Alexzander A. A. Asea
Ian R. Brown
Editors

Heat Shock Proteins Volume 3

Series Editors: Alexzander A.A. Asea · Stuart K. Calderwood

Heat Shock Proteins and the Brain: Implications for Neurodegenerative Diseases and Neuroprotection

Springer

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HEAT SHOCK PROTEINS

Volume 3

Series Editors:

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This book is dedicated to our children Colleen, Kitty and Heather (I.R.B.),
Alexzander Jr., Vanessa and Edwina (A.A.A.)

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PREFACE

With the prevalence of neurodegenerative diseases on the rise as average life expectancy increases, the hunt for effective treatments and preventive measures for these disorders is a pressing challenge. Neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis have been termed 'protein misfolding disorders' that are characterized by the neural accumulation of protein aggregates. Manipulation of the cellular stress response involving the induction of heat shock proteins offers a therapeutic strategy to counter conformational changes in neural proteins that trigger pathogenic cascades resulting in neurodegenerative diseases. Heat shock proteins are protein repair agents that provide a line of defense against misfolded, aggregation-prone proteins.

Heat Shock Proteins and the Brain: Implications for Neurodegenerative Diseases and Neuroprotection reviews current progress on neural heat shock proteins (HSP) in relation to neurodegenerative diseases (Part I), neuroprotection (Part II), extracellular HSP (Part III) and aging and control of life span (Part IV). Key basic and clinical research laboratories from major universities and hospitals around the world contribute chapters that review present research activity and importantly project the field into the future. The book is a must read for researchers, postdoctoral fellows and graduate students in the fields of Neuroscience, Neurodegenerative Diseases, Molecular Medicine, Aging, Physiology, Pharmacology and Pathology.

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PART I

HEAT SHOCK PROTEINS AND NEURODEGENERATIVE DISEASES

CHAPTER 1

CHAPTERONES AND POLYGLUTAMINE EXPANSION DISORDERS

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Abstract: Polyglutamine expansion disorders are caused by the misfolding of proteins with abnormally long polyglutamine regions. This misfolding produces toxicity and leads to the dysfunction and ultimately to the demise of neurons in affected individuals. The molecular basis for polyglutamine toxicity is unclear and the number and complexity of documented cellular pathways involved in polyglutamine expansion disorders is daunting. However, the use of effective experimental model systems is rapidly advancing our understanding of polyglutamine misfolding and the cellular factors that govern the related toxicity. Molecular chaperones, the central regulators of cellular protein quality control, improve polyglutamine misfolding and hence ameliorate polyglutamine toxicity. Additionally, polyglutamine expansion proteins overwhelm molecular chaperones and thereby reduce their capacity to execute protein quality control. Hence, dysfunctional cellular protein quality control presents a very basic and fundamental problem of protein misfolding and therefore of polyglutamine toxicity. Thus, the elucidation of the interplay between polyglutamine expansion proteins and molecular chaperones contributes profoundly to our understanding of polyglutamine expansion disorders and offers great promise for developing effective therapeutic strategies in the treatment of these devastating maladies

Keywords: Neurodegeneration; protein misfolding; protein aggregation; stress response; heat shock proteins; neuroprotection

INTRODUCTION

Nine different neurodegenerative diseases are caused by expansions of polyglutamine regions in different proteins: Huntington's disease (HD); the Spinocerebellar Ataxias 1, 2, 3, 6, 7, and 17 (SCA1, 2, 3, 6, 7 and 17); Spinal and Bulbar Muscular Atrophy (SBMA, also known as Kennedy's disease); and Dentatorupallidolysian Atrophy (DRPLA) (Cummings and Zoghbi, 2000; Zoghbi and Orr, 2000). In each of

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these disorders, polyglutamine expansions arise from genetic mutations that generate the expansion of repeated CAG codons (encoding the amino acid glutamine) in the genes encoding the disease-proteins. Except for their polyglutamine regions, the nine disease-proteins differ profoundly in amino acid composition, subcellular localization and function. Consequently, the nine polyglutamine expansion disorders present distinct pathologies and affect different neurons in different regions of the brain (Zoghbi and Orr, 2000; La Spada and Taylor, 2003; Cowan and Raymond, 2006). For example, in HD medial spiny neurons of the striatum are primarily, and most severely, damaged by the polyglutamine expanded protein huntingtin (Graveland et al., 1985; DiFiglia et al., 1997; Cowan and Raymond, 2006) whereas Purkinje cells of the cerebellum and brain stem neurons are most affected by a polyglutamine expanded ataxin-1 protein in SCA1 (Gilman et al., 1996).

In spite of these differences, all nine polyglutamine disorders share important basic features (Ordway et al., 1999; Zoghbi and Orr, 2000; Gatchel and Zoghbi, 2005). All of them are late onset diseases, i.e. they usually manifest in midlife, or even at advanced age, and they are progressive, possessing symptoms that increasingly worsen throughout the course of the disease. Most polyglutamine expansion disorders share a threshold of about 40 glutamines in the disease-proteins: individuals who express disease-proteins with polyglutamine expansions beneath this threshold do not develop the disease, while individuals harboring a polyglutamine expansion above this threshold will eventually develop the disease. Strikingly, in each of the nine polyglutamine expansion disorders, the longer the polyglutamine expansion is, the earlier is the disease onset and the more severe the disease-phenotype. Finally, for each disorders, inclusions formed by the polyglutamine expanded proteins are found in specific types of neurons (Ross et al., 1999). These shared features imply that – at least at a very fundamental level – all polyglutamine expansion disorders are based upon a common pathological mechanism.

Polyglutamine expansion disorders can be regarded as protein conformational, or better, protein misfolding diseases. This term indicates that the conversion of a protein from its functional, benign conformation into an aberrant (misfolded), toxic conformation constitutes the molecular basis for the diseases. While the conversion from a benign into a toxic protein is triggered by discrete genetic mutations resulting in polyglutamine expansions, several other common protein misfolding diseases of the nervous system, such as sporadic cases of Alzheimer's disease and Parkinson's disease, are not caused by single mutations. In these diseases, the conversion of disease-proteins from benign into toxic conformations is mostly triggered by stochastic protein misfolding events that are elicited by stressful environmental conditions and aging (Soto, 2001; Forman et al., 2004; Selkoe, 2004).

From a cell biological perspective, the conversion of a disease-protein from a benign into a toxic conformation in protein misfolding diseases can be attributed to the failure of cellular protein quality control (Sherman and Goldberg, 2001; Welch, 2004). Cellular protein quality control consists of two major branches. One branch facilitates the production and preservation of accurately folded and

functional proteins. This task is mainly carried out by molecular chaperones (Hartl and Hayer-Hartl, 2002; Young et al., 2004). This process generally occurs through the prevention of undesired interactions of hydrophobic domains with other proteins or folding intermediates (Hartl and Hayer-Hartl, 2002; Young et al., 2004). This is an essential task in the highly crowded molecular environment of cells with concentrations of 50 to even 250 mg of protein per ml. In addition, protein remodeling factors, can refold proteins that have attained an aberrant conformation resulting from harsh environmental conditions, such as a heat shock (Weibezahn et al., 2004). The second major branch of protein quality control degrades damaged and misfolded proteins and therefore averts their deleterious functions. This task is carried out by the ubiquitin-proteasome system (UPS) and lysosome-mediated autophagy (Rubinsztein, 2006).

Numerous pathological and experimental findings clearly document the tight connection between cellular protein quality control and polyglutamine expansion disorders (Sherman and Goldberg, 2001; Muchowski, 2002; Barral et al., 2004; Hinault et al., 2006). Here, I describe research on the role of molecular chaperones in polyglutamine expansion diseases. Specifically, I will focus on the work carried out in experimental model systems (e.g. cultured cells, yeast, worms, flies, zebra fish and mice) because this work has allowed deep insights into both the mechanistic interplay between chaperones and polyglutamine expanded proteins, and its cellular consequences. The factors involved in protein quality control, including molecular chaperones, are mostly conserved between these model organisms and human neurons. Hence, the experiments in these models not only reveal basic molecular mechanism of polyglutamine expansion disorders, but they also elucidate fascinating aspects of the basic cell biology of protein quality control. Further, manipulating the interaction of polyglutamine expansion proteins with molecular chaperones presents a promising therapeutic strategy for the treatment of polyglutamine expansion disorders and protein misfolding disorders in general – because it targets a universal and basic molecular feature of each of these maladies (Muchowski and Wacker, 2005; Herbst and Wanker, 2006).

POLYGLUTAMINE TOXICITY – A DAUNTING PROBLEM

Although substantial progress in the understanding of polyglutamine expansion disorders has been made in the past ten years, the molecular basis for polyglutamine toxicity still remains an enigma. Alterations in several different cellular pathways, including gene transcription (Okazawa, 2003; Helmlinger et al., 2006), intracellular trafficking (Feany and La Spada, 2003; Gunawardena and Goldstein, 2005; Morfini et al., 2005), and mitochondrial function (Beal, 1998; Sawa, 2001) have been documented to be involved in polyglutamine expansion disorders.

However, at this point it is unclear whether any of these features initiate the diseases or merely represent downstream effects of cells expressing polyglutamine expansion proteins. It is quite possible that specific combinations of dysfunctional cellular pathways are responsible for different pathologies. To add even more

complexity to the problem, we must consider that in each of the nine polyglutamine expansion disorders, the polyglutamine expansions are imbedded in different proteins with different functions (La Spada and Taylor, 2003). Consequently, we should assume that specific cellular pathways are affected to different degrees in each polyglutamine expansion disorder.

From a structural viewpoint the understanding of polyglutamine toxicity has also reached a high level of intricacy. Traditionally, large insoluble protein aggregates (called inclusions) in the cytosol or nuclei of neurons of affected individuals were regarded as the toxic protein species (Ross et al., 1999; Rubinsztein et al., 1999). Lately, this concept has been challenged by several enticing experimental results that strongly correlate the presence of smaller, more soluble polyglutamine protein species (often referred to as oligomers), with cytotoxicity (Klement et al., 1998; Saudou et al., 1998; Kayed et al., 2003; Glabe and Kayed, 2006). Consequently, the function of large polyglutamine inclusions has been reconsidered to represent either a benign epiphenomenon or even a protective protein species.

Yet no experimental evidence exists to rule out the possibility that the presence of different polyglutamine expansion protein species have different consequences at different stages of the disease. For instance, large protein inclusions might initially serve a cytoprotective role by disfavoring the formation of highly toxic oligomers. However, at a later stage, the inclusions might grow too large, thereby sequestering too many essential proteins causing the large inclusions to become very toxic. Further, oligomers might differentially cause toxicity in a subset of cell types while big inclusions are toxic in different cell types. Again, this feature could be very distinct in each of the nine polyglutamine expansion disorders. This means that a certain protein species, say an oligomer, that has been documented to be toxic in the context of one specific polyglutamine expansion protein, might not be toxic in the context of a different polyglutamine expansion protein.

On the whole, the complexity of the current experimental data regarding polyglutamine toxicity seems daunting. Yet we should keep in mind that at the heart of all nine polyglutamine expansion disorders lie the expansions of a polyglutamine regions in proteins that promotes their misfolding. This common misfolding most likely precedes other structural conversions and all deleterious cellular consequences. Thus, protein quality control – as carried out by molecular chaperones – can be viewed as the first cellular line of defense against polyglutamine toxicity. Consequently, the challenge to protein quality control presented by a polyglutamine expansion protein might very well be the only molecular pathological commonality of all nine polyglutamine expansion diseases and might therefore explain their pathological similarities.

POLYGLUTAMINE EXPANSION PROTEINS TIP THE BALANCE OF CELLULAR PROTEIN QUALITY CONTROL

Work by Morimoto and coworkers illustrates a very fundamental and enticing aspect of how polyglutamine expansion proteins disrupt cellular protein control (Gidalevitz et al., 2006). In their experiments the authors use worms (*Caenorhabditis elegans*)

that harbor conditional mutations in several different genes. These mutations lead to the expression of metastable proteins: at lower (permissive) temperatures the expression of these metastable proteins does not have any phenotypic consequences while at higher (restrictive) temperatures, the metastable proteins misfold and hence cannot perform their cellular functions. This loss of function can be measured easily by the different phenotypes that the mutant animals exhibit.

Remarkably, when these mutant worms express polyglutamine expansion proteins, the mutant phenotypes are revealed even at the permissive temperature. This finding indicates that the presence of the polyglutamine expansion proteins uncovers the metastability of the mutant proteins. Notably, numerous conditional mutants in unrelated proteins that function in distinct cellular pathways are affected by the expression of the polyglutamine expansion protein. Thus, the polyglutamine expansion protein does not seem to interfere with specific cellular pathways. Rather, it weakens protein quality control in a general, basic manner. In consequence, metastable proteins misfold and lose their activity.

The authors conclude that polyglutamine expansion proteins can uncover latent phenotypes of protein folding mutations that are not detected in the absence of a polyglutamine expansion protein. These findings may explain the variability in the progression and the varying age of onset in the polyglutamine expansion disorders. Different individuals possess polymorphic genomes: some of these polymorphisms may elicit protein folding defects. These polymorphisms, combined with the expression of a polyglutamine expanded protein, cause variations in disease phenotypes. The progressive, age-dependent nature of polyglutamine disease can be interpreted in a similar manner: damaged proteins with the propensity to misfold accumulate throughout the course of aging. In the presence of polyglutamine expansion proteins, the accumulation of misfolded proteins increases and enhances toxicity. This combination of age-damaged proteins and polyglutamine expansion proteins results in the amplification of polyglutamine toxicity during aging.

POLYGLUTAMINE EXPANDED PROTEINS WEAKEN THE CELLULAR STRESS RESPONSE IN A BASIC MANNER

Cellular viability is frequently challenged by harmful environmental conditions, commonly referred to as stress. These stressful conditions include the exposure to elevated temperatures, i.e. a heat shock. Cells react to a heat shock via the activation of a highly conserved and universal cellular program, the heat shock response (Lindquist, 1986). The heat shock response comprises the expression of a specific set of proteins, the heat shock proteins, which overlap to a large extend with molecular chaperones. Molecular chaperones balance the defects in protein folding that occur in response to a heat shock. Importantly, some proteins that are activated in order to cope with a heat shock, are also essential components of other stress responses, such as the response to oxidative stress (Cotto and Morimoto, 1999). The inability of neurons expressing polyglutamine expansion protein to respond

adequately to a heat shock or other related stress conditions has been speculated to be an important contributor to polyglutamine toxicity.

Indeed, the expression of polyglutamine expansion proteins sensitizes cells to a heat shock (Wyttenbach et al., 2000). Exposure to elevated temperatures or to the proteasome inhibitor lactacystin, a potent inducer of the heat shock response, enhances the aggregation of polyglutamine expansion protein when compared to untreated cells.

Further, Cowan et al. demonstrate that cultured animal cells expressing polyglutamine expansion proteins are more likely to die following a heat shock and other stressors (Cowan et al., 2003). Strikingly, under the stress conditions tested, cells expressing proteins with a short polyglutamine region showed hardly any effects. In contrast, under the same stress conditions, cells expressing polyglutamine expansion proteins display a much greater rate of survival than cells expressing polyglutamine expansion proteins. The reduced viability of cells expressing polyglutamine expansion proteins correlates with a redistribution of polyglutamine inclusions from the cytosol into the nucleus after a heat shock. Also, in cells expressing polyglutamine expansion proteins, the activation of the molecular chaperone Hsp70 (a central molecular chaperone, see below) is delayed when compared to cells expressing a control protein with a short polyglutamine region. In a mouse model for polyglutamine toxicity, the levels of several molecular chaperones in the brain are notably reduced when compared to wild-type animals (Hay et al., 2004). Intriguingly, the polyglutamine-dependent reduction in molecular chaperones is not due to a block in their expression, rather the chaperones are mislocalized to nuclear polyglutamine inclusions.

Similarly, using a fly model, Huen et al. present evidence for a dynamic decline in the expression of molecular chaperones in the presence of polyglutamine expansion proteins (Huen and Chan, 2005). While levels of Hsp70 are increased in early stages of development in flies expressing polyglutamine expansion proteins, Hsp70 expression declines during ongoing development, and specifically during aging. The same study demonstrates that flies expressing a polyglutamine expansion protein are not able to activate the expression of stress-regulated version of Hsp70, indicating a polyglutamine expansion-mediated block in Hsp70 activation.

In lymphoblastoid cells from SCA7 patients carrying ataxin-7 with long polyglutamine expansions, the levels of Hsp27 (a small heat shock protein, see below) and Hsp70 are significantly reduced (Tsai et al., 2005). Interestingly, this reduction seems quite specific to Hsp27 and Hsp70 because the levels of other chaperones remain unaltered.

Tagawa et al. provide evidence that Hsp70 is differentially regulated in different types of neurons in response to the expression of polyglutamine expansion proteins (Tagawa et al., 2007). Specifically, neurons expressing polyglutamine expanded ataxin-1 that is most severely affected in SCA1 show lower Hsp70 levels. In contrast, the neurons that are insensitive to the expression of polyglutamine expanded ataxin-1 display elevated Hsp70 levels. Similar results are observed for polyglutamine expanded huntingtin in different neuronal cell types in HD. These results

demonstrate the strong negative correlation in the degree of polyglutamine toxicity and the expression level of Hsp70: the more Hsp70 that is present in cells expressing polyglutamine expansion proteins the less toxic they are. These results also begin to explain the cell-type specific effects of different polyglutamine expansion proteins in distinct neuronal cell types in each of the nine polyglutamine expansion disorders.

In summary, the reports presented above attest to the fact that cells expressing toxic polyglutamine expansion proteins display reduced chaperone activity and hence an impaired stress response. Importantly, neurons are frequently exposed to stress conditions as part of the normal physiology and even more so throughout the course of aging. Thus, impaired stress management in distinct neurons expressing polyglutamine expansion proteins might be an important contributor to polyglutamine toxicity.

In the following sections I will discuss the interplay between specific molecular chaperones or protein remodeling factors and polyglutamine expansion disorders.

HSP70S AND HSP40S SOLUBILZE POLYGLUTAMINE EXPANSION PROTEINS AND REDUCE THEIR TOXICITY

Molecular chaperones of the Hsp70 class are central players in protein quality control. They are involved in a plethora of different cellular functions, including co-translational protein folding, protein transport, protein disaggregation, and protein degradation (Hartl and Hayer-Hartl, 2002). Hsp70 consists of an amino-terminal ATPase domain and a carboxy-terminal substrate-binding domain. The identity of the nucleotide that is bound to Hsp70 determines its substrate interactions: in the ATP-bound state substrates interchange rapidly while in the ADP-bound state substrates are tightly bound to Hsp70 (Hendrick and Hartl, 1995).

Hsp40s function as co-chaperones for the Hsp70s (Kelley, 1998). Hsp40s contain a conserved amino-terminal J-domain that activates ATPase hydrolysis of Hsp70s (Hartl and Hayer-Hartl, 2002). The other domain(s) of Hsp40s are not as well conserved as the J-domain but generally functions in substrate recognition and specification (Hennessy et al., 2005).

Together, Hsp70 and Hsp40 engage in a substrate binding cycle: the protein folding substrate is first recognized and bound by Hsp40. This Hsp40:substrate complex then closely interacts with an ATP-bound Hsp70. Hsp40 stimulates ATP-hydrolysis and departs from the Hsp70-substrate complex. After nucleotide exchange, the substrate is released from Hsp70. Sometimes the substrate leaves the cycle as a completely folded protein, but it may also re-enter another cycle of Hsp40 and Hsp70 to obtain a more complete folding status. Alternatively, a partially folded substrate that exits the Hsp40–Hsp70 cycle can engage in folding reactions performed by yet other molecular chaperones (Bukau et al., 2006).

Numerous studies in diverse experimental models have documented the colocalization of Hsp70 and Hsp40 with inclusions formed by different polyglutamine expansion proteins, including ataxin-3 (Warrick et al., 1998, 1999; Chai et al., 1999; Chan et al., 2000), huntingtin-fragments (Jana et al., 2000; Chuang et al., 2002;

Schiffer et al., 2007), ataxin-1 (Cummings et al., 1998) and the androgen receptor (Stenoien et al., 1999; Kobayashi et al., 2000; Bailey et al., 2002; Adachi et al., 2003). This might suggest that Hsp70 and Hsp40 recognize the polyglutamine expansions as binding substrates – regardless of their flanking amino acid sequences. However, this colocalization does not reveal the functional relation of Hsp70/Hsp40 and polyglutamine expansion proteins because – as discussed above – the role of inclusions in polyglutamine expansion disorders remains unclear.

