. 1997; Goto et a. 1997, Meißlitzer et al. 1997). Like other DNA helicases, WRNp was found exclusively in the nucleus, as could be shown by fluorescence microscopy

and a nuclear localisation signal was detected close to the C-terminus by site-directed mutagenesis (Matsumoto et al. 1997).

Helicases are involved in unwinding of double-stranded DNA in order to create a single-stranded template for such vital processes as replication, transcription, recombination and DNA repair (Tuteja and Tuteja 1996). The question as to what special function the WRN helicase may serve may be answerable by the observation of homologous proteins in other systems which mutate and by consideration of the most striking features of the Werner syndrome phenotype.

2.4.3 Functions of Proteins of the RecQ Subfamiliy Indicate the Involvement of WRN Helicase in the Prevention of DNA Destabilization

2.4.3.1 The Wildtype Proteins Mutated in Werner Syndrome and Bloom`s Syndrome Prevent Hyperrecombination

Werner syndrome cells exhibit chromosomal instability and spontaneous hypermutability. This "mutator" phenotype is characterized by unusually large deletions. (Fukuchi et al. 1989; Monnat Jr. 1992). These deletions are not the result of inefficient but of inaccurate ligation as was shown by an in vivo DNA ligation assay in which linearized plasmid DNA was transfected into WS cells and into those from normal donors. The recovered plasmid DNA was tested for circularization and mutations in bacteria. It became obvious that passage through WS host cells raised the mutation frequency significantly due to an inaccurate ligation (Rünger 1994). This tendency for nonhomologous or illegitime recombination demonstrated in Werner syndrome cells is also seen in yeast SGS1p mutants (Watt et al. 1996). SGS1 also belongs to the RecQ subfamily with a helicase domain homologous to the human WRN and BLM proteins (Ellis et al. 1995).

BLM is the protein mutated in Bloom`s syndrome, an inherited disease characterized by a high incidence of sister-chromatid exchanges in somatic cells due to an increased rate of somatic recombination. Homozygotes for mutations of the BLM protein have a "mutator" phenotype with mutations all over the genome, a great number of breaks, and interchanges between homologous chromosomes. This DNA instability gives rise to an unusual cancerproneness. That all three proteins (SGS1,WRN,BLM) participate in the maintainance of genome stability by preventing hyperrecombination and illegitime recombination could be demonstrated by Yamagata et al. (1998). CDNAs coding for wildtype proteins BLM and WRN, respectively, were introduced into yeast strains mutated in the SGS1 protein. With the aid of plasmids recombination was measured in these constructs and compared

to SGS1 mutant strains. The genome stabilizing effect of BLM and WRN could be verified.

2.4.3.2 The WRNp May be Involved in DNA Repair Mechanisms

A high chromosomal instability has been found in Werner syndrome patients and, to a lesser extent, in heterozygote siblings (Weirich et al. 1996). This instability was, in contrast to earlier reports, inducible by X-irradiation (Meißlitzer et al. 1997) pointing to a repair deficiency of oxidative damages. These findings are in line with those from Gebhart et al. (1988) and Ogburn et al. (1997) who reported the hypersensitivity of Werner syndrome patients and carriers to 4-nitro-quinoline-1-oxide. Webb et al. (1996) found a repair deficiency for UV-induced thymine-dimers in lymphoblastoid WS cells but not in primary fibroblasts. Weirich-Schwaiger et al. (1994) had previously analysed the capacity of Werner syndrome fibroblasts to repair a transfected, extracellularly UV-damaged plasmid. This eukaryotic expression plasmid, carrying the bacterial chloramphenicol-acetyltransferase as reporter gene was less effectively repaired by WS than by normal cells. Deficiency in mismatch repair, on the other hand, was reported in lymphoblastoid but not in fibroblastoid cell lines (Bennett et al. 1997). The authors suspect that this discrepancy may reflect a cell- or tissue-type specific function of the WRNp. Taken together, these results provide evidence for a reduced repair capacity of WS cells and contradict the earlier reports arguing, that repair in WS cells is not impaired (Stefanini et al. 1988; Fujiwara et al. 1977).