Strong evidence for a beneficial role of Hsp70/Hsp40 in polyglutamine expansion disorders comes from genetic experiments carried out in such diverse model organisms as flies (*Drosophila melanogaster*) and mice for polyglutamine toxicity. In the fly model the polyglutamine expanded proteins are expressed in the fly eye where it causes a strong degenerative phenotype that can be easily scored (Bonini, 2001). The overexpression of Hsp40s and Hsp70 strongly suppresses this degenerative phenotype as detected in both unbiased genetic screens and directed experiments (Warrick et al., 1998; Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000). Hsp70 and Hsp40 suppress toxicity in fly models expressing different polyglutamine expansion proteins, again indicating that this suppression is independent of the amino acids flanking the polyglutamine expansions. The suppression of polyglutamine toxicity by Hsp70/Hsp40 in various other experimental systems, including mouse models, has been confirmed (Cummings et al., 1998; Chai et al., 1999; Stenoien et al., 1999; Chan et al., 2000; Jana et al., 2000; Kobayashi et al., 2000; Muchowski et al., 2000; Wyttenbach et al., 2000; Bailey et al., 2002; Chuang et al., 2002; Adachi et al., 2003; Hansson et al., 2003; Howarth et al., 2007; Schiffer et al., 2007). Thus, Hsp70 and Hsp40 suppress a common and very basic aspect of polyglutamine toxicity. It is noteworthy that a study by Howarth et al. provides evidence that overexpression of Hsp40 reduces inclusion formation by polyglutamine expanded androgen receptor in tissue culture models of SBMA (Howarth et al., 2007). Notably, in this case, Hsp40 promoted the UPS-dependent degradation of the polyglutamine expanded androgen receptor.

Amazingly, the overexpression of Hsp70 and Hsp40 does not alter the microscopic appearance of the inclusions formed by the polyglutamine expansion proteins in the nuclei of the fly cells (Warrick et al., 1998). In light of this observation we must ask what mechanism underlies the Hsp70/Hsp40- mediated suppression of polyglutamine toxicity. Experiments using purified proteins in vitro and in a yeast (*Saccharomyces cerevisiae*) model for polyglutamine aggregation and toxicity have started to answer this question (Muchowski et al., 2000). In vitro, purified polyglutamine expansion proteins spontaneously form highly insoluble protein aggregates, termed amyloids, that appear as fibers when observed under an electron microscope. In the presence of Hsp70 and Hsp40 amyloid fiber formation is strongly inhibited. Notably, the combined action of Hsp70 with Hsp40 antagonizes amyloid fiber formation much more effectively then either chaperone alone.

In yeast, the expression of polyglutamine expansion proteins causes polyglutamine-length dependent formation of insoluble aggregates (Krobitsch and Lindquist, 2000). This aggregate formation can be followed either by microscopy

or by biochemical methods. The co-expression of either Hsp70 or Hsp40 with the polyglutamine expansion protein in yeast reduces the formation of these insoluble aggregates. In contrast, when inspected by immunofluorescence microscopy, the inclusions formed by the polyglutamine expansion protein are not converted into diffusely localized proteins by Hsp70 or Hsp40 overexpression. Rather, the morphology of the aggregates is altered (Muchowski et al., 2000). Wacker et al. analyze this Hsp70/Hsp40 induced alteration of aggregates in great structural detail and find that these chaperones partition monomers of polyglutamine expansion proteins and thereby antagonize the formation of spherical and annular oligomers which might present highly toxic polyglutamine protein species (Wacker et al., 2004).

Together, these results indicate that the molecular chaperones Hsp70 and Hsp40 can antagonize polyglutamine toxicity by inhibiting the formation of a toxic protein conformation. These experiments also document that it can be very important to employ different experimental methods to elucidate mechanistic aspects of the aggregation of polyglutamine expansion proteins by different methods: proteins aggregates that look identical under the light microscope can reveal a very distinct nature when analyzed by biophysical methods. However, it will be very important to reproduce the *in vitro* results presented above in a cellular system for polyglutamine aggregation to assure relevance for the disease.

Experiments performed by Schaffar et al. suggest a different mechanism for how Hsp70 and Hsp40 antagonize polyglutamine toxicity (Schaffar et al., 2004). In their experimental system, the basis for polyglutamine toxicity is the sequestration of an essential protein (a major transcription factor) into aggregates formed by polyglutamine expanded proteins. They show that Hsp70/Hsp40 can prevent this toxic sequestration thereby restoring viability.

Importantly, the two different modes for the beneficial effects of Hsp70 and Hsp40 on polyglutamine toxicity could be complementary: Hsp70 and Hsp40 might alter the conformation of polyglutamine expansion proteins, resulting in differently structured proteins that do not engage in toxic protein interactions. Alternatively, Hsp70 and Hsp40 might prevent polyglutamine toxicity by altering aggregation and preventing toxic protein interactions in an additive manner.

CHIP PROMOTES THE DEGRADATION OF POLYGLUTAMINE EXPANSION PROTEINS

The protein CHIP (Carboxy terminus of Hsc70 Interacting Protein) connects the activity of the molecular chaperones Hsp70 and Hsp40 with protein degradation mediated by the ubiquitin-proteasome systems (UPS). CHIP is characterized by three functional domains: an amino-terminal tetratricopeptide (TPR) domain that mediates protein–protein interactions, especially interactions between several molecular chaperones (McDonough and Patterson, 2003); a carboxy-terminal U-Box domain that provides E3 ubiquitin ligase activity and thereby facilitates the ubiquitination and ensuing degradation of target proteins; and an intervening charged

domain. The fact that CHIP contains both a domain usually found in molecular chaperones (the TPR domain) and a domain typical for members of the UPS (the U-box domain) places it is at the crossroad of these two different branches of protein quality control. Indeed, CHIP passes protein substrates from the folding cycle of Hsp70/Hsp40 to a pathway of ubiquitination and proteasomal degradation (McDonough and Patterson, 2003).

Four different studies provide convincing experimental evidence that CHIP antagonizes polyglutamine toxicity (Jana et al., 2005; Miller et al., 2005; Al-Ramahi et al., 2006; Choi et al., 2007). CHIP co-localizes with aggregates formed by polyglutamine expanded proteins in tissue culture models. CHIP overexpression solubilizes polyglutamine aggregates as observed by light microscopy. Moreover, overexpressed CHIP facilitates the degradation of polyglutamine expansion proteins which correlates with reduced polyglutamine toxicity in cultured cells and in a zebra fish model (Miller et al., 2005). Importantly, both the TPR and the U-box domain are indispensable for CHIP-mediated reduction in polyglutamine toxicity. It also appears that CHIP haploinsufficiency enhances polyglutamine toxicity: CHIP+/− mice display increased inclusion formation and enhanced toxicity (Miller et al., 2005).

The important role of CHIP in polyglutamine expansion toxicity is confirmed in a fly model expressing a polyglutamine expanded ataxin-1 protein (Choi et al., 2007). This indicates that – like Hsp70/Hsp40 – CHIP most likely interacts with polyglutamine expansion proteins and antagonizes their toxicity – regardless of their overall protein context. Notably, CHIP expression is induced in cultured cells expressing polyglutamine expanded ataxin-3, indicating that CHIP might be a part of the naturally occurring cellular defense systems against polyglutamine toxicity (Dikshit and Jana, 2007).

TRIC – A CHAPERONIN DEFIES POLYGLUTAMINE TOXICITY

TRiC (TCP-1 Ring Complex, also termed CCT) is a member of a class of molecular chaperones called chaperonins (Spiess et al., 2004). Chaperonins are large multi-domain protein complexes with a cylindrical shape and a central cavity. Protein substrates bind to TRiC in this central cavity during a folding cycle that is governed by ATP binding and hydrolysis. TRiC facilitates the folding of a very specific suite of substrate proteins, including actin and tubulin (Spiess et al., 2004). Curiously, TRiC substrates are not able to obtain their accurate conformation through the assistance of other chaperones, which makes TRiC an essential component of the protein folding machinery. This substrate specificity also implies a unique folding mechanism that cannot be carried out by other chaperones.

The first evidence for the interaction of polyglutamine expanded proteins with TRiC came from a screen for modifiers of polyglutamine aggregation and toxicity in worms (*C. elegans*) (Nollen et al., 2004). In this screen RNAi-mediated knock down of the expression of six different genes encoding chaperonin proteins were identified as enhancers of polyglutamine aggregation and toxicity.

In 2006 three publications independently documented that the TRiC alters the aggregation of polyglutamine expansion proteins and thereby antagonizes polyglutamine toxicity (Tam et al., 2006). In these studies yeast and/or tissue culture models for polyglutamine aggregation and toxicity and in vitro experiments with purified proteins have been exploited to investigate the role of TRiC in polyglutamine aggregation and toxicity. Collectively, these studies show that the depletion of TRiC enhances polyglutamine aggregation and toxicity. Notably, using fluorescence correlation spectroscopy (FCS) and sucrose gradient fractionation, Kitamura et al. illustrate that TRiC depletion increased the amount of a more soluble, oligomeric species of polyglutamine expansion proteins (Kitamura et al., 2006). Presumably, this soluble polyglutamine oligomer presents a highly toxic species. Further, all three studies document that TRiC overexpression decreases the formation of insoluble polyglutamine inclusions and oligomers as well as polyglutamine toxicity. Specific subunits of TRiC are more effective in this regard than others. Importantly, using size-exclusion chromatography, Behrends et al. demonstrate that TRiC overexpression reduces the fraction of soluble polyglutamine oligomers (Behrends et al., 2006). These authors also provide evidence that TRiC teams up with Hsp70s and Hsp40s to modify polyglutamine aggregation and toxicity.

In summary, TRiC reduces the aggregation and toxicity of polyglutamine expanded proteins. It is crucial to emphasize that the studies described above identify an oligomeric yet soluble protein species formed by polyglutamine expanded proteins that might present highly toxic conformations. Only a wide variety of experiments, including fluorescence microscopy techniques and biophysical methods, have allowed the identification of this oligomeric species.

HSP104 – DISAGGREGATING POLYGLUTAMINE EXPANSION PROTEINS WITH A YEAST PROTEIN REMODELING FACTOR

Hsp104 is a protein remodeling factor from the yeast *S. cerevisiae* which belongs to the Clp/Hsp100 family of AAA+ proteins (ATPase Associated with a variety of cellular Activities (Weibezahn et al., 2004)). Hsp104 is dispensable for the life of yeast cells under normal growth conditions, however yeast cells that harbor deletions of Hsp104 have strongly reduced survival rates following a heat shock (Glover and Tkach, 2001). Hsp104 possesses the exceptional ability to dissolve protein aggregates in the yeast cytosol and nucleus such as those produced following a thermal insult (Parsell et al., 1994). Hsp104 plays a crucial role in the maintenance and propagation of yeast prions, which present protein conformation-based entities of inheritance (Tuite and Lindquist, 1996). In its active state Hsp104 is assembled into a two-tired, cylindrical hexamer. Cycles of ATP hydrolysis promote Hsp104-dependent protein refolding through an incompletely understood mechanism. No close homologues of Hsp104 have been identified in animal cells.

Morimoto and co-workers demonstrate that Hsp104 antagonizes inclusion formation of a polyglutamine expanded protein in *C. elegans* (Satyal et al., 2000). Hsp104 also reduces toxic phenotypes associated with the expression of polyglutamine expansion proteins in this model. Using a yeast model, Cashikar et al. show that Hsp104 potently solubilzes SDS-resistant aggregates formed by a polyglutamine expanded huntingtin fragment which also strongly reduces its toxicity (Cashikar et al., 2005).

These results were recapitulated in cultured cells, including mouse and rat models for polyglutamine expansion protein associated aggregation and toxicity (Carmichael et al., 2000; Vacher et al., 2005; Perrin et al., 2007). Curiously, behavioral analyses of transgenic mice do not provide evidence that coexpression of Hsp104 with a polyglutamine expanded fragments of huntingtin ameliorates behavioral phenotypes and there is no change in weight loss (Vacher et al., 2005). However, Hsp104 prolongs the lifespan of mice expressing a polyglutamine expanded huntingtin fragment and antagonizes inclusion formation. Intriguingly, in a rat model, Hsp104 did not reduce the number of microscopically detectable inclusions (Perrin et al., 2007). Rather, the inclusions occur more frequently yet with an altered morphology with a hitherto elusive biophysical nature.

Experiments in yeast show that Hsp104 antagonizes polyglutamine aggregation and toxicity most effectively in collaboration with small heat shock proteins (sHsps, see below) indicating a close functional connection between two different chaperone families (Cashikar et al., 2005). Further, the combined expression of polyglutamine expanded huntingtin fragments with Hsp104 increases the levels of endogenous sHsps and Hsp70 in rat brains (Perrin et al., 2007). These results place Hsp104 in an interaction network with other molecular chaperones that antagonizes polyglutamine toxicity.

TORSIN – A NOTORIOUS TROUBLE MAKER AMELIORATES POLYGLUTAMINE TOXICITY

Torsin is another member of the AAA+ protein family; torsin's regular cellular function remains poorly defined. Despite the lack of mechanistic insight, torsin is infamous for its role in human disease: mutations in torsin cause the most common form of the inherited movement disorder dystonia (Breakefield et al., 2001).

Intriguingly, a report by Caldwell et al. characterizes torsin in a much more benevolent role (Caldwell et al., 2003). Using a *C. elegans* model for polyglutamine aggregation and toxicity, their work shows that wild-type torsin can reduce the formation of inclusions and the toxicity of a polyglutamine expanded protein. Torsin might therefore play an important role in the cellular defense against toxic misfolded proteins. Thus, polyglutamine aggregation and toxicity can provide insight into the basic cellular functions of proteins with poorly described functions and thereby contribute to the understanding of diseases that are unrelated to polyglutamine expansion disorders.

VCP – A MULTIFUNCTIONAL PROTEIN GETS INVOLVED WITH POLYGLUTAMINE

VCP (also called p97) is not a canonical chaperone but certainly performs several protein remodeling functions (Dreveny et al., 2004). Like Hsp104 and torsin, VCP is a member of the AAA proteins and is active as a hexamer with cylindrical shape and a central cavity. VCP is involved in very diverse cellular processes including protein degradation, Golgi reassembly and spindle assembly for the exit from mitosis (Dreveny et al., 2004). A chaperone activity of the yeast homologue of VCP, CDC48, has also been reported (Thoms, 2002).

Hirabayashi et al. describe VCP as a polyglutamine interacting protein in a tissue culture model and in inclusion formed in brains of patients suffering from HD (Hirabayashi et al., 2001). Work carried out in a *C. elegans* model shows that overexpression of VCP reduces polyglutamine inclusion formation (Yamanaka et al., 2004). Also, Kobayashi et al. demonstrate that siRNA-mediated reduction in VCP levels diminishes the clearance of polyglutamine-aggregates in cultured cells (Kobayashi et al., 2007). Accordingly, VCP might antagonize polyglutamine aggregation and presumably also polyglutamine toxicity in a similar manner as its AAA-protein cousins Hsp104 and torsin.

The role of VCP in SCA3 is analyzed in two independent publications. One describes how wild-type ataxin-3 interacts with VCP during the degradation of ER-proteins (ERAD) (Zhong and Pittman, 2006). When ataxin-3 carries a polyglutamine expansion, its interaction with VCP is abnormally enhanced and the efficiency of ERAD is reduced. This work contributes greatly to our understanding of the regular cellular function of ataxin-3. It also implies that while mostly polyglutamine expansions are thought to be gain-of function mutations the loss of the regular cellular function of a polyglutamine expanded protein might also contribute to pathology.

The other publication on ataxin-3 and VCP focuses on the effect of VCP on the aggregation of polyglutamine expanded ataxin-3 (Boeddrich et al., 2006). The authors find that VCP interacts with ataxin-3 in human brains and in cultured cells. This interaction strictly depends on an arginine/lysine-rich motif in ataxin-3. Remarkably, VCP modulates the aggregation of polyglutamine expanded ataxin-3: low levels of VCP enhance aggregation whereas high VCP concentrations decrease aggregation. This effect on polyglutamine expanded ataxin-3 aggregation strongly correlates with its toxicity in a fly model. Overexpression of VCP markedly reduces toxic phenotypes associated with the expression of polyglutamine expanded ataxin-3.

SHSPS – DIRECT AND INDIRECT RESCUE FROM POLYGLUTAMINE TOXICITY

Small heat shock proteins comprise a huge and quite diverse group of molecular chaperones (Haslbeck et al., 2005). Most of them share a conserved alpha-crystallin domain, a small molecular mass (between 12 and 43 kDa), and the capacity to

form big oligomeric complexes. sHsps often have a flexible quaternary structure and their expression is typically only induced under stressful conditions. sHsps have the exceptional ability to bind to non-native substrate proteins and hence keep them in a folding-competent state. This averts irreversible protein aggregation and in concert with other molecular chaperones, such as Hsp70 and Hsp104, sHsps can facilitate the refolding of aggregated proteins. sHsps protect against oxidative stress, have anti-aging effects and perform neuroprotective functions (Sun and MacRae, 2005).

As mentioned before, sHsps – in concert with other chaperones – can solubilze polyglutamine expansion protein and reduce their toxicity. Yet Wyttenbach et al. describe how sHsps reduce polyglutamine toxicity by a different mechanism (Wyttenbach et al., 2002). Using a tissue culture models, the authors observe that sHsps antagonize polyglutamine toxicity without altering the microscopic appearance of aggregates. They determine that the expression of polyglutamine expanded huntingtin fragments induced oxidative stress. This is indicated by a steep increase in proteins that are damaged by reactive oxygen species (ROS) in cells expressing polyglutamine expanded huntingtin fragments. When sHsps are co-expressed with polyglutamine expanded huntingtin fragments, the levels of ROS-damaged proteins are reduced. Thus, sHsps protect from polyglutamine toxicity by reversing a downstream effect of the expression of polyglutamine expanded proteins, i.e. oxidative stress, rather than modulating the polyglutamine expansion protein.

Curiously, when tested in a mouse model, this sHsp-mediated suppression of polyglutamine toxicity is not recapitulated (Zourlidou et al., 2007). In the mouse model, sHsp overexpression did not alter any of the phenotypes associated with polyglutamine toxicity, including inclusion formation and polyglutamine-induced oxidative stress. Further experiments show that sHsps are found mainly in an inactive conformation and that they can not be activated by a heat shock in mice expressing a polyglutamine expanded huntingtin fragment. The authors conclude that sHsps might be very effective in acute models for polyglutamine toxicity, such as the tissue culture models, but they are ineffective in chronic disease models, such as their mouse model, with ongoing expression of the polyglutamine expansion protein.

These two studies on sHsps document the profound differences between different models of polyglutamine toxicity and the consequences these differences might have. These conceptual discrepancies should always be considered when interpreting the data generated in different models of polyglutamine toxicity, especially in regard to the relevance in the human diseases.

HSP90 – INHIBITING A REGULATOR OF CELL SIGNALING ANTAGONIZES POLYGLUTAMINE EXPANSION TOXICITY

The family of Hsp90 chaperones is well conserved from bacteria to man. Hsp90s are unusual in their substrate specificity: they interact with numerous proteins involved in cellular signal transduction such as hormone receptors and kinases. Unlike other

chaperones, Hsp90 interacts with its substrates when they are in a quasi-native conformation, i.e. very late in their folding process. A constitutive homodimer, Hsp90 very often functions in association with multiple other chaperones (Young et al., 2001).

A connection between Hsp90 and polyglutamine expansion protein associated inclusion formation is observed following Hsp90 inhibition. When cultured cells that express a polyglutamine expanded protein are treated with the Hsp90-inhibitor geldanamycin, inclusion formation is strongly reduced (Sittler et al., 2001). This effect can be attributed to the activation of the Hsp90-dependent transcription factor HSF, the heat shock factor. Hsp90 is constitutively bound to HSF and thereby maintains it in an inactive conformation. Following exposure to geldanamycin, Hsp90 is inhibited and it dissociates from HSF. HSF is activated and induces the transcription of heat shock proteins including numerous molecular chaperones (Young et al., 2001). The elevated levels of chaperones – including Hsp70s and Hsp40s – in cells treated with geldanamycin are proposed to solubilze the polyglutamine expansion protein.

An interesting case of a polyglutamine expansion protein and its relation to Hsp90 is presented by SBMA. Here, the polyglutamine expansion is located in the androgen receptor, which is a natural client of Hsp90. When a tissue culture model for SBMA is treated with the geldanamycin analogue, 17-AAG, the HSF-mediated expression of chaperones is potently induced (Waza et al., 2005). Moreover, the steady-state levels of both monomeric and aggregated polyglutamine expanded androgen receptor are significantly reduced. The authors further document that 17-AAG modulates Hsp90 function so that it can effectively and specifically promote the degradation of the polyglutamine expanded androgen receptor. Notably, the reduction in polyglutamine expanded androgen receptor levels following 17-AAG treatment correlates strongly with a reduction in polyglutamine-associated toxicity in a mouse model of SMBA.

A study by Thomas et al. suggests a somewhat different mechanism for how Hsp90 inhibition antagonizes the toxic accumulation of the polyglutamine expanded androgen receptor (Thomas et al., 2006). Following Hsp90 inhibition, the intracellular trafficking of the polyglutamine expanded androgen receptor is altered, which in turn enhances its degradation. In these experiments Hsp90 inhibition does not induce the expression of chaperones. Hence, the alteration in polyglutamine expanded androgen receptor trafficking and degradation does not depend on increased levels of chaperones.

The discrepancy between these two studies might be explained by different sensitivities of the respective models to the applied concentrations of Hsp90-inhibitors. However, the results might also indicate that different degrees of Hsp90-inhibtion can result in a different cellular response in regard to polyglutamine aggregation and toxicity. Thus, further experiments evaluating effective Hsp90 inhibitors are needed to discern between these two alternate explanations.

CHEMICAL CHAPERONES – TREHALOSE AMELIORATES POLYGLUTAMINE TOXICITY

Chemical chaperones are small molecules, such as certain amino acids and sugars that can stabilize proteins (Yancey, 2005). For instance, the sugar trehalose can protect yeast cells from stressful environmental conditions, including a heat shock or cold shock, by preventing permanent protein misfolding (Singer and Lindquist, 1998).

Trehalose was identified as a potent inhibitor of polyglutamine aggregation in a screen for molecules that inhibit the aggregation of a purified polyglutamine expanded protein in vitro (Tanaka et al., 2004). When trehalose is added to cells expressing polyglutamine expanded huntingtin fragments it reduces both its aggregation and toxicity. Likewise, in a mouse model expressing a polyglutamine expanded huntingtin fragment, trehalose antagonizes both aggregation and polyglutamine toxicity. These results indicate that chemical chaperones present a promising strategy to combat polyglutamine expansion disorders. The current challenge clearly is to identify chemicals that execute effective chaperoning functions even at therapeutically feasible concentrations.

CONCLUSION

All the experimental results discussed herein document that molecular chaperones have a remarkable capacity to antagonize polyglutamine toxicity. Nearly all known classes of molecular chaperones appear to elicit this beneficial impact on polyglutamine expansion proteins. Molecular chaperones generally seem to complete this task by converting polyglutamine expansion proteins from toxic to benign species. Notably, this action of molecular chaperones extends to different polyglutamine expansion proteins.

It also becomes obvious that polyglutamine expansion proteins produce neurotoxicity via impeding cellular protein quality control, specifically by reducing the efficiency of molecular chaperones to assist in protein folding. Future experiments will clarify what precise role that impaired protein quality control plays in the different polyglutamine expansion disorders.

Several studies are currently investigating the impact of molecular chaperones in protein misfolding diseases beyond the group of polyglutamine expansion disorders such as Parkinson's disease and Alzheimer's disease. It will be fascinating to learn whether and how molecular chaperones act in the context of these diseases and whether there are common unifying mechanisms in all protein misfolding diseases. Such mechanistic commonalities would potentially be of great benefit to devise effective therapeutic strategies to treat these horrible maladies that plague the increasingly aging population in our societies.

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CHAPTER 2

HEAT SHOCK PROTEINS, UNFOLDED PROTEIN RESPONSE CHAPERONES AND ALZHEIMER'S DISEASE

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Abstract: Molecular chaperones interact with cellular proteins to ensure proper folding and transport between or into organelles. They also associate with mature proteins that have unfolded (and become prone to aggregation) because of an environmental insult such as heat shock. There is a large body of evidence that protein quality control mechanisms involving the HSP family of molecular chaperones, as well as proteasomal and lysosomal functions, become impaired with aging and contribute to a variety of neurodegenerative diseases. Promising therapeutic approaches tested in animal models of Parkinson's and polyglutamine diseases include the up-regulation of molecular chaperones to prevent protein misfolding and aggregation and to facilitate clearance mechanisms. In spite of a slow start, the role of molecular chaperones in Alzheimer's disease is increasingly being elucidated at the molecular level. This chapter summarizes the nature of the cellular stress response that is induced in Alzheimer's disease and examines current research related to the function of molecular chaperones in the cellular metabolism of tau and β-amyloid peptide

Keywords: β-amyloid; tau; Alzheimer's disease; neurodegeneration; heat shock proteins; protein misfolding

HEAT SHOCK PROTEINS, THE AGING PROCESS AND NEURODEGENERATION

Sophisticated quality control mechanisms are required to ensure the proper synthesis, folding, post-translational modifications, and transport of proteins within a highly crowded macro-molecular, intracellular environment that favors protein

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misfolding and aggregation (Ellis, 2001). Over the past few years, considerable attention has been focused on a highly conserved family of proteins, termed chaperones, which helps to fold proteins into their native conformations. Historically, chaperones are referred to as heat shock proteins (Hsp), if they respond to heat shock, or glucose regulated proteins (Grp), if they respond to metabolic stress, such as glucose insufficiency. Hsp are generally cytoplasmic, while the corresponding Grp are components of the unfolded protein response (UPR) of the endoplasmic reticulum and Golgi.

Protein misfolding results in the exposure of hydrophobic domains normally buried in the interior of the native structure. Interactions between these exposed hydrophobic regions lead to the formation of toxic aggregates, which include oligomers, protofibrils and fibrillar deposits having the chemical signature of amyloid. Hsp not only detect and refold denatured proteins, but are also actively involved in the triage of unsalvageable products to the major cellular protein degradation system, the proteasome. Thus, Hsp in the role of chaperones are key components of the machinery that maintains a delicate balance between natively folded functional proteins and aggregation-prone misfolded proteins. The latter may form acutely to some cell stressors or build-up over a lifetime and lead to cell death.

The accumulation of misfolded proteins is one hallmark of aging. During the life-span of a stable protein, various post-translational modifications occur (Harding et al., 1989). In some cases, for instance oxidative damage, these modifications induce conformational changes that impair protein function, and cannot be reversed by Hsp. Therefore, the only solution to protect the cell from these misfolded proteins is their elimination or sequestration. Aging is accompanied by decreases in proteasome activity (Conconi et al., 1996) as well as autophagic lysosomal protein degradation or "autophagy" (Cuervo and Dice, 2000). When the chaperone-degradation system fails at any of several steps, abnormal proteins accumulate as aggregates or inclusions. The problem may also be amplified when Hsp and other protective chaperones get trapped over time in these insoluble inclusions, therefore reducing their cellular levels and leaving the cell more susceptible to further stresses. Indeed, as cells age they also lose their ability to fully activate the unfolded protein stress response or UPR as defined below (Liu et al., 1989; Fargnoli et al., 1990; Sherman and Goldberg, 2001). Thus, aging rats display reduced levels of endoplasmic reticulum chaperones, increased ubiquitination and impaired activation of the stress response in the hippocampus (Paz Gavilan et al., 2006).

Dysfunction of the protein folding and degradation machinery is also believed to contribute to a variety of human diseases (Cummings et al., 1998; Lam et al., 2000; Bence et al., 2001; morley and moromoto, 2004; Lindsten et al., 2002). Thus, subtle impairments in the chaperone system that may occur with aging, together with increases in abnormally folded client protein expression or production, may result in aberrant accumulation and aggregation of cytotoxic proteins and neurodegeneration (Cohen et al., 2006). Increasing evidence points to a critical role for molecular chaperones in neurodegenerative diseases (DeArmond and Prusiner, 1995; Bonini, 2002; Sakahira et al., 2002). Several neurodegenerative

diseases also appear to involve an early impairment of the stress response (Batulan et al., 2003; Magrane et al., 2005), further compromising the function and survivability of neurons. Since they are post-mitotic, dilution of cytotoxic misfolded proteins by cell division is not an option. For instance, it has been recently shown that abnormal S-nitrosylation of protein disulfide isomerase, an endoplasmic reticulum chaperone, is found in brain samples of sporadic Parkinson's and AD cases. The S-nitrosylation modification blocks protein disulfide isomerase protective function that is part of the endoplasmic reticulum stress response (Uehara et al., 2006). In another case of endoplasmic reticulum chaperone dysfunction in neurons, a mutation in the gene *sil1* that encodes a co-chaperone of Grp78, a crucial endoplasmic reticulum chaperone, leads to protein accumulation and neurodegeneration (Zhao et al., 2005). Importantly, mutations that compromise the activity of Hsp family members lead to several rare syndromes, such as hereditary spastic paraplegia (mitochondrial Hsp60), desmin-related myopathy (αB-crystallin), Marinesco-Sjogren syndrome (Sil1), axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy (Hsp27), and distal motor neuropathy (Hsp22) (Vicart et al., 1998; Hansen et al., 2002; Evgrafov et al., 2004; Irobi et al., 2004; Anttonen et al., 2005; Senderek et al., 2005).

In broad terms, most of the neurodegenerative diseases can be considered as disorders of protein misfolding, referred as "foldopathies" (Kosik and Shimura, 2005). Several age-related disorders such as AD, Parkinson's disease, amyotrophic lateral sclerosis, prion diseases and the polyglutamine expansion diseases share a common pathology related to protein misfolding and progressive intracellular accumulation of specific but unrelated toxic proteins that target select cell populations. Each protein abnormality also triggers a different cellular response within the protein folding machinery and the degradation pathways. In this chapter, we will review the nature of this stress with respect to AD and examine some recent studies related to the function of heat shock and stress related proteins in the cellular metabolism of tau and β-amyloid peptide.

APP PROCESSING AND INTRACELLULAR EVENTS IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common neurodegenerative disorder and the leading cause of dementia. It is usually diagnosed through recognition of a restricted impairment in memory that then expands to involve other cognitive process. AD is associated with a characteristic combination of morphological brain alterations and most often arises sporadically from the combination of genetic risk factors and unknown environmental/ aging processes (sporadic AD) or much less often (2%) directly from heritable defects in key proteins (familial AD, FAD). While mutations in either the amyloid precursor protein (*APP*), presenilin-1 (*PS1*) or presenilin-2 (*PS2*) genes cause the majority of early-onset FAD, the molecular basis for the later onset sporadic forms of AD remains largely unknown. However, evidence implicates oxidative stress (Markesbery, 1997) and cardiovascular risk factors

(de la Torre, 2002), as well as from the increased incidence of AD in individuals possessing specific apolipoprotein E (APOE4) or sortilin-related receptor (SORL1) genotypes (Cedazo-Minguez and Cowburn, 2001; Rogaeva et al., 2007).

The major pathological hallmarks in the brains of AD patients are extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs). However, amyloid angiopathy and inflammatory changes also occur in most cases. Amyloid deposits are derived from the amorphous aggregation of a number of proteins, of which a small peptide referred to as β-amyloid (Aβ) is the main component. The other observable pathological structures, NFTs, are paired helical filaments derived from the aggregation of hyperphosphorylated forms of the microtubule-associated protein tau (τ). It is now widely accepted that Aβ peptide has a primary role in the development of AD (Hardy and Allsop, 1991; Hardy and Selkoe, 2002; Wirths et al., 2004). However, in the order of events leading to AD, the exact roles played by intracellular Aβ and τ are still to be elucidated. Nevertheless, increasing evidence both from transgenic mice expressing familial AD mutations and human AD patients supports an early role for intracellular Aβ accumulation (LaFerla et al., 2007). Indeed, although the formation of NFTs closely parallels the progression and anatomic distribution of neuronal loss in AD, intraneuronal Aβ accumulation precedes the deposition of amyloid plaques and the appearance of NFTs, and correlates with the first indications of cognitive deficits (Gouras et al., 2000; D'Andrea et al., 2001; Wirths et al., 2001; Oddo et al., 2003; Billings et al., 2005; Oakley et al., 2006; Knobloch et al., 2007). It is worth mentioning that intracellular Aβ has also been clearly associated with Down's syndrome and the human muscle disease inclusion body myopathy (Tseng et al., 2004). Several observations also suggest that both intracellular amyloid and τ pathologies may be causally linked (Blurton-Jones and Laferla, 2006). Recent studies have shown that the accumulation of intracellular Aβ in vivo inhibits the proteasome, and that proteasome impairment leads to the buildup of abnormally phosphorylated τ protein (Oddo et al., 2004; Tseng et al., 2007).

The Aβ peptide is generated by endoproteolysis of the APP, a single pass, type I membrane protein. Three different groups of enzymes, termed α-, β-, γ-secretases, sequentially cleave APP in two alternate processing pathways. In the most common non-amyloidogenic pathway, membrane proximal cleavage by α-secretases (at a position 83 amino acids away from the carboxy-terminus of APP) precludes generation of Aβ peptide. A large amino-terminal domain (sAPPα) is secreted into the extracellular medium, and the resulting C83 fragment is then cleaved by a γ-secretase complex cleavage (composed of presenilin 1 or 2, nicastrin, anterior pharynx defective and presenilin enhancer 2) to generate a short fragment named p3. In the amyloidogenic pathway, cleavage of APP by β-site APP cleaving enzyme 1 (BACE1; at an extracellular position 99 amino acids away from the C-terminus of APP) results in the release of sAPPβ into the extracellular space, and subsequent cleavage of the resulting C99 fragment by the γ-secretase complex results in the generation of Aβ peptide. Processing of APP by β-secretase occurs in endosomes following cell surface receptor recycling, but can also occur in the endoplasmic

reticulum/trans-Golgi network, prior to APP secretion, or at the plasma membrane (Hardy and Selkoe, 2002; Kins et al., 2006; Vetrivel and Thinakaran, 2006). Thereby, generation of Aβ peptide is likely to occur in several intracellular compartments.

Several Aβ variants have been described which differ in their length. The most abundant Aβ peptide is 40 residues long (Aβ40), whereas a small proportion is 42 residues long (Aβ42). This longer variant is more hydrophobic and prone to aggregation, and is the predominant form found both intracellularly and in cerebral plaques (Gouras et al., 2000). The Aβ40 isoform is predominant in congophilic angiopathy. Aβ can be found in different assembly states (monomers, oligomers, protofibrils and fibrils), which have important physiological and pathological effects. While monomeric Aβ appears to be the less neurotoxic species, oligomers and protofibrils are the most pathological forms (Walsh et al., 2002; Cleary et al., 2005; Lesne et al., 2006). Similar conclusions were drawn in a number of other neurodegenerative diseases (Caughey and Lansbury, 2003). Aβ oligomerization first occurs intraneuronally, when associated with synaptic pathology (Walsh et al., 2000; Takahashi et al., 2004; Oddo et al., 2006). Although not clear yet, fibril formation appears to result from β sheet formation and linear cross-stacking, since the Aβ42 peptide contains exposed hydrophobic domains that can seed abnormal protein aggregation (Bitan et al., 2003; Kayed et al., 2003).

Consistent with clinical observations on the importance of intracellular accumulation, we and others have reported on the toxic effects of intraneuronal Aβ accumulation in model systems (LaFerla et al., 1995; Zhang et al., 2002; Magrane et al., 2004). Observations made when using synthetic Aβ peptides applied to cell cultures do not necessarily reflect what happens intracellularly when Aβ accumulates (Zhang et al., 2003; Magrane et al., 2005). Accumulation of intracellular Aβ42 may affect a variety of signal transduction pathways including Akt and MAPK family members that have important roles in neuronal function (Yuan and Yankner, 2000). We previously reported that intracellular Aβ42 deposition disrupts signaling through the Akt pathway, both in vitro and in vivo (Magrane et al., 2005). Others observed that the MAPK pathway may also be affected in an in vivo model of intracellular Aβ accumulation (Echeverria et al., 2004). We first described that the down-regulation of the Akt survival pathway caused a suppression of the stress response (Magrane et al., 2005). Moreover, when τ becomes abnormally phosphorylated, it aggregates and loses its ability to maintain stability of the axonal microtubules, which are the conduits for intracellular protein traffic (Mandelkow et al., 1995). Thus, both aberrant processing of APP and/or post-translational modifications affecting τ, generate species susceptible to aggregation and shown to be neurotoxins. Several other common neurodegenerative diseases have pathological features similar to AD, all characterized by inclusions of misfolded proteins. Indeed, it has been shown that soluble oligomers from Alzheimer's, Parkinson's, polyglutamine and prion diseases share a common structural feature that is recognized by a single antibody, an observation that points to the highly related nature of these diseases (Kayed et al., 2003).

While many mechanisms have been suggested to explain the starting point of AD pathogenesis, it is clear that neurons first fail in function and then die for lack of ability to buffer multiple metabolic stresses arising from overproduction and/or failure to clear neurotoxic amyloidogenic proteins. In the sequence of intraneuronal events that lead to AD pathogenesis, alterations in APP processing, Aβ turn-over and τ phosphorylation factor early in the disease progression. One promising role for Hsp in this process is to hasten Aβ removal. When clearance mechanisms become overwhelmed, Aβ oligomers eventually form insoluble fibrils that are deposited as amorphous inclusions that can include Hsp (Muchowski and Wacker, 2005). Hsp have been shown to accumulate in senile plaques and to be up-regulated in AD brain (Hamos et al., 1991; Perez et al., 1991; Yoo et al., 2001; Sahara et al., 2005). While several members of the Hsp family are shown to interact with key players of AD pathogenesis, not many studies have explored the role of the stress response in AD. Most of them have focused on abnormal τ phosphorylation and not until recently, has the role of heat shock and stress proteins in Aβ accumulation been addressed at the molecular level.

THE UPR AND CYTOPLASMIC CHAPERONE RESPONSES TO β-AMYLOIDOGENESIS

All proteins destined to the plasma membrane, including APP, first translocate from ribosomes into the endoplasmic reticulum(ER), where a group of chaperones work together to ensure the proper folding and assembly of nascent proteins, so that trafficking towards the secretory and the endocytic/lysosomal pathways can proceed. These chaperones include: BiP/Grp78 (glucose-regulated protein 78), Grp94, Grp170/ORP150, Grp58/ERp57, peptidyl prolyl isomerase, ERp72, calnexin, calreticulin, EDEM, Herp, protein disulfide isomerase and co-chaperones Sil1 and p58IPK. When misfolding and aggregation occurs in the endoplasmic reticulum, a specific ER stress response, known as the unfolded protein response (UPR) is activated. First, the response is initiated by the dissociation of BiP/Grp78 from unfolded protein "stress sensors" PERK (pancreatic ER serine/threonine kinase) and Ire-1. Then, expression of endoplasmic reticulum-resident chaperones (such as Grp78 and Grp94) is increased. Additionally, general protein synthesis is attenuated by translational shut-down. A third arm of the stress response is the activation of an endoplasmic reticulum-associated degradation (ERAD) pathway, by which misfolded proteins are retrotranslocated into the cytosol for ubiquitination and degradation (Bonifacino and Weissman, 1998; Ellgaard et al., 1999; McCracken and Brodsky, 2003). When the problem persists, C/EBP homologous protein (CHOP) and other factors are activated to induce apoptosis (Rao et al., 2004).

Experimental evidence points to endoplasmic reticulum being an important cellular compartment in which Aβ generation can occur (Hartmann et al., 1997; Wild-Bode et al., 1997; Tomita et al., 1998; Greenfield et al., 1999). Moreover, endoplasmic reticulum-resident chaperones interact with APP and APP proteolytic fragments (Yang et al., 1998b; Fonte et al., 2002; Hoshino et al., 2007) and thereby

could affect the generation of Aβ. It has been suggested that mutations in the *PS1* or *PS2* genes contribute, in part, to neuronal vulnerability through down-regulation of the UPR signaling pathway and impaired Grp78 induction (Guo et al., 1999; Katayama et al., 1999, 2001, 2004; Niwa et al., 1999). Of note, not all studies show such changes (Sato et al., 2001). This would have the undesirable effect of impairing the neuron's ER-based capability to prevent the accumulation of toxic proteins. The phenomenon of decreased ER chaperone expression in familial AD brain, (e.g. Grp78 levels (Kudo et al., 2002)), resembles what has been proposed to occur during aging (Sherman and Goldberg, 2001). In sporadic AD brains on the other hand, increased expression of proteins of the UPR has been observed in neurons without signs of neurodegeneration or NFTs (Hamos et al., 1991; Chang et al., 2002; Onuki et al., 2004; Hoozemans et al., 2005). This is in contrast to other studies where a decrease in Grp78 or no changes were observed (Katayama et al., 1999; Sato et al., 2000; Kudo et al., 2002). In another study, BiP/Grp78 is increased in AD brain coincident with down-regulation of cell cycle proteins and G1 phase arrest (Hoozemans et al., 2006). To reconcile these studies, it is plausible that initial activation of the UPR in viable AD neurons can be neuroprotective, while sustained activation lead to failure and heralds neurodegeneration (Ghribi et al., 2001; Chen et al., 2004; Tessitore et al., 2004; Brewster et al., 2006). Involvement of the UPR has been demonstrated in other neurodegenerative conditions such as juvenile Parkinson's disease and in Pelizaeus-Merzbacher disease (Imai et al., 2001; Southwood et al., 2002).