A very recent report (Huang et al. 1998) analysed the non-helicase N-terminus of wildtype WRN and, using site-directed mutagenesis, found a 3'–5' exonuclease activity. This activity may serve repair functions.

2.4.3.3 WRNp May function on Telomeres and Nucleoli as Reported for Yeast SGS1p

Telomere instability seems to be an important mechanism for the induction of cellular senescence as soon as one telomere reaches a critical length (see 2.3.3). This shortening may be brought about by telomerase inhibition and by damage to telomeric DNA. Certain proteins are attached to telomere sequences, regulating the length by inhibiting telomerase (TRF1), protecting the ends (TRF2), and avoiding double strand breaks (Ku80). These proteins seem to be stringently controlled as was recently shown for tankyrase (Smith et al. 1998). This enzyme was shown in vitro to ADP-ribosylate TRF1, thereby reducing the protein`s ability to bind to telomeres, thus rendering telomer ends accessible for telomerase. Poly-ADP-ribosyl-transferase is an

enzyme type that posttranscriptionally modifies proteins and hereby seems to critically influence repair mechanisms (Schweiger et al. 1987; reviewed by Oei et al. 1998).

In Werner syndrome cells telomere shortening was expected to be accelerated. Abnormal telomere dynamics were actually found in those cells. WS strains showed both drastic shortening as well as lengthening of telomeres and they terminated their lifespans at a wider range of telomere length than normal cells did (Schulz et al. 1996; Tahar et al. 1997). This may indicate that in the absence of WRNp hyperrecombination is also active in telomeres. Non-homologous recombination may result in duplicated but also in deleted telomere regions such that critically shortened telomeres signal proliferation arrest prematurely.

Werner helicase has been localized to transcriptionally active nucleoli of replicating cells (Gray et al. 1998). Considering the situation in yeast (Sinclair et al. 1997), WRNp might be targeted to the nucleoli during replication and transcription of rDNA in order to prevent illegitime recombination of the highly repetitive DNA and/or to repair damaged rDNA to enable transcription. Also, as verified for yeast SGS1 helicase, WRN helicase may interact with topoisomerases (Lebel and Leder 1998), thereby influencing chromosomal condensation / decondensation and the faithful segregation of chromosomes. Segregation without prior disentanglement leads to chromosomal breaks and non-disjunctions (reviewed by Watt and Hichson 1994).

2.4.3.4 Werner Helicase May be an Integral Part of the "Replication Foci"

Besides DNA instability, the most striking feature of Werner syndrome cells in culture is their limited proliferation capacity and the prolongation of the S-phase of the cell-cycle. In fission yeast the helicase Rqh1+, a member of the RecQ subfamily with homologies to the Werner helicase, prevents hyperrecombination that otherwise would lead to irreversible S-phase arrest (Stewart et al 1997). Thus, rqh1 null cells became sensitive to DNA damage by UV- and X-irradiation. Overexpression of the wild-type gene also exhibited hypersensitivity to these agents associated with the inability to control and to regulate the exit from S-phase checkpoint. In summary, this protein appears to couple chromosomal integrity to cell cycle progression suggesting a similar function for WRNp (Davey et al. 1998).

DNA replication starts from many origins of replication in eukaryotic cells. These origins contain 300-1000 DNA loops and a number of proteins to form a replication center or focus. One of the main proteins involved is RP-A (review: Wold 1997) a single stranded DNA binding protein, that is localized at the foci before, during and after replication. This fact raised the idea,

that the foci may be stable cellular structures which are not only important for replication but also for DNA-repair and -recombination. Yan and Newport (1995) described a protein with focus-forming ability (FFA-1). Further research indicated, that FFA-1 is a stable component of the foci with binding sites for RP-A.