After the discovery that secretases are present in different compartments of the secretory and the endosomal/lysosomal pathways, and that APP C-terminus bearing fragments (CTF) and Aβ are generated intracellularly (Busciglio et al., 1993; Wertkin et al., 1993; Cook et al., 1997; Hartmann et al., 1997; Skovronsky et al., 1998), attention was put on potential ER quality control mechanisms that could alter APP processing and Aβ production/removal. First, interactions between APP and chaperones were revealed in the endoplasmic reticulum, where APP folding and maturation occurs. Therein, holoAPP directly interacts with Grp78, a resident chaperone that transiently associates with normally maturing polypeptides and more stably with misfolded or incompletely assembled proteins. When Grp78 is over-expressed, APP translocation from the endoplasmic reticulum to the Golgi is inhibited, APP maturation fails, and the levels of CTF and Aβ released into the medium decrease (Yang et al., 1998b; Kudo et al., 2006; Hoshino et al., 2007). Grp78 protects against excitotoxic and amyloid cell death (Yu et al., 1999). Next, the over-expression of certain other chaperones in the endoplasmic reticulum, have similar activities on APP processing. Thus, Grp170/ORP150 (oxygen-regulated protein 150) decreased the levels of both Aβ40 and 42 released into the medium, whereas calnexin decreased the release of only Aβ42 (Hoshino et al., 2007). Transient interaction with calreticulin, which is involved in the maturation of glycoproteins in the secretory pathway, is also required for APP trafficking and maturation through the endoplasmic reticulum and early cis-Golgi. It is known that holoAPP/calreticulin complex formation requires both prior binding

to Grp78 and N-glycosylation to occur (Johnson et al., 2001). Despite this interaction, calreticulin does not affect Aβ release into the medium (Hoshino et al., 2007). Although it is unclear how these different effects on wild type APP processing may occur, one possible explanation is that sublocalization of the various ER chaperones leads to a differential ability to activate the ERAD pathway. The data also suggests that expression of mutant APP, and in particular Aβ production, activates the UPR (Hoshino et al., 2007), although the role of intracellular Aβ accumulation was not explored. Induction of certain endoplasmic reticulum chaperones could therefore be therapeutically beneficial for the treatment of AD.

We turn attention now onto the cytoplasmic chaperone system. On the cytosolic site of the endoplasmic reticulum, APP has been shown to interact with heat shock cognate (Hsc) 73 (Kouchi et al., 1999). Although the significance of this interaction remains unclear, it presumably facilitates APP ubiquitination and degradation. In fact, the proteasome has been shown to be involved in the catabolism of APP and its secretase cleavage products (Marambaud et al., 1997; da Costa et al., 1999; Nunan et al., 2001; Flood et al., 2005; Kumar et al., 2007). This is reminiscent of the increase in Hsc/Hsp70 bound to polyubiquitinated hyperphosphorylated τ that was observed in the presence of proteasome inhibitors (Petrucelli et al., 2004; Shimura et al., 2004b). Moreover, CHIP (carboxy terminus of the Hsc70-interacting protein) over-expression increases cellular APP levels and promotes both APP and phospho-τ ubiquitinations (Petrucelli et al., 2004; Shimura et al., 2004b; Kumar et al., 2007) in accordance to a proposed role of CHIP to act as a molecular triage center (Connell et al., 2001).

Aβ-targeted to the secretory pathway was found to activate the cytosolic stress response and to interact with cytosolic signal and chaperone proteins (Suhara et al., 2003; Magrane et al., 2004, 2005; Zhang et al., 2004; Kumar et al., 2007). A stress response involving up-regulation of Hsp70 levels in AD and Down's syndrome temporal cortex confirms the in vitro data (Yoo et al., 1999). It is unclear how Aβ exits the endoplasmic reticulum, although reverse translocation through the ERAD pathway is a likely explanation. Aβ may also gain access to the cytosol due to leakage from lysosomes (Yang et al., 1998a; McCracken and Brodsky, 2003). Cytosolic Aβ has been shown to be highly cytotoxic to primary neurons (Zhang et al., 2002), and Aβ expressed intracellularly in an in vivo model of AD was found to interact directly with HSP70 family members (Fonte et al., 2002). Since Hsp70 has roles in preventing protein aggregation and promoting protein degradation (Muchowski et al., 2000; Dul et al., 2001; Chan et al., 2002; Dou et al., 2003), it is plausible that Hsp70 is critical in the proteasomal handling or in the sequestration of intraneuronal Aβ. Interestingly, over-expression of some Hsp reduced intracellular Aβ levels and, consequently, Aβ-induced neuronal death (Kumar et al., 2007). Other mechanisms involving loss of Hsp function in AD are apparent. For instance, mortalin (mtHsp70/Grp75) is a heat non-inducible mitochondrial protein that when oxidatively damaged in AD, results in reduced mitochondrial import of essential proteins (Yaguchi et al., 2007).

REMOVAL OF HYPERPHOSPHORYLATED tau (τ)

Tau (τ) is normally a highly soluble and natively unfolded protein that undergoes continuous turnover in neurons (Dickey et al., 2006). It remains unclear how the quality control system is able to distinguish between normal phospho-τ and aberrantly hyperphosphorylated τ species, although it has been demonstrated that early in AD pathogenesis, a combination of abnormal phosphorylation and certain conformational changes to τ serves as the misfolding event that is recognized by the chaperone machinery (Weaver et al., 2000; Ghoshal et al., 2001). Hyperphosphorylated τ is toxic to neurons in vitro and in vivo and has been clearly implicated in AD progression (Gomez-Isla et al., 1997; Gamblin et al., 2003; Kobayashi et al., 2003; Roberson et al., 2007). The mechanisms underlying τ-mediated neurotoxicity however remain unclear. Those isoforms phosphorylated by the kinases glycogen synthase kinase 3beta (GSK3-β) and Cdk5 (Lucas et al., 2001; Cruz et al., 2003; Noble et al., 2003) are particularly suspect. Many of these sites are dephosphorylated by PP2A, and a deficiency of phosphatase activity has also been implicated in τ hyperphosphorylation and impairment of behavior performance in rats (Sun et al., 2003). As is the case with Aβ entities, soluble τ oligomers and/or protofibrils probably mediate τ-associated neurodegeneration (Dickey et al., 2006). To reduce phosphorylated τ concentrations and τ-associated cellular toxicity, a variety of protective mechanisms involving the stress response are activated. They include binding of abnormal τ to Hsp70 to prevent toxic conformations of the protein, ubiquitination of τ for degradation by the proteasome, segregation of τ aggregates from the cellular machinery, and recruitment of anti-apoptotic molecules. Thus one mechanism for τ accumulation may be insufficient Hsp-mediated phospho-τ ubiquitination and degradation.

Several studies have explored the role of Hsp in hyperphospho-τ degradation, and focused on a complex comprised of Hsp70/Hsc70, Hsp90 and the E3 ubiquitin ligase CHIP (carboxy terminus of Hsc70-interacting protein). The Hsp/CHIP complex is a highly sensitive and tightly regulated quality control mechanism, involving multiple players that may also compete for the refolding or degradation of the abnormal protein (Johnson et al., 1998; Grenert et al., 1999; Liou et al., 2003). CHIP works together with BAG-1, an ubiquitin domain co-chaperone protein that accepts substrates from Hsc/Hsp70 and presents them to the CHIP-ubiquitin conjugation machinery and onto the proteasome (Luders et al., 2000; Demand et al., 2001; Qian et al., 2006). The recognition of τ by Hsc70 and Hsp90 suggests that phosphorylation may serve to disrupt the native structure of τ, targeting it for processing by the Hsc70/CHIP complex. The current view in the field is that if Hsc/Hsp70 system is unable to restore proper folding of τ, then the ubiquitin domain protein BAG-1 and the ubiquitin ligase CHIP, in collaboration with the E2 conjugating enzyme UbcH5B, can shift the chaperone activity of Hsc/Hsp70 from protein folding to an assist role in degradation (Petrucelli et al., 2004; Shimura et al., 2004b; Dickey et al., 2007). CHIP appears to ubiquitinate phosphorylated τ, not only to promote its proteasomal degradation but also to sequester it into insoluble filamentous aggregates and in so doing prevent cell death (Shimura et al., 2004b; Dickey et al., 2006).

Correspondingly, deletion of CHIP in mice results in accumulation of soluble phosphorylated τ in the brain (Dickey et al., 2006). CHIP also acts as a stress sensor (Qian et al., 2006) to positively regulate heat shock factor (HSF)-1 activity (Dai et al., 2003) and to terminate HSP70 through degradation when misfolded protein levels are returned to acceptable levels. HSF1 is a key transcriptional factor that controls the levels of the constituents of the cellular stress response.

Induction of a stress response by increasing levels of Hsp70 (and 90) in τ-transfected cell cultures prevents insoluble τ aggregates and τ phosphorylation, increases the solubility of τ and promotes the normal association of τ with microtubules (Dou et al., 2003). The same authors also demonstrated that Hsp70 (and Hsp90) bind τ and that both Hsp levels are lower in the brains of AD patients bearing τ aggregates and in transgenic mice expressing a mutant form of human τ that is responsible for fronto-temporal dementia (Dou et al., 2003). Over-expression of Hsp70 alone in vivo appears to reduce steady state levels and attenuate partitioning of τ into the high molecular weight detergent insoluble fraction (Petrucelli et al., 2004).

Hsp27 has also been shown to bind directly to phosphorylated τ and attenuate its toxicity by facilitating its degradation (through an ubiquitin-independent pathway) and/or dephosphorylation (Shimura et al., 2004a). Hsp27 plays a critical role in neuronal metabolism and survival, and can inhibit caspase activation (Concannon et al., 2003).

Hsp90 is involved in the folding and stabilization of multiple client proteins (Zhao and Houry, 2005). A central role for Hsp90 in the development of AD and associated tauopathies (Dickey et al., 2007) has resulted in the identification of several Hsp90 inhibitors as potential therapeutic tools in neurodegenerative diseases (Dickey et al., 2006; Waza et al., 2006a). Inhibition of Hsp90 by blockade of the refolding pathway promotes degradation of proteins bound to Hsp90 and usually causes the activation of the HSF1. Thus it has been recently reported that inhibition of Hsp90 actually leads to a decrease in phosphorylated τ levels. However, this action proved independent of HSF1 activation. The mechanism is via increased τ turnover and degradation mediated by CHIP (Dickey et al., 2006). Although somewhat alternative to the HSP90 results of Dou et al. presented above, this is reminiscent of the proteasome-dependent reduction in mutant polyglutamine expanded androgen receptor levels by a geldanamycin-like inhibitor of HSP90 (Waza et al., 2006b).

SMALL HSP, CHAPERONINS AND OTHER STRESS-RELATED PROTEINS IN AD

Although the role of other stress-related proteins in AD remains largely unexplored, some advances have been made more recently, specially related to small Hsp (sHsp), chaperonins and Hsp104. The sHsp are a family of chaperones, with subunit molecular masses ranging from 15 to 40 kDa, that bind to exposed hydrophobic residues but lack active refolding capabilities (ATP-independent chaperones). They

recognize proteins in the early stages of denaturation and help to maintain unfolded proteins in a folding-competent state (Lee et al., 1997). Subsequent refolding is thought to occur by Hsp70 and/or chaperonin function. In humans, the sHsp family comprises 10 members, among which αB-crystallin, Hsp27, Hsp20, HspB2 and HspB8 (Kappe et al., 2003).

αB-crystallin is the prototypical sHsp and contains a characteristic highly conserved carboxy-terminus "α-crystallin domain" that defines the members of this sHsp subfamily. αB-crystallin is the main component of the eye lens where it maintains a clear amorphous composition by preventing denatured proteins from aggregating to form opaque inclusions (Horwitz, 2000). Indeed, Aβ has been observed to accumulate in the cataracts of AD patients and to colocalize with αB-crystallin (Goldstein et al., 2003). αB-crystallin is expressed in both neurons and glia of the normal brain, and in various neurodegenerative diseases, including AD (Iwaki et al., 1992; Mao et al., 2001; Yoo et al., 2001). Changes in expression of αB-crystallin and Hsp16, among others, in response to intracellular Aβ42 expression have been observed (Link et al., 1999, 2003). In addition, αB-crystallin was shown to directly interact with Aβ both in cell culture and in a transgenic *C. elegans* model that expresses human Aβ intracellularly (Stege et al., 1999; Liang, 2000; Fonte et al., 2002). As in the case with Hsp70/CHIP, this sHsp associates with ubiquitinated τ (Goldbaum and Richter-Landsberg, 2004). αB-crystallin has been shown to increase the neurotoxicity of Aβ, possibly by preventing its aggregation into insoluble fibrils (Stege et al., 1999; Raman et al., 2005; Narayanan et al., 2006).

Hsp27 is expressed both in normal and AD brains, and appears to be associated with amyloid plaques and NFT (Renkawek et al., 1994; Stege et al., 1999; Wilhelmus et al., 2006a). Increased expression of Hsp27 has been found in AD brains (Renkawek et al., 1993; Stege et al., 1999) and in Dementia with Lewy Bodies (Outeiro et al., 2006). Furthermore, Hsp27 directly interacts with Aβ and inhibits fibril formation in vitro, possibly by interfering with the nucleation process in the early phase of amyloidogenesis (Kudva et al., 1997). In addition, Hsp27 binds to hyperphosphorylated τ and promotes its degradation by a proteasome-independent pathway and, when over-expressed, prevents hyperphosphorylated τ-mediated cell death (Shimura et al., 2004a).

Although direct interaction between Aβ and some sHsp remains to be demonstrated in vivo, and their potential role in intraneuronal Aβ aggregation has not been proved, it has recently shown in vitro that Hsp20, Hsp 27 and αB-crystallin, but not HspB2, bind to Aβ, prevents Aβ aggregation and attenuate toxicity (Lee et al., 2005, 2006; Wilhelmus et al., 2006b). While the usual function of sHsp is in intracellular surveillance, they are also expressed by reactive astrocytes and their cytoprotective role against experimental Aβ toxicity seems to be explained by interference with extracellular oligomerization at the cell surface (Wilhelmus et al., 2006b). Immunohistological studies have found that extracellular HspB2 is strongly expressed in fibrillar amyloid deposits around the cerebral vessels, and that Hsp20 is mainly associated with non-fibrillar Aβ in diffuse senile plaques (Wilhelmus et al., 2006a).

In addition, another member of the sHsp family, HspB8, binds to distinct Aβ species and inhibits the aggregation of mutated huntingtin (Carra et al., 2005; Wilhelmus et al., 2006c). The results suggest that while sHsp may have different affinities for the various Aβ species and other amyloidotic proteins, they have potential to play a significant role in Aβ deposition in AD brains.

Chaperonins (Cpn) are a family of sequence-related proteins of about 60 kDa that are classified in two groups: group I chaperonins are found in bacteria and organelles (mitochondria and chloroplasts), and group II chaperonins are found in the cytosol of eukaryotes and Archaea. The mitochondrial chaperonin Cpn60 is primarily found in the mitochondrial matrix. Both synthetic Aβ peptide treatment and intraneuronal Aβ accumulation reduced levels of mitochondrial Cpn60, without similarly affecting other Hsp levels, such as Hsp70 and Hsp90 (Zamostiano et al., 1999; Veereshwarayya et al., 2006). Mitochondrial Cpn60 overexpression protected components of the electron transport chain and enzymes of the mitochondrial matrix from Aβ expression-induced toxicity, although it had no effect on Aβ levels or oligomerization (Veereshwarayya et al., 2006). Similarly, reduced expression of mitochondrial Cpn60 has been reported in cells derived from individuals with Down's syndrome, which shares a number of characteristic lesions with AD including intraneuronal Aβ accumulation (Bozner et al., 2002). Similarly, cytoplasmic levels of Cpn60, a specific chaperonin for actin and tubulin, also decrease in AD-affected neurons leaving the cytoskeletal proteins deficient and aggregated (Schuller et al., 2001).

Another chaperone-related family of proteins is the class I family of Clp/Hsp100 AAA^+ ATPases, to which Hsp104 belongs. Hsp104 does not associate with any protease or ligase, unlike other Clp1 proteins, and does not protect from denaturation, but it acts as a molecular chaperone to rescue proteins from an aggregate state, with the help of the Hsp70 system (Parsell et al., 1994; Lee et al., 2004). Hsp104 was shown to inhibit both Aβ and α-synuclein aggregate formation in vitro (Kong et al., 2005).

NEUROPROTECTION AND THERAPEUTIC STRATEGIES

Several studies have convincingly demonstrated that increased chaperone expression can suppress the neurotoxicity caused by accumulation of neurotoxic proteins. Interventions that enhance or boost a deficient stress response may have therapeutic value in limiting the neuronal dysfunction and loss that defines neurodegenerative disease states (Rochet, 2007). Thus, Hsp can delay the onset and the outcome of protein-misfolding diseases, such as in transgenic models of Parkinson's and polyglutamine diseases such as spinocerebellar ataxia (Warrick et al., 1999; Chan et al., 2000; Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000; Cummings et al., 2001; Auluck et al., 2002). Recent findings suggest that Hsp can also be neuroprotective in AD, but this area of research remains largely unexplored having been focused on in vitro and cell culture studies. The lack of

suitable animal models that accurately replicate the characteristics of the human disease and the complex etiology of AD are possible reasons for this state. Hsp have been shown to modulate the aggregation of Aβ peptide in a cell-free system (Evans et al., 2006; Wilhelmus et al., 2006b) and enhance clearance of exogenous Aβ42 in rat hippocampus in vivo (Takata et al., 2003). We demonstrated that the endogenous modest activation of the neuronal stress response was insufficient to prevent Aβ42-induced cell death and that over-expression of Hsp70 reversed Aβ42 toxic effects (Magrane et al., 2004), a result confirmed by others (Zhang et al., 2004). Intraneuronal Aβ42 accumulation was shown to compromise neuron survival by impairing full expression of the stress response. Thus, when inherent cellular protection mechanisms were boosted, Aβ42-induced neuronal death was prevented (Magrane et al., 2005). More recently, over-expression of Hsp70 and Hsp90, together with CHIP, reduced intracellular Aβ levels and, consequently, Aβ-induced neuronal death (Kumar et al., 2007). Additionally, it has been suggested that induction of certain endoplasmic reticulum chaperones can be therapeutically beneficial for the treatment of AD since it decreases Aβ production (Hoshino et al., 2007).

In keeping with the potential for direct increase in Hsp levels, stimulation of HSF1 maybe similarly beneficial. Down-regulation of HSF1 in transgenic worms that over-express intracellular Aβ42 resulted in accelerated paralysis and increased protein aggregation (Cohen et al., 2006). Inhibition of HSF-1 also accelerates aging in wild type (Garigan et al., 2002) and decreases longevity in *c.elegans* in which an age-1 gene mutation in the insulin signaling pathway extends lifespan (Morley and Morimoto, 2004). Although never tested in models of AD, activation of HSF1 and the downstream expression of the stress response has been proven to be therapeutically effective in several neurodegenerative diseases. Thus, the Hsp90 inhibitor geldanamycin activates HSF1 and inhibits α-synuclein aggregation and toxicity both in vitro and in vivo (McLean et al., 2004; Auluck et al., 2005). Furthermore, geldanamycin and radicicol, another Hsp90 inhibitor, suppressed huntingtin aggregation and toxicity in organotypic cultures derived from huntingtin transgenic mice (Hay et al., 2004). Similarly, celastrol, a recently identified drug compound and a component of Chinese herbal medicines, was reported to induce the cellular stress response by activating HSF1 (Westerheide et al., 2004) and to improve memory in normal Sprague-Dawley rats (Allison et al., 2001). Arimoclomol, another HSF1 activator, improves neuronal survival in SOD1 mutant amyotrophic lateral sclerosis mice (Kieran et al., 2004). Acetyl-L-carnitine, a mitochondrial antioxidant, up-regulates Hsp in cortical neurons exposed to Aβ42-oxidative stress (Abdul et al., 2006).

Another relevant Hsp-directed therapeutic strategy would be to remove hyperphosphorylated τ. Reduction of insoluble τ aggregates and τ phosphorylation was achieved in culture by over-expression of Hsp70 and Hsp90, and in vivo by Hsp70 alone (Dou et al., 2003; Petrucelli et al., 2004). Recently, a novel Hsp90 inhibitor promoted selective decreases in phosphorylated τ in a mouse model of tauopathy (Dickey et al., 2007).

Table 1. Heat shock proteins in Alzheimer's disease: Cytoplasmic and mitochondrial chaperone families

Family	Function	AD mechanisms	References
Hsp100: 104–110	ATPase activity. Thermal tolerance, protein disaggregation and refolding. Works with Hsp70	Inhibits Aβ aggregation	Kong et al. (2005)
Hsp90	ATPase activity. Interacts with signal transduction molecules, nuclear hormone receptor maturation, stabilizes misfolded proteins, prevents aggregation of refolded peptides, and ensures correct assembly and folding of newly synthesized client proteins. Hsp90 inhibitors activate HSF1	Associated with Aβ plaques. Binds to APP and τ. Complexes with CHIP. Inhibition of Hsp90 hastens phospho-τ and polyglutamine protein degradation by the proteasome	McLean et al. (2004); Auluck et al. (2005); Zhao and Houry (2005); Dickey et al. (2006); and Waza et al. (2006a)
Hsc70–73 (non-inducible) Hsp70 (stress-inducible): cytoplasmic	ATPase activity. Regulates protein transport and cell cycle. Anti-apoptotic. Binds to, stabilizes and correctly folds nascent polypeptides, refolds denatured proteins, prevents aggregation. Down-regulates HSF1	Binds to APP, Aβ and phospho-τ in complex with CHIP. Reduces levels of phospho-τ, promotes its ubiquitination and increases levels of inert-insoluble τ. Reduces Aβ levels and reverses Aβ and τ toxicities	Kouchi et al. (1999); Fonte et al. (2002); Dou et al. (2003); Magrane et al. (2004); Petrucelli et al. (2004); Shimura et al. (2004b); Zhang et al. (2004); Magrane et al. (2005); and Kumar et al. (2007)
mitochondrial (mortalin)	ATPase activity. Mitochondrial import of proteins and energy production. Interacts with mtHsp60	Unknown	Yaguchi et al. (2007)
Hsp60 (chaperonins) cytoplasmic (TCP1)	Binds to partially folded polypeptides to assist in mature folding (e.g. actin, tubulin), refolds denatured proteins, facilitates degradation	Levels are reduced in Aβ-expression systems.	Zamostiano et al. (1999)
mitochondrial (Group 1)	Facilitates protein folding in the mitochondria. Interacts with mortalin	Protective vs. Aβ toxicity to PDH, αKGDH and electron transport components	Bozner et al. (2002); Veereshwarayya et al. (2006)

(*Continued*)

Hsp40	Co-chaperone to Hsp70, aiding ATP hydrolysis and the closing of the Hsp70 pocket to release folded substrate	No action reported in AD yet, but suppresses inclusion formation in mutant α-synuclein, SOD1 and polyglutamine models	Minami et al. (1996); Jana et al. (2000); McLean et al. (2002); Takeuchi et al. (2002); Klucken et al. (2004); Muchowski and Wacker (2005)
Small Hsp:	ATP independent.		
αB-crystallin	Cytoskeletal stabilization, suppresses aggregation of partial denatured proteins	Interacts with Aβ, APP and ubiquitinated τ, may increase Aβ toxicity	Stege et al. (1999); Liang (2000); Fonte et al. (2002); Narayanan et al. (2006)
Hsp27	Assembles into dynamic oligomers, binds to released cytochrome c and inhibits caspase activation, stabilizes intermediate microfilaments and actin. Suppresses aggregation and heat inactivation of proteins. Cell survival and metabolism homeostasis	Binds phospho-τ, promotes its dephosphorylation and is neuroprotective versus τ toxicity (ubiquitin-independent). Binds Aβ and prevents fibril formation. Increased expression in AD brain. Associated with plaques	Renkawek et al. (1993); Kudva et al. (1997); Stege et al. (1999); Concannon et al. (2003); Shimura et al. (2004)
Hsp20		Binds Aβ and prevents fibril formation, associated with plaques in AD brain.	Lee et al. (2005); Wilhelmus et al. (2006)
HspB2, B8		Suppresses Aβ toxicity in vitro. Associates with vascular Aβ	Wilhelmus et al. (2006)

CONCLUSION

Although still far from application to patients with AD, the sum of these studies provide a rationale for the development of novel therapeutic strategies designed to up-regulate endogenous Hsp levels in order to prevent or reverse protein misfolding and to boost aggregate-clearance mechanisms (see Table 1). Therapeutic stimulation of inducible chaperones like the HSP70 and the small Hsp27 hold promise to restore several proteolytic systems that become overwhelmed in neurodegenerative diseases. Desirable outcomes are to increase ubiquitin-proteasome throughput to reduce levels of soluble toxic proteins, as well as to promote aggresome formation (a centrosome-associated, membrane bound structure to sequester small aggregates) and lysosomal autophagy to clear the bulkier protein aggregates. Given that Hsp are located primarily in intracellular compartments, Hsp induction is likely to protect against Aβ and τ toxicity by binding oligomeric species inside the cell, in

distinction to effects on extracellular aggregates or plaques and intracellular inclusions. This view is supported by some studies that show inclusion body formation per se in α-synuclein and polyglutamine models is not affected by chaperone expression (Warrick et al., 1999; Cummings et al., 2001; Zhou et al., 2001; Auluck et al., 2002). This prediction however deserves to be tested in AD transgenic animals. Moreover, the consequences of possible feedback repression of HSF1 also need to be worked out. Further understanding of the cooperative interactions among molecular chaperones that affect APP processing, intraneuronal Aβ accumulation and abnormal τ phosphorylation would strengthen the chances to translate present knowledge into a practical drug. Delineation of the mechanism behind the impact of the aging process on the stress response will go a long way to develop therapeutic approaches to reverse the toxic effects of aggregated Aβ peptides and τ proteins in AD. Various animal models support the notion that a critical link between aging and the response to misfolded protein stress is mediated by HSF-1 and its HSP transcriptional targets (Hsu et al., 2003; Morley and Morimoto, 2004). The inseparable relationship between AD and aging augers the same benefit.

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CHAPTER 3

CELLULAR AND MOLECULAR MECHANISMS UNDERLYING PARKINSON'S DISEASE: THE ROLE OF MOLECULAR CHAPERONES

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Abstract: Adult onset neurodegenerative diseases are thought to be caused by toxic misfolded proteins. Triggered by gene mutations and environmental events, toxic proteins cause cell death in various brain regions and accumulate in intraneuronal inclusion bodies. In Parkinson's disease (PD), Lewy bodies (LBs) containing the small acidic protein, α-synuclein, are found at autopsy making α-synuclein a candidate for the toxic protein in PD and other synucleinopathies such as dementia with Lewy bodies (DLB), where the pathology extends into the cortex. Normally, mutant proteins that fail to fold properly are either refolded by molecular chaperones or degraded by the proteasome or lysosome. The ineffectiveness of these quality control mechanisms in neurodegenerative diseases such as Parkinson's disease may be due, at least in part, to a natural decline in function of the aging brain. The result is the accumulation of misfolded protein and a disruption in cellular function. Here, we will examine the emerging role of heat shock protein molecular chaperones in Parkinson's disease and investigate the potential targeting of heat shock protein molecular chaperones as therapeutic drug targets

Keywords: α-synuclein; Parkinson's disease; dementia with Lewy bodies; heat shock proteins; protein misfolding; neurodegeneration

Abbreviations: PD, Parkinson's disease; DLB, dementia with Lewy bodies; LBs, Lewy bodies; HSPs, heat shock proteins; GA, geldanamycin

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MOLECULAR PATHOGENESIS OF PARKINSON'S DISEASE

Parkinson's disease (PD) is an incurable neurodegenerative disease affecting from ~1% of the worldwide population at the age of 65 to ~5% at the age of 85, making age the major risk factor for the development of the disease. PD is pathologically characterized by the depigmentation of the substantia nigra (SN), caused by the selective and progressive loss of dopaminergic (DA) neurons, and the presence of intraneuronal proteinaceous inclusions known as Lewy bodies (LBs) within the surviving neurons in the SN and other brain regions. LBs are enriched in fibrillar α-synuclein and other proteins and are often highly ubiquitinated (Kuzuhara et al. 1988; Spillantini et al. 1997). Loss of DA neurons leads to a depletion of dopamine within the striatum which causes dysregulation of the motor circuits that project throughout the basal ganglia, resulting in the clinical manifestations of PD. Clinical symptoms include slowness of movement (bradykinesia), muscular rigidity, resting tremor and postural instability. The symptoms can be partially alleviated by the exogenous administration of the dopamine precursor L-3,4-dihydroxyphenylalanine (L-dopa). The majority of cases of PD are sporadic but several genes have been identified that, when mutated, give rise to rare, familial forms of the disease.

There is strong evidence to suggest that α-synuclein accumulation is an early step in the pathogenesis of both sporadic and familial PD and related disorders such a dementia with Lewy bodies (DLB). Mutations in the α-synuclein gene (A30P, E46K, and A53T) are associated with rare familial forms of PD (Polymeropoulos et al. 1997; Krüger et al. 1998), and α-synuclein is abundant in Lewy bodies (LBs) even in sporadic PD (Irizarry et al. 1998; Spillantini et al. 1998). Moreover, increased expression of α-synuclein in the brain due to the duplication and triplication of the *SNCA* gene encoding α-synuclein, results in PD in rare cases (Singleton et al. 2003; Farrer et al. 2004). In animal models, overexpression of α-synuclein in transgenic mice (Masliah et al. 2000; Giasson et al. 2002), rat (Kirik et al. 2002; Klein et al. 2002; Lo Bianco et al. 2002) and drosophila (Feany and Bender 2000) leads to α-synuclein aggregation and toxicity in the dopaminergic system. Therefore, an entire class of neurodegenerative diseases, referred to collectively as the "synucleinopathies" (Spillantini and Goedert 2000) appears to result from the accumulation of α-synuclein aggregates in various central nervous system cell populations.

α-Synuclein is a natively unfolded molecule that can self-aggregate to form oligomers and fibrillar intermediates (Weinreb et al. 1996; Hashimoto et al. 1998; Uversky et al. 2002). LBs are small, round inclusions found in neurons at risk for degeneration in PD and DLB. They are predominantly localized in the substantia nigra pars compacta, but can also be found in widespread cortical and subcortical regions (Gomez-Tortosa et al. 2000). α-Synuclein is a major component of LBs and Lewy neurites (LNs) (Spillantini et al. 1997; Irizarry et al. 1998) and the protein is highly amyloidogenic and aggregates in vitro in a concentration-dependent manner to form fibrils reminiscent of those observed in LBs (Conway et al. 2000; Serpell et al. 2000). α-Synuclein is normally localized in the presynaptic compartment,

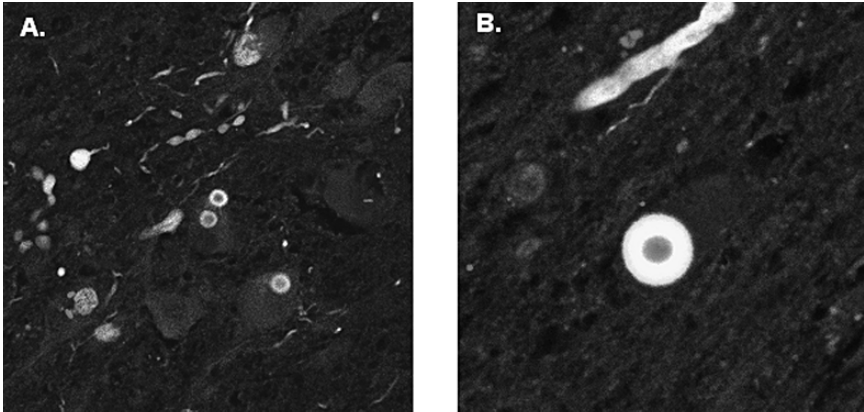

Figure 1. Confocal microscope images of SN in DLB. Immunostained for α-synuclein and ubiquitin. (**A**) The greatest diversity of α-synuclein inclusions is found in the SN region in DLB. (**B**) Classic LBs have a peripheral rim immunoreactive for α-synuclein, a middle ring where α-synuclein and ubiquitin colocalize and a central core immunopositive only for ubiquitin
Source: Adapted from Gomez-Tortosa et al. (2000)

however, in PD and DLB α-synuclein accumulates in aggregates with various morphologies within neurons (Gomez-Tortosa et al. 2000) (Figure 1). For the most part, these aggregates are densely compact, and can be immunostained for multiple additional components, including the α-synuclein interacting protein, synphilin-1 (Engelender et al. 1999; Wakabayashi et al. 2000), which has also been implicated as a substrate of the E3 ubiquitin activity of parkin (Chung et al. 2001). LBs are also immunopositive for ubiquitin, additional evidence that protein misfolding or clearance is altered in cells that develop LBs (Kuzuhara et al. 1988), and chaperone proteins like Hsp70, Hsp40 and Hsp27 (Auluck et al. 2002; McLean et al. 2002). The conformation of α-synuclein in LBs is significantly different than α-synuclein in the neuropil, as assessed by advanced imaging techniques such as fluorescence resonance energy transfer (FRET) (Sharma et al. 2001) and fluorescence lifetime imaging (FLIM) (Klucken et al. 2006a). Nonetheless, the conformation of α-synuclein found in PD tissue remains unknown, although it has been postulated that oligomeric species represent the toxic genus (Sharon et al. 2003).

PROTEIN MISFOLDING IN PARKINSON'S DISEASE

A common pathological feature among neurodegenerative diseases is the excessive accumulation of abnormal proteins. As a cell makes new proteins it must also make triage decisions. Mutant proteins that fail to fold properly are either refolded by molecular chaperones or destroyed by the proteasome or lysosome. The inability of these quality control mechanisms to work effectively in neurodegenerative diseases such as PD may be due, at least in part, to a natural decline in function of the aging brain (Keller et al. 2002). The result is the accumulation of misfolded protein

and a disruption in cellular function. At present it is still unclear which conformation of mutant proteins represent the toxic species (Figure 2). The conformations of abnormal proteins range from monomers, to protofibrils, to fibrils and finally inclusion bodies. Although for many years the end-stage inclusions such as LBs were viewed as toxic, recent data has raised the question that other species may mediate toxicity as well and, in some cases, the inclusions may be protective, or innocuous, with prefibrillar oligomeric forms taking primacy as the toxic moieties. Therefore, a therapeutic strategy for protein aggregating neurodegenerative diseases could involve lowering the levels of toxic species by either preventing them from forming or by clearing them from the cell once formed.

Whether the LBs themselves are toxic, or simply represent one end of a spectrum of misfolded, toxic proteins remains unknown and is a topic of intense investigation in the synucleinopathies. In studies of trinucleotide repeat diseases, it is possible to dissociate aggregate formation from toxicity (see (Paulson et al. 2000) for review); data point towards a similar phenomenon in cell culture studies of α-synuclein, where an equal amount of toxicity is observed under conditions where α-synuclein forms visible aggregates as when it does not (Klucken et al. 2004b). Research studies aimed at developing tools and assays to distinguish which conformations of α-synuclein are associated with toxicity are particularly topical at present.

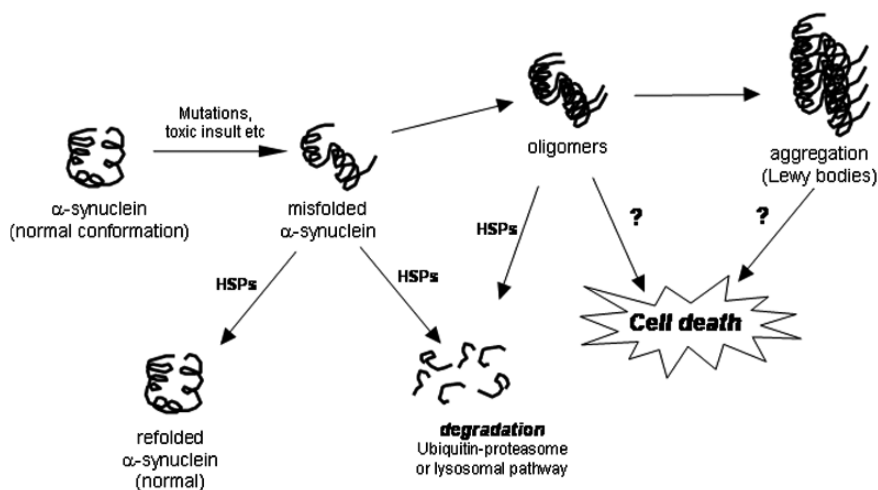

Figure 2. Proposed role of HSPs in α-synuclein-induced cell death. α-Synuclein becomes misfolded due to the presence of a mutation or a toxic insult that may result in post-translational modifications such as phosphorylation. Misfolded α-synuclein can potentially be refolded or targeted for degradation by HSPs or go on to form prefibrillar oligomers which may represent a toxic intermediate. Alternatively, the end stage fibrillar aggregates may represent the toxic species and causes cell death. To date it is unclear which conformation of α-synuclein leads to neurodegeneration. Toxic oligomeric species could also be targeted for degradation by the proteasome by enhancing the levels of HSPs in the cell – thus representing an important target for therapeutic drug development

Given that the aggregation of α-synuclein is a key factor in the development of PD and related synucleinopathies, molecules that inhibit fibril formation or disaggregate fibrils of α-synuclein may lead to therapies to prevent or control PD as well as to a better understanding of the process. Fibrillization of α-synuclein initiates with the dimerization of partially folded monomers (Uversky et al. 2001; Krishnan et al. 2003), followed by the formation of β-sheet rich nonfibrillar oligomeric intermediates, also known as protofibrils. Protofibrils are transient β-sheet-containing oligomers that are formed during fibrillization. If protofibrils are pathogenic in PD, then it is possible that PD-associated mutations may enhance the formation of them. Pathogenic mutations in α-synuclein increase rates of self-assembly and fibrillization and it is thought that the soluble, oligomeric forms of α-synuclein represent the toxic species rather than the insoluble fibrils that make up a LB (Conway et al. 2000). Although the mechanisms of α-synuclein fibrillization and its related cytotoxicity remain elusive, it is thought that oxidative stress may trigger oligomerization, a first step towards formation of insoluble aggregates. Therefore molecules with anti-aggregation/anti-oxidant properties may be potential therapeutic agents.

MOLECULAR CHAPERONES IN PARKINSON'S DISEASE

Heat shock proteins (HSPs), also known as molecular chaperones, are a general class of proteins that can help prevent protein misfolding and/or aggregation or direct proteins towards degradation, and thus help maintain normal protein structure and function. HSPs have been reported to play important roles in the folding of nascent proteins, in guiding the renaturation of misfolded proteins, as well as in the degradation of misfolded client proteins. HSPs are categorized into six different families according to their respective molecular weights. They are HSP100, 90, 70, 60, 40, and the small heat shock family that includes Hsp27.

The evidence for HSPs playing a role in PD has a strong parallel in studies of trinucleotide repeat diseases (Sakahira et al. 2002). In in vitro models, transgenic mouse models, and drosophila models, overexpression of Hsp70 provides protection against aggregation and/or toxicity of multiple types of polyglutamine aggregates and polyalanine aggregates (Chai et al. 1999; Warrick et al. 1999; Jana et al. 2000; Krobitsch and Lindquist 2000; Kobayashi and Sobue 2001; Wyttenbach 2004; Muchowski and Wacker 2005).

In postmortem DLB brain tissue, protein expression levels of molecular chaperones Hsp90, Hsp40 and Hsp27 correlate with the amount of total or triton X-100 soluble α-synuclein (bioavailable fraction) but not in control brain tissue. Surprisingly, no clear association with Triton X-100 insoluble α-synuclein fraction was found suggesting an interaction of HSP molecular chaperones mainly with bio-available fractions of α-synuclein (Klucken et al. 2006b). Similarly, mRNA levels of Hsp70 and Hsp27 were found to be increased in DLB brain compared to control (Cantuti-Castelvetri et al. 2005; Outeiro et al. 2006).