Surprisingly, Yan et al (1998) identified the FFA-1 protein from Xenopus laevis extracts to be a homologue of the Werner syndrome gene product. This protein does not only belong to the RecQ family due to its helicase domains and its ATPase activity but it also shows similarity in the N-terminus to RNaseD and exhibits 3`-5`exonuclease proofreading activity reminiscent of DNA-polymerase I from E. coli. Experiments characterizing the biochemical properties of WRN protein showed that the helicase activity was most extensively stimulated by human RP-A. (Shen et al. 1998)

Meanwhile, the promoter structure of the human WRN gene has been characterized. Investigations by Wang et al. (1998) revealed features of an housekeeping promoter with a dramatically reduced activity in WS cells. The results from Yamabe et al. (1998) give insight into some possible regulation that may allow preliminary speculations about the part the WRN protein plays in chromosomal instability, cell cycle progression and senescence: Regulatory SP1-elements are modulated by the tumor suppressor proteins retinoblastoma (Rb) and p53.

These tumor suppressor proteins have been also implicated in regulation of cellular senescence by Stein et al. (1990) and Shay et al. (1991). P53 signals single-stranded breaks and induces the production of the proliferation inhibitor Sdi1 which inhibits phosphorylation of Rb. The unphosphorylated Rb, on his part, acts by cell cycle inhibition.

3 Conclusion

Chromosomal instability resulting from endogeneous and exogeneous DNA damaging agents, telomere shortening or illegitime recombination accumulate with age. Numerous repair mechanisms are provided to maintain genomic integrity. However, repair capacity decays during the life span of an individual depending on the excess of injuries accumulated, the genetically determined personal repair capacity and the amount of damage to genes coding for enzymes involved in repair processes. By means of cell cycle retardation cells acquire time for repair. Checkpoints in the cell cycle and inhibitors of proliferation like Sdi1 (Noda et al. 1994) are set up to arrest the cycle until all damages are removed, thereby avoiding replication of unrepaired chromosomal defects that would give rise to mutations and cancer. The age-related decay of repair capacities, however, entails the accumulation

of more and more injuries. No recovery of the cell cycle occurs and finally the cell dies.

Human premature aging syndromes are model systems to learn about mechanisms of normal aging. Werner syndrome is an inherited disease in which the genetic basis has been recently elucidated. Considering all results obtained until now, it appears as if the WRN protein would link the damage signal in the cell to the cell cycle to induce repair in order to avoid premature senescence.

Evidence from this review may favor the following model:
The WRN protein resides at the replication foci (like FFA-1) where it binds topoisomerases and generates binding sites for phosphorylated RP-A. RP-A phosphorylation is mediated by DNA damage that is recognized by p53. P53 interacts with RP-A thereby leading RP-A to the damage-site. RP-A, however, interacts with WRN as it does in Xenopus with FFA-1 and is attracted to the foci where it activates DNA helicases like WRN and topoisomerases, stimulating unwinding of double-stranded DNA and binding and stabilizing the single-stranded DNA exposed by WRN helicase. P53 initiates activation of Sdi1 that inhibits DNA synthesis during repair and inhibits phosphorylation of Rb. The unphosphorylated Rb also blocks the cell cycle. Repair enzymes are assembled by RP-A interaction. WRN exonuclease exerts proofreading activity. RP-A and WRN prevent illegitime recombination of unwound DNA. This is most important at repetitive DNAs such as rDNA, telomeres and streches of nucleotid repeats. WRNp, positioned at the site of replication, releases S-phase arrest if repair was successful. Rb becomes phosphorylated again and stimulates the expression of WRNp. If, however, defects remain unrepaired, p53 reduces the expression of WRNp drastically. Lack of WRN helicase raises the amount of illegitime recombination and finally makes S-phase arrest irreversible.

The elucidation of the gene-product responsible for features of premature senescence in Werner syndrome and the preliminary knowledge of the function of WRN protein emphazise the importance of DNA instability for aging. Unrepaired lesions may signal senescence by reducing WRN protein expression. As a result, hyperrecombination and a "mutator" phenotyp are promoted as is demonstrated in WRNp deficiency. Incorrect chromatin condensation retards cell-cycle progression, fascilitating further mutations. The replicative potential declines and the loss of cellular function gradually entails loss of function of the organism, a characteristic feature in the process of aging.

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