Table 1. Frequency of HSP colocalization with α-synuclein in LBs and LNs in vivo. Immunostaining was assessed for four cases of pathologically confirmed DLB for LBs and two cases for LNs. + 0–25%; ++ 25–50%; +++ >50%

Heat shock protein	Frequency of occurrence in DLB	
	LBs	LNs
Hsp27	+++	+
Hsp40 (HDJ-1)	+++	+
Hsp 40 (HDJ-2)	+++	++
Hsp60	++	+
Hsp70	+++	+
Hsp90	+	+
Hsp110	++	+

(Adapted from McLean et al. 2002)

It is likely that Hsp70 and co-chaperone proteins have a special role in processing misfolded or aggregated proteins. In a screen for genetic factors modifying the polyglutamine-mediated eye degeneration in drosophila, only two suppressors, HDJ-1 and a tetratricopeptide repeat protein 2 (both of which contain chaperone-related J domains), were isolated (Kazemi-Esfarjani and Benzer 2000). Others have shown that Hsp40, Hsp70, and Hsp104 are important in yeast responses to polyglutamine inclusions (Glover and Lindquist 1998; Muchowski et al. 2000). Thus it is possible that Hsp70 and related co-chaperones may play an important role in α-synuclein misfolding in PD. Most recently, it has been shown that Hsp70, Hsp40 and Hsp27 are all upregulated in SN following targeted viral vector-mediated overexpression of α-synuclein in a mouse model of PD (St Martin et al. 2007).

There is strong evidence that HSPs are prominent components of LBs (Table 1), and can modify α-synuclein aggregation in vitro (McLean et al. 2002; Klucken et al. 2004b; Outeiro et al. 2006). How specific molecular chaperones contribute to PD pathogenesis and modify α-synuclein misfolding is discussed in more detail in the following sections.

HSP70 IN PD

Hsp70 is the major molecular chaperone induced in the cytoplasm under stress conditions, while its constitutively expressed homologue, Hsc70, may play a greater role in normal protein folding (Fink 1999); Hsp70 and its homologues have been characterized by crystallography, and its structure has been studied extensively (Zhu et al. 1996; Sriram et al. 1997; Sondermann et al. 2001). There are two major domains: an N terminal ATPase domain, and a C terminal ligand binding domain. Hsp70 exists in cells in two conformations: ATP-bound, or ADP-bound, with the latter having enhanced chaperone activity. Yet, Hsp70 does not act alone. Based on analogy to *E coli* and yeast systems, the interactions of mammalian

HSPs are a complex interplay in which various molecular co-chaperones are critical for HSP functions. Hsp70 forms a complex with Hsp40s (including HDJ-1 and HDJ-2, human homologues of the HDJ family) that contain J domains that interact with Hsp70 and enhance chaperone activity. Hsp70 can also form a complex with Hsp90 (mediated by hop (heat shock protein organizing protein, p60)) that includes Hsp70, Hsp90, hop, p23, and a cyclophilin (Scheufler et al. 2000). Other co-chaperones are especially important to direct proteins towards the proteasome, using both ubiquitination and non-ubiquitin dependent mechanisms. For example, CHIP (carboxy terminus Hsp70 interacting protein) appears to be an E3 ubiquitin ligase (Connell et al. 2001; Imai et al. 2002).

Recent data in a PD fly model suggest that overexpression of the molecular chaperone Hsp70 protects against α-synuclein-induced degeneration (Auluck et al. 2002; Bonini 2002). There is strong evidence that Hsp70, its co-chaperones of the HSP40 class, HDJ-1 and HDJ-2, as well as other potentially important molecular chaperone proteins Hsp27, Hsp90 and the putative chaperone protein torsinA are associated with Lewy bodies (McLean et al. 2002). Data also suggest that over-expression of Hsp70 and related molecules can prevent α-synuclein aggregates from forming in vitro (McLean et al. 2002), is protective against the toxic effects of α-synuclein expression in cell culture, and that overexpression of Hsp70 diminishes the high molecular weight (HMW) smear of α-synuclein found in α-synuclein overexpressing transgenic mice. Moreover, the ansamycin antibiotic and Hsp90 inhibitor, geldanamycin was found to upregulate Hsp70 and protect against α-synuclein-induced toxicity in mammalian cell culture as well (McLean et al. 2004).

The chaperone function of Hsp70 requires the N-terminal ATPase domain in coordination with the C-terminal substrate binding domain. A single amino acid substitution (K71S) in the ATP-binding domain in *Drosophila melanogaster* results in loss of function in an in vitro clathrin uncoating assay (Elefant and Palter 1999). Likewise, the same single amino acid substitution in the ATPase domain of mammalian Hsp70 results in a loss of the Hsp70 refolding activity and interferes with its ability to prevent α-synuclein induced toxicity (Klucken et al. 2004a). However, Hsp70 K71S still mediates α-synuclein degradation and prevents aggregation, suggesting a dissociation between toxic and aggregating α-synuclein molecules.

HSP27 AND SMALL HEAT SHOCK PROTEINS

Small HSPs share a number of features, including low monomeric molecular mass, formation of large oligomeric complexes, and the presence of an αB-crystallin domain that occurs near the C-terminal region (de Jong et al. 1988). Along with Hsp70, Hsp27 is one of the main inducible HSPs in the nervous system and it has been shown to be neuroprotective against a variety of stresses and stimuli, including heat stress and oxidative conditions (Pauli et al. 1990; Benjamin and McMillan 1998; Wagstaff et al. 1999). It has also been shown to interfere with

apoptosis by preventing caspase activation (Bruey et al. 2000; Rane et al. 2003). Notably, Hsp27 prevents polyglutamine-induced toxicity in a cellular model of huntington's disease, where it acts upon the oxidative stress response caused by overexpression of mutant huntingtin (Wyttenbach et al. 2002). Hsp27 mRNA is upregulated in DLB brain compared to control (Outeiro et al. 2006) and Hsp27 and αB-crystallin are found in LBs in DLB and in glial cytoplasmic inclusions in multiple system atrophy (McLean et al. 2002; Pountney et al. 2005). Hsp27 has been found to be protective against a variety of stresses in α-synuclein overexpressing cells (Zourlidou et al. 2004) and can prevent aggregation and α-synuclein induced cytotoxicity in a neuroglioma cells in culture and primary dopaminergic neurons (Outeiro et al. 2006). Therefore, increasing levels of Hsp27 in the brain may be a protective strategy to cope with misfolded and/or aggregated α-synuclein and pharmacological elevation of Hsp27 may hold potential as a therapeutic strategy for PD and other synucleinopathies.

COCHAPERONES: CARBOXY-TERMINAL HSP70 INTERACTING PROTEIN (CHIP) AND ST13

Impaired proteasomal degradation of α-synuclein leads to enhanced aggregation and toxicity both in vitro and in vivo after proteasome inhibition (McLean et al. 2001; McNaught et al. 2002a, b; Rideout and Stefanis 2002). Additional support for this idea comes from consideration of another gene implicated in PD: Mutations in parkin, an E3 ubiquitin ligase, underlie juvenile onset PD (Kitada et al. 1998), and a mutation in ubiquitin C-terminal hydrolase is a rare cause of inherited PD (Leroy et al. 1998). HSPs are important in both refolding activities and in directing proteins towards proteasomal degradation (Cyr et al. 1994; Pirkkala et al. 2000; Bailey et al. 2002). The role of HSPs in either refolding or ubiquitination of proteins is partly dependent on interacting proteins such as CHIP (carboxyl terminus Hsp70 interacting protein), a tetratricopeptide repeat protein that negatively regulates Hsp70 and Hsp90 mediated refolding of proteins. Instead, CHIP directs proteins towards degradation (Ballinger et al. 1999; Connell et al. 2001). CHIP inhibits the ATPase activity of Hsp70 and can also bind to an Hsp90 complex, causing the release of p23 (a necessary co-chaperone for refolding) and, likely at least in part through an interaction with Bag-1, directs the misfolded protein towards degradation. CHIP has been shown to be a U-box containing ubiquitin E3 ligase, and is responsible for ubiquitinating misfolded mutant cystic fibrosis transmembrane conductance regulator (CFTR) (Meacham et al. 2001). CHIP also interacts directly with parkin (Imai et al. 2002), providing another level of regulation of the cell decision towards refolding or proteasomal degradation, and a direct link to PD phenomenon. CHIP immunoreactivity is observed in LBs in human tissue and an interaction of CHIP with α-synuclein that leads to increased degradation via both the proteasome and lysosome in a domain dependent manner (Shin et al. 2005). These data support the hypothesis that chaperone-mediated pathways involved in handling misfolded proteins play a role in Parkinson's disease.

An Hsp70 cofactor that is structurally related to CHIP is ST13. Also known as Hip (Hsp70 interacting protein), ST13 was found to be underexpressed in blood cells of PD patients and may thus be dysregulated in PD (Scherzer et al. 2007). The discovery of genes that are dysregulated in PD compared to healthy controls, regardless of their expression changes in other neurodegenerative diseases, may provide biological insights into the molecular pathology underlying PD-related changes in blood cells and may offer a robust noninvasive means of identifying PD-related changes in blood.

OTHER MOLECULAR CHAPERONES: DJ-1

DJ-1 is a familial Parkinson's disease-related gene that has been associated with autosomal recessive Parkinsonism (Bonifati et al. 2003). DJ-1 is a small protein member of the highly conserved *ThiJ* domain family which has associated chaperone and protease activities (Wilson et al. 2005). DJ-1 deficiency sensitizes dopaminergic neurons to oxidative stressors (Shendelman et al. 2004) and DJ-1 overexpression appears to be protective against oxidative toxic insults (Martinat et al. 2004). Oxidized DJ-1 appears to be broadly active as a chaperone towards a variety of protein substrates including, α-synuclein (Shendelman et al. 2004). Furthermore, DJ-1 can inhibit fibrillization of recombinant α-synuclein in a cell-free in vitro assay (Shendelman et al. 2004) and in vivo (Zhou et al. 2006). DJ-1 can interact with α-synuclein in intact living cells which suggests a mechanism whereby DJ-1 could inhibit α-synuclein fibrillization (Mclean et al., unpublished observation). Furthermore, PD-associated mutations in DJ-1 prevent homo-dimer formation and decrease the stability of the protein (Moore et al. 2003). Taken together, it is reasonable to propose that DJ-1 can reduce α-synuclein-mediated toxicity by sequestering toxic species and that targeting DJ-1 stabilization may be an important therapeutic strategy.

PHARMACOLOGICAL UPREGULATION OF CHAPERONE FUNCTION

The observations outlined in the preceding sections suggest that enhancing the function of HSPs is a useful strategy to inhibit toxic aggregate formation in PD and related protein-misfolding diseases. Thus, modulators of chaperone activities are a new and emerging field of drug development. Data suggest that it may be possible to modulate HSPs using one of several pharmacologic strategies. Inhibition of Hsp90 has emerged as a very promising tool to combat various forms of cancer whereas the induction of Hsp70 has proven to be an efficient help in the recovery from large number of diseases including ischemic heart disease, diabetes and more recently neurodegeneration.

Hsp90 is an ATP dependent chaperone that is involved in the folding and stabilization of many client proteins. The unique ATP binding site allows the development of specific Hsp90 inhibitors. Recently if was shown that Hsp90 contains

a second nucleotide binding site at it C-terminal domain, which may open new possibilities for the inhibition of this chaperone. Chaperone-based inhibitors do not interact with the effector proteins, but instead inhibit the ability of the associated chaperones to maintain their activation-competent conformation. As a result, the client proteins become degraded by the proteasome (Schulte et al. 1997). In contrast to most inhibitors, Hsp90 inhibitors diminish the level of the client protein targets in parallel, which increases overall toxicity and may narrow the therapeutic window.

The most well-characterized Hsp90 inhibitors are the naturally occurring ansamycin antibiotic geldanamycin (GA) and it's less toxic analogs, 17-allyamino- 17-demethoxy-geldanamycin (17-AAG), 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), and radicicol, its more stable oxime derivative (Figure 3). 17-AAG, 17-DMAG, and radicicol all have higher affinity for Hsp90 than GA. Recently, new GA analogs and a third class of Hsp90 inhibitors, the purine-scaffold inhibitors, have been developed. GA inhibits Hsp90 chaperone function by reducing ATPase activity which has two functional consequences: (a) enhanced degradation of client proteins bound to Hsp90 and (b) activation of heat

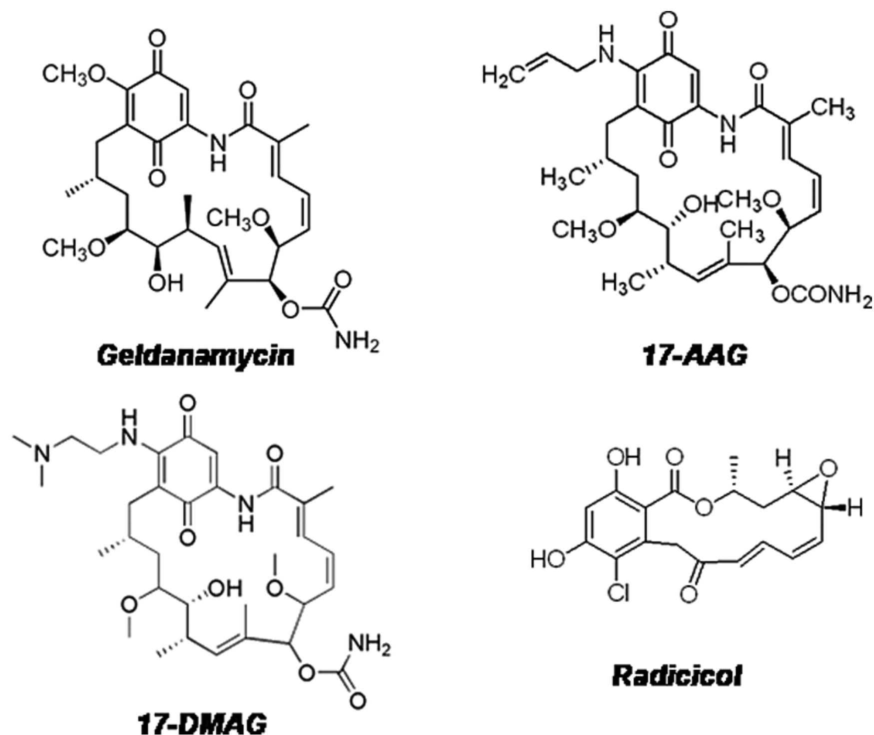

Figure 3. Structures of the most common Hsp90 inhibitors geldanamycin, 17-AAG, 17-DMAG, and radicicol

shock factor 1 (HSF1), a transcriptional activator of other stress-induced chaperone proteins such as Hsp70.

In neurodegenerative disease, studies have shown that Hsp90 inhibitors have potential neuroprotective effects. By taking advantage of the HSP-inducing effects of Hsp90 inhibitors, neuroprotection has been demonstrated in models of huntington's disease, tauopathies and PD. In tauopathies, Hsp90 inhibitors were shown to selectively decrease phosphorylated tau levels in the brains of human tau overexpressing mice (Dickey et al. 2007). Consistent with these results, treatment with GA was shown to inhibit α-synuclein toxicity in a cell-culture model (McLean et al. 2004) and in transgenic *Drosophila* (Auluck and Bonini 2002). The protective effect of GA occurred in parallel with a decrease in α-synuclein aggregation in culture, whereas it correlated with an increase in detergent resistant, presumably nontoxic α-synuclein species in the brains of transgenic flies. GA was also found to protect against dopaminergic neurotoxicity in MPTP treated mice (Shen et al. 2005). In polyglutamine disorders, GA and another Hsp90 inhibitor, radicicol, suppressed Huntington toxicity and aggregation in organotypic slice cultures derived from huntingtin transgenic mice (Hay et al. 2004). The data from all of these studies support a model in which Hsp90 inhibitors inhibit the formation of toxic protein aggregates by upregulating Hsp70.

In addition to the Hsp90 inhibitors, other small molecules have been shown to induce the heat shock response, especially upregulating Hsp70, which may also have therapeutic value in a variety of diseases. There are various approaches to induce Hsp70. Stannous chloride has been shown to be a nontoxic efficient inducer of Hsp70 (House et al. 2001) as have geranyl-geranyl acetone (Yasuda et al. 2005) and carbenoxolone (Nagayama et al. 2001). Other drugs, such as aspirin (Jurivich et al. 1992) have been described to induce Hsp70 via their chaperone co-inducer effects. It remains to be determined if induction of Hsp70 via means other than Hsp90 inhibition will be effective against α-synuclein aggregation and toxicity in PD and related disorders.

Taken together, upregulation of HSPs may represent a promising therapeutic strategy to target PD, DLB and other neurodegenerative diseases where protein misfolding is central to their pathogenesis.

CHAPERONE-MEDIATED AUTOPHAGY IN PARKINSON'S DISEASE

Chaperone-mediated autophagy (CMA) is a selective pathway for the degradation of cytosolic proteins in lysosomes (reviewed in (Kaushik and Cuervo 2006)). CMA declines with age because of a decrease in the levels of lysosome-associated membrane protein (LAMP) type 2A, a lysosomal receptor for this pathway (Cuervo and Dice 2000). The substrate proteins are recognized by a chaperone–cochaperone complex which delivers them to the lysosomal membrane (Chiang et al. 1989). Here they bind to LAMP-2A (Cuervo and Dice 1996), and after unfolding, the substrate proteins are translocated across the lysosomal membrane assisted by a lysosomal – resident chaperone, following which they are degraded in the hydrolase-rich lumen

(Kaushik and Cuervo 2006). CMA is unique in that it selectively degrades cytosolic proteins which contain a CMA-targeting motif.

Both cytosolic and lysosomal chaperones are required for completion of CMA. The cytosolic chaperone, Hsc70, recognizes and binds to the targeting motif in the substrates. The interaction between Hsc70 and the substrate is modulated by other cytosolic co-chaperones including Hsp90, Hsp40, hip, hop, and bag-1 (Agarraberes and Dice 2001).

CMA has been predicted to play a role in PD and related synucleinopathies. Wild-type α-synuclein is efficiently degraded in lysosomes by CMA, but the pathogenic α-synuclein mutations, A53T and A30P are poorly degraded by CMA despite a high affinity for the CMA receptor (Cuervo et al. 2004). Mutant α-synucleins block the lysosomal uptake and degradation of other CMA substrates. CMA blockage then results in a compensatory activation of macroautophagy which cannot maintain normal rates of degradation under these conditions (Cuervo et al. 2004). Impaired CMA of pathogenic α-synuclein may favor toxic gains-of-functions by contributing to its aggregation. Mutant α-synuclein also inhibits degradation of other long-lived cytosolic proteins by CMA, which may further contribute to cellular stress, perhaps causing the cell to rely on alternate degradation pathways or to aggregate damaged proteins (Dauer and Przedborski 2003).

Targeting therapeutics to enhance CMA may prove beneficial for PD therapies. Indeed trehalose, a disaccharide and novel mammalian target of rapamycin (mTOR)-independent autophagy enhancer, was found to enhance the clearance of the autophagy substrates mutant huntingtin and mutant α-synuclein (Sarkar et al. 2007). Furthermore, trehalose and the mTOR inhibitor rapamycin together exerted an additive effect on the clearance of these proteins due to increased autophagic activity and protected cells against pro-apoptotic insults. The dual protective properties of trehalose (chemical chaperone and inducer of autophagy) combined with rapamycin may be relevant to the treatment of PD where mutant proteins are autophagy substrates (Cuervo et al. 2004).

CONCLUSIONS

Thus converging information implicate HSPs in PD, DLB and other synucleinopathies that involve α-synuclein misfolding or aggregation. (1) α-Synuclein misfolds and aggregates in LBs. (2) HSPs are present in LBs. (3) Hsp70 prevents α-synuclein mediated toxicity in a fly model overexpressing α-synuclein, as well as in multiple models of trinucleotide disease-related aggregation. (4) HSPs diminish aggregation of α-synuclein in both an in vitro model of α-synuclein aggregation and in α-synuclein overexpressing transgenic mice. (5) HSPs prevent toxicity in mammalian in vitro models of α-synuclein overexpression, independent of aggregate formation, suggesting that HSP mediated refolding may be critical to prevent toxicity. (6) Hsp90 inhibitors are effective in inhibiting α-synuclein toxicity and aggregation cell culture and animal models. Taken together the further

investigation of the links between HSPs and α-synuclein in neurodegenerative diseases is warranted.

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CHAPTER 4

HEAT SHOCK PROTEINS AS THERAPEUTIC TARGETS IN AMYOTROPHIC LATERAL SCLEROSIS

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Abstract: Amyotrophic lateral Sclerosis (ALS) is a progressive neurodegenerative disorder characterized by the loss of motoneurons in the motor cortex, brainstem and spinal cord, resulting in paralysis and death within 1–5 years of diagnosis. Although the precise etiology of ALS remains elusive, approximately 20% of cases are known to be familial and of these approximately 10%–20% are due to mutations in the ubiquitously expressed human Cu/Zn superoxide dismutase (SOD1) gene. Transgenic mice that over-express the mutant human SOD1 (mSOD1) protein exhibit a phenotype and pathology that resemble that observed in ALS patients. ALS is widely regarded as a motoneuron-specific disorder but increasing evidence indicates that non-neuronal cells also play a significant role in disease pathogenesis. Some characteristics of the disease observed in mice and patient tissue, such as the presence of insoluble protein aggregates containing heat shock proteins (Hsps) as well as the apoptotic degeneration of motoneurons, suggest that manipulation of the heat shock response (HSR) may be a successful strategy for the treatment of ALS. In this chapter evidence for the involvement of the various Hsp families in disease pathology and their therapeutic potential is reviewed based on the molecular characteristics of the Hsp sub-families

Keywords: Motoneuron degeneration; motor neuron disease; protein aggregation; apoptosis; co-chaperones; HSF-1

INTRODUCTION

Motor neuron diseases (MND) are a group of progressive disorders involving the nerve cells responsible for innervating voluntary skeletal muscles. There are four main types of MND: (i) Amyotrophic Lateral Sclerosis (ALS); (ii) Progressive Muscular Atrophy; (iii) Progressive Bulbar Palsy and (iv) Primary Lateral Sclerosis, although there can be a great deal of clinical and pathological overlap between

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each form. ALS is the generic term widely used in the USA, where it is also known as Lou Gehrig's disease, whereas MND is used more widely in Europe. ALS was first described in 1869 by the French neurologist, Jean-Martin Charcot (Charcot and Joffroy, 1869). It is a fatal, adult onset neurodegenerative disease that affects the upper and lower motoneurons in the brain and spinal cord. The disease is characterized by weakness and paralysis of voluntary skeletal muscles due to the progressive loss of motoneurons, ultimately leading to respiratory failure and death, usually within 2–5 years of diagnosis. The incidence of ALS is approximately 2 per 100,000 of the population, with prevalence at any point in time of 6 per 100,000. The vast majority of ALS cases are sporadic, with around 10% of cases which are familial (inherited). Approximately 120,000 new cases of ALS are diagnosed worldwide each year, and although it can strike at any age it is more commonly found in the 40–70 year age group. Despite intensive research, particularly during the past 10 years, there remains no effective treatment for this devastating disease. It is possible that the development of an effective disease-modifying therapy for ALS has been hindered by our relatively poor understanding of the pathogenesis of ALS. A major breakthrough came in 1993, with the discovery that approximately 10%–20% of the familial forms of ALS (FALS) were due to a mutation in the Cn/Zn Superoxide Dismutase 1 (SOD1) gene (Rosen et al., 1993) and to date, more than 100 mutations in the SOD1 gene have been discovered that are linked to ALS. Shortly following the discovery of mutant SOD1(mSOD1)-linked ALS, a transgenic mouse over-expressing the same mutant form of the human SOD1 protein was developed and this has become an indispensable tool for research not only investigating the pathogenesis ALS but also as an animal model for preclinical testing of potential therapeutic agents (Gurney et al., 1994).

MECHANISMS UNDERLYING ALS PATHOLOGY

It is now clear that ALS is a multi-factorial disease in which a number of pathological mechanisms contribute to the selective and progressive degeneration of motoneurons. The sporadic and familial forms of ALS show both phenotypical and pathological similarities and so an understanding of the mechanisms involved in the death of motoneurons in SOD1 mice, which model the familial form, are likely to also be of relevance to the majority of sporadic cases of ALS (see Shaw and Eggett, 2000; Bruijn et al., 2004; Shaw, 2005; Boillee et al., 2006). Motoneurons have some specific functional and morphological characteristics that may actually contribute to their selective vulnerability in ALS. For example, as a consequence of their high metabolic load, motoneurons are particularly susceptible to excitotoxic insults and oxidative damage (Shaw, 2005), so that even healthy motoneurons are more susceptible to activation of AMPA receptors than other neuronal populations (Carriedo et al., 1996). Motoneurons also have particularly high energy demands (Briese et al., 2005) which makes them susceptible to mitochondrial dysfunction. Indeed, altered mitochondrial morphology, increased mitochondrial Ca^{2+} levels, mitochondrial deposits of mutant SOD1 protein and reduced complex IV activity

have all been observed in tissue from both ALS patients and SOD1 mice (Siklos et al., 1996; Borthwick et al., 1999; Jaarsma et al., 2001; Menzies et al., 2002; Liu et al., 2004). Motoneurons also have some of the longest axons of all neurons in the human body, so that mutations or abnormal assembly of cytoskeletal neurofilament proteins will have a great impact on the function of motoneurons and, for example, alterations in neurofilament structure in motoneurons may lead to impaired axonal transport. Indeed, in approximately 1% of ALS cases a mutation in the neurofilament heavy chain has been identified and a number of neurofilament mutations have been shown to result in ALS-like symptoms in mice (Cote et al., 1993; Xu et al., 1993; Lee and Cleveland, 1994). Furthermore, functional deficits in axonal transport have also been observed in SOD1 mice, and these can be present prior to the onset of disease symptoms (Williamson and Cleveland, 1999; Ligon et al., 2005). In fact, deficits in axonal transport are present in motoneurons even during very early development and can be detected in E13 motoneurons cultured from embryonic SOD1 mice (Kieran et al., 2005). These deficits in axonal transport represent some of the very earliest pathological changes reported in SOD1 mice to date, suggesting that axonal transport defects may play a key role in ALS pathogenesis. Another hallmark feature of ALS which is also characteristic of other neurodegenerative disorders is the presence of intracellular ubiquitinated protein aggregates (Ince et al., 1998; Watanabe et al., 2001). However, although it has generally been accepted that such aggregates are toxic to neurons, recent results have suggested that aggregates may in fact either be inert by-products of the disease or may represent an effort of the part of cell to sequester and isolate toxic proteins (Gispert-Sanchez and Auburger, 2006). In either case, the presence of aggregates is suggestive of proteasome dysfunction, a result of which is the accumulation of ubiquitinated proteins that would have been otherwise degraded by the proteasome (Puttaparthi et al., 2003; Kabashi et al., 2004; Cheroni et al., 2005). There is evidence that aggregated mSOD1 can sequester otherwise vital proteins such as anti-apoptotic Bcl-2, cytoplasmic dynein and Hsps into the aggregates and so such aggregates can have toxic effects, even if only indirectly (Pasinelli et al., 2004; Matsumoto et al., 2005, 2006; Deng et al., 2006).

Although previously regarded as a motoneuron-specific disorder, surprising experimental evidence has recently established that ALS is a non-cell autonomous disease that requires multiple cell types and multiple disease factors to act together in order for the disease to manifest. The role of non-neuronal cells in ALS pathogenesis has been established in a number of elegant experiments which showed that expression of mSOD1 in either motoneurons or glia alone does not lead to disease (Gong et al., 2000; Pramatarova et al., 2001; Lino et al., 2002; Clement et al., 2003). Furthermore, increasing the proportion of healthy glia surrounding mSOD1-expressing motoneurons can protect the motoneurons from mSOD1 toxicity (Beers et al., 2006; Boillee et al., 2006). Activated microglia and astroglia mediate activation of inflammatory pathways (Kawamata et al., 1992; Alexianu et al., 2001). Microglial activation leads to the release of potentially neurotoxic reactive oxygen species (ROS), nitric oxide and proinflammatory cytokines (Elliott, 2001; Almer et al., 2002; Hensley et al., 2002). Astrocytes also play a critical role in ALS

pathogenesis and recent results have shown that mSOD1-expressing astrocytes release soluble factors that are toxic to motoneurons (Di Giorgio et al., 2007; Nagai et al., 2007). It has been suggested that ALS consists of distinct disease phases where pathological events in neurons play a key role in initiatiating disease onset whereas surrounding microglial cells influence disease duration (Boillee et al., 2006). However, the precise role of glial cells may be even more complex than currently suggested, as these cells are capable of not only initiating neurotoxic mechanisms but can also activate neuroprotective cascades (for review see Benarroch, 2005).

Ultimately, in ALS, pathological events involving non-neuronal cell populations and intracellular abnormalities within motoneurons eventually lead to the activation of the apoptotic cascade and subsequent death of motoneurons. In view of the multiple pathological pathways and cell populations that are involved, it is likely that the most effective therapeutic strategy for the treatment of ALS will involve targeting of multiple cellular sites and pathways. Increasing attention has recently focused on the possibility that manipulating ubiquitously expressed endogenous cellular defence pathways may be a successful approach to take in the treatment of neurodegenerative disorders. The heat shock response (HSR) forms part of such a cytoprotective mechanism that is present within all cells but until recently received little attention in the field of neurodegeneration in general and ALS in particular (Muchowski and Wacker, 2005). Heat shock proteins (Hsps) are a highly conserved, ubiquitiously expressed family of stress response proteins which are expressed at low levels under normal physiological conditions. However, in response to cellular stress the expression of Hsps is dramatically increased. Hsps can function as molecular chaperones, facilitating protein folding, preventing protein aggregation, or targeting improperly folded proteins to specific degradative pathways. However, some Hsps also play a role in regulating apoptosis by interacting directly with key components of the apoptotic pathway. Hsps are classified into subfamilies according to their molecular weight. In this Chapter the current understanding of the role of individual Hsps in the progression of ALS will be summarized, with a particular focus on the possible therapeutic implications that manipulation of this pathway may have for the treatment of ALS.

MEMBERS OF THE SMALL HEAT SHOCK PROTEIN (SHSP) FAMILY OF STRESS PROTEINS

Intracellular Effects of Small Heat Shock Proteins

Members of the small molecular weight Hsp family are more heterogeneous in their molecular weight than any other Hsp family, ranging from 16 to 40 kDa. The defining feature of this Hsp subfamily is the α-crystallin domain which is highly conserved from prokaryotes to mammals and which is thought play a crucial role in sHsp oligomerization following stress (de Jong et al., 1998; Haslbeck et al., 2005). Features common to all sHsps are firstly, that they can all be phosphorylated at

several sites and secondly, that they tend to form large oligomeric complexes, aggregates with a size of between 300 and 800 kDa. There are several reports that suggest that the phosphorylation state of sHsps influences their oligomeric size (Ehrnsperger et al., 1997). The phosphorylation of sHsps favors the dissociation of multimers (Rogalla et al., 1999; Hollander et al., 2004) in which form they act as molecular chaperones, providing protection against the negative effects of a diverse range of stress factors (Ciocca et al., 1993; Kato et al., 1994; Lelj-Garolla and Mauk, 2006). Interestingly, although there are numerous genes which are responsible for sHsp proteins, unlike other Hsp families and subfamilies, no specific "cognate" and "inducible" isoforms have yet been identified. sHsps are also distinct from other families of Hsps in that they do not bind and hydrolyse ATP in order to chaperone client proteins. Instead sHsps act as large chaperone oligomers, stably binding unfolded proteins. However, refolding of these non-native proteins requires the contribution of additional chaperone proteins, such as Hsp70.

The neuroprotective properties of sHsps are attributed not only to their ability to chaperone large proteins but to another intracellular function that is independent of their chaperoning activity. SHsps can bind to elements of cytoskeletal proteins such as intermediate filaments and microtubular proteins, particularly under conditions of stress, thereby stabilizing their cellular structure (Head and Goldman, 2000). SHsps participate in the process of actin polymerization/depolymerization, modulating the assembly of intermediate filament proteins and inhibiting the aggregation of tubulin. Un-phosphorylated Hsp27 is thought to act as an actin-capping protein, inhibiting actin polymerization when bound (Lavoie et al., 1993). In its un-phosphorylated form, Hsp27 also binds and sequesters actin monomers, making them unavailable for further polymerisation (During et al., 2007). Upon stress, Hsp27 is phosphorylated by p38 MAPK (Casado et al., 2007) causing a conformational change in the structure of Hsp27, resulting in the dissociation of Hsp27 from the barbed ends of actin filaments as well as from actin monomers. These monomers are then available in the cytosol for polymerisation with actin filaments (Pichon et al., 2004; During et al., 2007). This interaction of Hsp27 with cytoskeletan proteins is related to its protective function in a manner that promotes reorganization of actin filaments and stabilizes fibres when cells are exposed to stress (Beck et al., 2001; Head and Goldman, 2000). Another member of this family of sHsps, αB-crystallin, has also been shown under conditions of stress to be phosphorylated by p38 MAPK and to protect cytoskeletal filaments from destruction (Singh et al., 2007).

Another key role of sHsps is their anti-apoptotic function within normal cells (Tezel and Wax, 2000). Under conditions of stress and in the absence of Hsp27, markers of the apoptotic cascade such as cytochrome c and p38 phosphorylation rapidly increase (Schepers et al., 2005). However, in the presence of Hsp27, interaction between Apaf-1 and procaspase-9 is inhibited as a consequence of the binding of Hsp27 to cytochrome c (Garrido et al., 1999; Bruey et al., 2000; Garrido, 2002). There is also evidence that suggests that Hsp27 can inhibit the release of mitochondrial cytochrome c (Gorman et al., 2005; Schepers et al., 2005) and association

with Daxx, thereby inhibiting the motoneuron-specific molecular death pathway of Fas-Ask1-p38 mediated apoptosis (Charette et al., 2000; Raoul et al., 1999, 2002, 2005, 2006).

Distribution of Small Heat Shock Proteins in Healthy Tissues

αB-crystallin was first identified as a structural protein in the lens of the eye, but it is now known to have a wider physiological role. For example, αB-crystallin is a component in the central nervous system, where it is expressed in oligodendrocytes and astroglia (Clayton and Truman, 1968; Iwaki et al., 1989). Other members of the sHsp family, such as Hsp27 and the mouse homologue Hsp25, are also constitutively expressed in the adult nervous system, predominantly in neurons (Plumier et al., 1997; Armstrong et al., 2001; Franklin et al., 2005). During embryonic and early postnatal development, neurons gradually acquire the ability to express Hsp25 and Hsp27 and this expression has been shown to be essential for the survival of sensory and motoneurons (Gernold et al., 1993; Kalmar et al., 2002a). Indeed, in a number of models of neurodegeneration, a loss of sHsp immunoreactivity within neurons is thought to contribute to their degeneration (Pieri et al., 2001; Suzuki et al., 2007). Interestingly, this reduction in neuronal expression of sHsps is accompanied by an increased expression of sHsps in glial cells (Pieri et al., 2001; Suzuki et al., 2007).

Role of Small Heat Shock Proteins in ALS Progression

Although there is a normal pattern of expression of Hsp25 and Hsp27 in presymptomatic SOD1 mice (Strey et al., 2004), immediately prior to disease onset there is a transient loss in Hsp25 and Hsp27 immunoreactivity in spinal cord motoneurons (Maatkamp et al., 2004; Strey et al., 2004). However, by the late stages of disease, Hsp27 and αB-crystallin are up-regulated in the spinal cord of SOD1 mice (Vleminckx et al., 2002; Strey et al., 2004) although Hsp27 is predominantly present in the cytoplasm and nuclei of glial cells and some neurons and αB-crystallin is only present in the cytoplasm of reactive glial cells (Vleminckx et al., 2002; Wang et al., 2007). The absence of αB-crystallin and Hsp27 immunoreactivity in motoneurons in the late stages of disease, together with the fact that mSOD1 protein co-immunoprecipitates with Hsp25 (Strey et al., 2004), suggests that as disease progresses in SOD1 mice, sHsps become depleted in vulnerable motoneurons. This absence of Hsp27 in motoneurons during the later stages of disease in SOD1 mice is likely to contribute to the death of motoneurons and at the very least will render them more vulnerable to the high levels of stress that motoneurons are under, particularly during the later stages of disease. Indeed, it has been shown that the expression and phosphorylation of Hsp27 is essential for the survival of sensory and motoneurons in stressed conditions (Costigan et al., 1998; Benn et al., 2002; Kalmar et al., 2002a). A reduction in Hsp27 will increase the vulnerability of motoneurons

to factors that have been implicated in ALS pathogenesis including protein aggregation, axonal transport defects and apoptosis mediated by the FasL-ASK1-Daxx pathway, all characteristic cellular processes in which sHsps have been implicated.

Therapeutic Potential of Small Heat Shock Proteins

There are a number of studies that describe the beneficial effects of sHsp up-regulation in models of neurodegenerative diseases including Parkinson's, Alzheimer's and Huntington's disease (Outeiro et al., 2006; Perrin et al., 2007). Since these neurodegenerative disorders are characterised by protein aggregation, it would seem likely that the beneficial effects of increased levels of sHsps in these models is due to their chaperoning capabilities. However, there is evidence that suggests that the neuroprotective effects of sHsps in models of neurodegeneration are in fact related to their anti-apoptotic and other cytoprotective capabilities including their ability to reduce neuroinflammation and to protect against oxidative damage and excitotoxicity (Wagstaff et al., 1999; Wyttenbach et al., 2002; Akbar et al., 2003; Kalwy et al., 2003; Masilamoni et al., 2006). Hsp27 is a potent promoter of regeneration and has the ability to support actin reorganization and therefore axonal growth (Hirata et al., 2003; Williams et al., 2006; Dodge et al., 2006). In vitro, up-regulation of Hsp27 is more effective at protecting SOD1 motoneurons against cellular stress when other Hsps such as Hsp70 and Hsp40 are also up-regulated (Patel et al., 2005; Batulan et al., 2006). However, in an in vitro model of mSOD1 toxicity, although transfection with Hsp27 did not provide any cellular protection, mild heat shock that resulted in massive up-regulation of Hsp27 saved these cells against a subsequent thermal stress (Krishnan et al., 2006). These controversial results are reflected in vivo, where it was found that over-expression of Hsp27 in transgenic mice prevents the extensive death of motoneurons that would normally occur following neonatal nerve injury (Sharp et al., 2006).

The majority of studies investigating the effects of increased sHsp expression employ transgenic over-expression models to increase intracellular levels of Hsps. However, genetic manipulation of Hsps may not be the most effective approach to take when attempting to develop a successful therapy for ALS. Firstly the difficulty of ensuring efficient delivery to motoneurons within the CNS in humans must be overcome. Moreover, although genetic manipulation may result in an increased expression of the protein, it may also disturb the fine balance that exists between Hsp expression and interaction with their co-chaperones and client proteins as well as, in case of sHsps, their phorphorylation level. Furthermore, for sHsps, the level of phosphorylation determines the size of intracellular oligomers and thereby determines their cellular function. In short, simply increasing the amount of sHsps such as Hsp27 within motoneurons may be futile if the sHsp is unable to carry out its cellular function. It may therefore be a more successful approach to pharmacologically manipulate or modify those intracellular functions which are critical for the survival and function of diseased neurons. An example of such a pharmacological agent is a small peptide construct called 35b that has been tested in

a model of Alzheimer's and shown to affect intracellular Ca^{2+} levels, mitochondrial membrane potential, cytochrome c release as well as increasing Hsp27 expression (Faden et al., 2003, 2004). Thus, it appears that strategies that aim to simply increase levels of Hsp27 alone are likely to be less successful at rescuing motoneurons from cell death than an approach that aims to up-regulate Hsp27 in conjunction with other cytoprotective Hsps such as Hsp70.

THE HSP70 FAMILY OF STRESS PROTEINS

Role and Distribution of Hsp70 in the Healthy Nervous System

Members of the Hsp70 family are found in most cellular compartments including the cytosol, ER (grp78 or also known as BiP) and mitochondria (grp75). The cytosolic homologue consists of at least two isoforms: a constitutively expressed 73 kDa cognate form, called Hsc73 and an inducible 72 kDa isoform. There is little biochemical or biological/functional difference between the constitutive Hsp73 isoform and the highly inducible Hsp72 isoform, apart from different signals for their synthesis. Most Hsp70s possess ATP binding and hydrolysis activity in their N-terminal domain. Hsp70 family members bind to nascent proteins via their carboxyterminal domain (Hightower and Li, 1994). Members of the Hsp70 family differ primarily in their protein binding sites and their binding affinity to the nascent proteins. Hsp70 forms complexes with co-chaperone proteins that help to exert their activity and at the same time act as regulators of chaperone activity. The main positive regulator protein of Hsp70 is called Hip, which binds to the ATP-ase domain, upon which it is converted to the ADP-bound state. For ADP binding, the assistance of Hsp40 is necessary since Hsp70 has only a weak ATPase activity when not associated with Hsp40. Two other proteins, CHIP and Bag-1 have been described as negative regulatory proteins of Hsp70, inhibiting the ATPase-chaperoning activity of Hsp70 and instead redirecting client proteins towards the proteasome (Nollen et al., 2001; Song et al., 2001; Takayama and Reed, 2001). Thus, Hsc70 not only chaperones its client proteins but, with the help of its co-chaperones, it also assists in intracellular sorting, transport and degradation of proteins. Hence Hsc70 is also named as clathrin-uncoating ATPase, indicating its role in protein translocation within the cell. In the presence of ATP, Hsp70 protein binds to the clathrin coat of endocytotic vesicles, contributing to the removal of clathrin from the proteins. Hsp70 also capable of inducing to induce disaggregation of proteins, a widely accepted intracellular role of Hsp70 (Pelham, 1990). Another housekeeping function attributed to Hsp70 proteins relates to the translocation of proteins across membranes (Kang et al., 1990). By binding to unfolded nascent proteins, Hsp70 maintains the soluble form of the protein while being transported between organelles such as ER or mitochondria (Craig, 1990). This finding is confirmed by other studies showing that Hsp73 has a prolonged interaction with polypeptides that cannot fold properly, suggesting a role for members of the Hsp70 family in preventing aggregation of precursors and in stabilizing them in an unfolded

and therefore translocation-competent form (Becker and Craig, 1994). As expected of a housekeeping protein, Hsp70 is present in neurons and glial cells of the spinal cord throughout embryonic and postnatal development, but at relatively low levels which do not change with age (D'Souza and Brown, 1998; Loones et al., 2000; Kalmar et al., 2002a).

Hsp70 in Cellular Stress

Among heat shock proteins, the inducible Hsp72 is thought to be the main cytoprotective protein, protecting cells against the damaging effects of a variety of stressors. Whilst Hsc73 levels are high in unstressed nervous tissue, levels of the inducible Hsp72 isoform are almost indetectable. There are a number of stress conditions that induce the expression of the inducible Hsp72 protein including hyperthermia (Brown, 1983; Manzerra et al., 1993; Satoh and Kim, 1994; Xia et al., 1998), CNS injury (Brown et al., 1989), axotomy (New et al., 1989; Tedeschi and Ciavarra, 1997), hypoxia (Kitamura, 1994), glutamate excitotoxicity (Armstrong et al., 1996), oxidative stress (Dastoor and Dreyer, 2000; Paschen et al., 2001) and ischemia (Bertrand et al., 2000). Induction of inducible Hsp70 synthesis is thought to be the most important mechanism by which mild heat shock or ischemia protects cells from subsequent and more toxic insults (Rordorf et al., 1991; Currie et al., 2000; Kelly, 2005; for review see Yenari, 2002). Following exposure to cell stress, the chances of cell survival are related to the ability of the cell to elevate Hsp70 synthesis (Chen and Brown, 2007). Indeed, in a model of Huntington's disease it has been shown that variations in the ability of different neuronal populations in the brain to increase Hsp70 expression in response to the expression of the expanded polyglutamine tract played a critical role in determining their vulnerability to cell death (Tagawa et al., 2007). For example, these authors found that Hsp70 induction was greatest in cerebellar granule cells that are resistant in Huntington's disease and that silencing Hsp70 in these cells results in an increased vulnerability of this neuronal population to extended polyglutamine tract toxicity.

In spite of the accumulating in vitro and in vivo evidence for the role of Hsp70 in cellular protection, the mechanism by which this cytoprotection is generated is still not clearly understood. Much of our current understanding about the mechanism of Hsp70 mediated neuroprotection comes from cell lines or transgenic mice that overexpress Hsp70. Evidence accumulated using a number of stress models including models of injury, ischemia, polyglutamine tract toxicity and Aβ-toxicity, all suggest that the neuroprotective effects of increased expression of Hsp70 are a consequence of increased chaperoning activity by Hsp70 (Uney et al., 1994; Amin et al., 1996; Plumier et al., 1997; Fink et al., 1997; Cummings et al., 2001; Kelly et al., 2001; Hoehn et al., 2001; Fujiki et al., 2003; Magrane et al., 2004). It is thought that most stress conditions, including pathological states, compromise the structural integrity of proteins, exposing otherwise hidden structural domains. The binding of Hsp70 may protect these functionally crucial domains, keeping the protein chaperoned and thereby preventing its aggregation (Becker and Craig, 1994). However, there is also

evidence that suggests vulnerable neurons do not necessarily benefit simply from increasing the levels of neuronal Hsp70. Studies on transgenic mice over-expressing the inducible Hsp70 have shown that Hsp70 over-expression does not protect against ischemic insults (Olsson et al., 2004). Similarly, Hsp70 over-expressing astroglia are no more resistant to oxygen–glucose deprivation injury than normal astroglia (Lee et al., 2001). However, these results do not necessarily signify that Hsp70 does not have a cytoprotective role, but rather they may highlight the likely importance of the concerted interaction of Hsp70 with a number of co-chaperones, an interaction that may be essential if Hsp70 is to exert its optimal cellular effects. Indeed, co-expression of Hsp70 in combination with its co-chaperone Hsp40 has been used successfully to reduce aggregate formation (Kobayashi et al., 2000; Bailey et al., 2002).

On the other hand, the intracellular effects of Hsp70 go beyond its ability to maintain proteins in a folding-competent state by keeping them chaperoned. It has recently been recognized that Hsp70 interacts with members of the cellular machinery that regulates the cell cycle and members of the apoptotic cascade, and therefore plays an important role in the maintenance and survival potential of cells. Thus, Hsp70 is also an anti-apoptotic protein and this function is independent from its chaperoning activity. In addition, through interaction with one of its co-chaperones, Bag-1, Hsp70 can also regulate cell growth. Bag-1 can interact with the anti-apoptotic protein Bcl-2 and the growth regulatory protein Raf-1 (Takayama and Reed, 2001). Hsp70 competitively inhibits Bag-1 and Raf-1 interaction by binding and sequestering Bag-1. This competitive inhibition can be very strong, particularly following heat shock, when levels of Hsp70 are elevated (Hohfeld and Jentsch, 1997; Townsend et al., 2003). An even more direct regulatory role of Hsp70 in cell survival and cell death decisions involves Hsp70 binding to Apaf-1 (apoptotic protease activating factor-1). Apaf-1 is normally activated by cytochrome c released from mitochondria and this activation leads to the formation of the apoptosome and the downstream activation of caspase-9 (Beere et al., 2000; Saleh et al., 2000; Li et al., 2000; Matsumori et al., 2006; for review see Beere and Green, 2001). It is therefore clear that a sufficient expression and functional activity of members of the Hsp70 family is essential for the maintenance of normal cellular homeostasis and cell survival under normal and stressful conditions.

Hsp70 also has intracellular actions which are independent of its chaperoning and anti-apoptotic roles. Increasing evidence suggests a complementary regulation between Hsp70 and inflammatory mediators such cyclooxygenase-2 (COX-2) and the transcription factor NFkappaB. In models of inflammation and stroke, exposure to a preconditioning stress or up-regulation of Hsp70 has been found to reduce the expression of COX-2 and production of nitric oxide (NO) (Feinstein et al., 1996; Van Molle et al., 2002; Ialenti et al., 2005; Jo et al., 2006; Zheng et al., 2008). These anti-inflammatory actions of Hsp70 are mediated by the binding of Hsp70 to NFkappaB and its subsequent inhibition. On the other hand, inflammation is itself a stimulus for increased Hsp70 production (Rockwell et al., 2000; Ianaro et al., 2001). A negative feedback mechanism exists between some inflammatory mediators and

Hsp70. For example, COX-2 over-expression inhibits Hsp70 synthesis following heat shock, and this inhibition of Hsp70 expression can be reversed by exposure to inhibitors of COX-2 (Ethridge et al., 1998). Moreover, in a model of cellular stress in which pharmacological inhibition of the proteasome results in Hsp70 up-regulation, the addition of NFkappaB inhibitors further increases Hsp70 synthesis, implying a negative regulatory role of NFkappaB in the machinery of the heat shock response (Rockwell et al., 2000). A number of studies using various pharmacological inducers of Hsps such as the immunosuppressant FK506, the Hsp70 inducer geranylgeranyl acetate (GGA) or the herbal drug celastrol, have all been shown to have anti-inflammatory effects (Pinna et al., 2004; Oltean et al., 2005; Sinn et al., 2007). Quite surprisingly, non-steroid anti-inflammatory drugs (NSAIDs) that inhibit COX-1 and COX-2 also increase Hsp70 synthesis. However, this action of NSAIDs is probably mediated by activation of heat shock factor 1 (HSF1) rather than by inhibition of NFkappaB (Housby et al., 1999). HSF1 is the transcriptional inducer of hsp gene expression in response to cell stress.

Hsp70 in ALS

In the majority of familial and sporadic cases of ALS, intracellular aggregates containing ubiquitin, proteasomal proteins and Hsc70 are present in spinal cord motoneurons (Namba et al., 1991; Garofalo et al., 1991; Watanabe et al., 2001) and astroglia (Kato et al., 1997). However, the pattern of expression of Hsp70 in ALS spinal cord is somewhat controversial. For example, a recent study found no Hsp70 up-regulation in either motoneurons or astroglia of ALS patients (Batulan et al., 2003), whereas in the spinal cord of SOD1 mice, clear Hsc70 immunoreca-tivity was observed in motoneurons, localized to inclusions (Watanabe et al., 2001; Howland et al., 2002). However, overall levels of Hsp70 within the spinal cord of SOD1 micer were not generally elevated (Vleminckx et al., 2002; Batulan et al., 2003).

There is overwhelming evidence that the SOD1 mutation alters the solubility of the protein, making it more prone to aggregation (Deng et al., 1993; Shinder et al., 2001.). The increased tendency of mSOD1 to aggregate suggests that Hsps in general and Hsp70 in particular, may play a crucial role in ALS pathogenesis. A number of studies have described the association of mSOD1 with Hsps and have also demonstrated the presence of Hsp27, Hsp40 and Hsp70 within mSOD1 aggregates (Shinder et al., 2001; Howland et al., 2002; Matsumoto et al., 2005). This finding supports the hypothesis that during disease progression in ALS, the sequestration of housekeeping Hsps into protein aggregates reduces the amount of Hsps available to undertake the other cellular functions that they are normally involved in, including cytoprotection, resulting in an increase in the vulnerability of motoneurons to cell death (Okado-Matsumoto and Fridovich, 2002). Furthermore, there is also a significant reduction in chaperoning activity in the spinal cord of SOD1 mice (Bruening et al., 1999). This is significant since it has been shown in in vitro models of mSOD1 toxicity, that resistance to mSOD1 toxicity is strongly

correlated with chaperoning activity and that over-expression of Hsp70 can significantly reduce markers of disease including aggregation (Bruening et al., 1999; Koyama et al., 2006).

However, motoneurons have a surprisingly high threshold for the activation of HSF-1 and as a consequence a significantly higher threshold for the induction of Hsps than other cells (Batulan et al., 2003). This inability to respond to stressful stimuli by activating this endogenous cellular defence mechanism, may in part explain why motoneurons are particularly vulnerable. For example, motoneurons are selectively susceptible to mutations in SOD1 which are ubiquitously expressed in neural and non-neural cells, but only result in the death of motoneurons. With an impaired ability to increase Hsp70 expression in response to stress, the vulnerability of mSOD1-expressing motoneurons is exacerbated by the fact that mSOD1 binds Hsp70 much stronger than wild-type SOD1, effectively depleting mSOD1-expressing motoneurons of the little Hsp70 that they have (Batulan et al., 2003; Matsumoto et al., 2005). Furthermore, interaction between mSOD1 and Hsp70 not only depletes the cell of Hsps but also inhibits the normal anti-oxidant functions of SOD1. Thus, it has been shown that the binding between mSOD1 and Hsp70 inhibits the uptake of SOD1 into mitochondria, causing an accumulation of reactive oxygen species (ROS) in mitochondria in the absence SOD1, a key antioxidant enzyme (Okado-Matsumoto and Fridovich, 2002). However, in an in vitro model of ALS using cell lines transfected with wild-type (wt) and mSOD1, exposure to oxidative stress modifies the conformation of wtSOD1 causing it to bind to Hsp70 in the same way as mSOD1. Thus, following exposure to oxidative stress, wtSOD1 can acquire toxic properties which are similar to mSOD1, which cause Hsp70 to be sequestered. This in turn results in inhibition of Hsp70-dependent cytoprotective mechanisms, under conditions of stress when Hsp70 chaperoning actions are most needed (Ezzi et al., 2007). These recent findings may be important for our understanding of the pathological mechanisms underlying the majority of sporadic, non-mSOD1 cases of ALS and suggest that SOD-1 dysfunction might also be involved in these cases. This also suggests that strategies that target SOD1 dysfunction may well be applicable to both familial mSOD–ALS as well as the sporadic forms of ALS.

Harnessing Hsp70 as a Therapeutic Target in ALS

Strategies that result in over-expression of Hsp70 have been shown to be neuroprotective in a number of models of stress such as thermal and ischemic damage as well as axotomy (Uney et al., 1994; Amin et al., 1996; Fink et al., 1997; Plumier et al., 1997; Hoehn et al., 2001; Kelly et al., 2001). The well known protective effects of a “preconditioning stress”, in which a mild stress is applied prior to a toxic and damaging insult, is mediated by up-regulation of protective Hsps, mainly comprising Hsp70 (Sato et al., 1996; Currie et al., 2000; Ahn and Jeon, 2006). In neurodegeneration, up-regulation of Hsp70 appears to be particularly successful in models of Huntingtons, Spino-Cerebellar-Ataxia (SCA) and

Kennedy's Disease (KD) (otherwise known as Spino-Bulbar Muscular Atrophy; SBMA), where neurodegeneration is known to be associated with an expanted polyglutamine tract in a specific protein (Kobayashi et al., 2000; Cummings et al., 2001; Bailey et al., 2002; Waza et al., 2005; Tagawa et al., 2007). In these models, neuronal death is preceded by the presence of well characterized protein aggregates which decrease in both size and formation following over-expression of Hsp70. Similar protective, anti-aggregate effects of Hsp70 over-expression have also been observed in models of other neurogenerative diseases such as Aβ-Amyoid and α-synuclein induced toxicity (Magrane et al., 2004; Zourlidou et al., 2004; Sahara et al., 2007).

There are several features of the pathomechanism of ALS that suggest that a therapy based on manipulation of Hsp70 may be particularly effective. Hsp70 can unload the proteasome by keeping stressed proteins in a folded state and this capability also enables Hsp70 to reduce protein aggregation. On the other hand, Hsp70 can also inhibit the apoptotic pathway that is eventually activated as a consequence of dysfunctional intracellular protein control in motoneurons in the later stages of disease. Since motoneurons have a reduced capacity to activate HSF-1 in response to stress, manipulation of Hsp-70 may be particularly effective in protecting motoneurons against stress (Batulan et al., 2003). Furthermore, manipulation of Hsp-70 levels in non-neuronal cells such as astroglia may also be effective in protecting motoneurons since it has been shown that even under normal conditions, motoneurons are supplied with essential Hsps by neighbouring astroglia (Hightower and Guidon, 1989; Guzhova et al., 2001). This hypothesis of a motoneuron–astroglia functional unit is supported by some evidence which shows that exogenously applied Hsp70 can be taken up by motoneurons (Robinson et al., 2005). Therefore, strategies that aim to increase Hsp-70 within motoneurons and astroglia may not only overcome the inherent inability of motoneurons to up-regulate Hsps in response to stress, but by increasing Hsp-70 expression within astroglia, such an approach will also support astroglial function, itself critical for motoneuron survival, and provide motoneurons with an additional source of Hsp-70 to be taken up as needed.

The experimental evidence for the beneficial effects of an Hsp70-based therapy for ALS are, however, very controversial. In in vitro models of mSOD1 toxicity, Hsp70 over-expression has been found to reduce aggregate formation, improve chaperoning activity and increase cell survival (Bruening et al., 1999; Koyama et al., 2006). These cytoprotective effects of Hsps are not restricted to Hsp-70 since over-expression of multiple Hsps, such as Hsp70, Hsp40 and Hsp27 is a more successful strategy to optimize the intracellular heat shock response in vitro (Takeuchi et al., 2002; Patel et al., 2005; Batulan et al., 2006). Unfortunately, these promising in vitro results have not always translated into successful in vivo strategies. Thus, although Hsp70 over-expression in vitro reduces aggregate formation and improves survival of primary motoneurons derived from SOD1 mice, increasing Hsp-70 expression in SOD1 mice in vivo, by crossing them with transgenic mice over-expressing Hsp70, has no effect on

disease progression or lifespan of SOD1 mice (Liu et al., 2005). Perhaps even more surprising was the finding that the Hsp70 over-expressing mice were as vulnerable to ischemic damage as wild-type mice (Olsson et al., 2004) and that astrocyte cultures from brains of Hsp-70 over-expressing mice were less resistant to hypoglycemia and oxygen–glucose deprivation than wild-type astroglia (Lee et al., 2001).

These findings suggest that transgenic models which have been generated to synthesize one particular Hsp behave differently and less effectively, from models in which the endogenous heat shock response is activated for example, following exposure to a preconditioning stress. However, all actions of Hsps involve a finely tuned interaction between Hsps and their co-chaperones and client proteins. It is therefore possibly more surprising that increased expression of a single Hsp, such as Hsp70, in the absence of targeting of co-chaperones, has any neuroprotective effects at all! Certainly, co-induction of multiple Hsps is a more successful strategy than up-regulation of individual hsps (Takeuchi et al., 2002; Patel et al., 2005; Batulan et al., 2006). Furthermore, since upregulation of most hsps is induced by HSF-1, strategies that activate this main heat shock transcription factor are likely to be particularly effective in protecting cells from stress. Under cellular stress HSF1, which normally resides in the cytosol, trimerizes and migrates to the nucleus. In the trimeric state, HSF1 has a high affinity for cis-acting DNA sequence elements, the heat shock elements (HSEs) in the promoter region of heat shock protein genes. The bound trimer forms a complex which is capable of activating transcription of the hsp gene. In vitro, transfection with a constitutively active HSF-1 offers significant protection against mSOD1-induced toxicity (Batulan et al., 2003). We have observed similar neuroprotective effects in vivo from pharmacological targeting of HSF-1 using a novel Hsp co-inducer called arimoclomol (Kieran et al., 2004). Arimoclomol is a member of a family of non-toxic hydroxylamine derivatives that have been shown to up-regulate Hsp40, Hsp70 and Hsp90, by prolonging the activation of HSF-1 (Vigh et al., 1997; Kalmar et al., 2002b; Hargitai et al., 2003; Kieran et al., 2004). We found that treatment of SOD1 mice with arimoclomol delays disease progression, increases motoneuron survival and significantly extends lifespan (Kieran et al., 2004). Celastrol is an herbal medicine that has a similar mechanism of action as arimoclomol and which has also been shown to be effective in extending the lifespan of SOD1 mice (Kiaei et al., 2005). However, although the results of these studies confirm that activation of HSF-1 can have significant neuroprotective effects, it is unlikely that these effects are simply the result of an up-regulation in the expression of specific Hsps such as Hsp70 and Hsp90. Indeed, it is likely that the neuroprotective effects of HSF-1 activation are the result of a number of diverse effects of HSF-1 that are independent of its ability to induce Hsp expression, which may help to explain why pharmacological manipulation of Hsp induction by activation of HSF-1 is more successful in protecting motoneurons than genetic manipulation of the expression individual Hsps (Liu et al., 2005).

THE HSP90 FAMILY OF STRESS PROTEINS

Most members of the Hsp90 family are cytosolic. There are two Hsp90 isoforms, Hsp90α (Hsp84) and Hsp90β (Hsp86). Hsp90α is the more inducible isoform, whereas Hspβ, sometimes called Hsc90, is less inducible and is mainly expressed constitutively as a cognate form. The N terminal domain of Hsp90 contains an ATP binding site, a sequence that is structurally homologous with the type II topoisomerase DNA gyrase B (Pearl and Prodromou, 2000). The binding site for geldanamycin, a functional blocker of Hsp90 ATPase is also located in the N terminal domain (Prodromou et al., 1997). The primary binding site of Hsp90 for other co-chaperones is located on the C terminal region of the protein. This site binds to the partner protein containing the sequence called tetratricopeptide repeat, TPR (Pearl and Prodromou, 2000). Hsp90 exists in the cell as phosphorylated dimer (Rose et al., 1987) and this dimerisation is essential for its physiological function. The protein has an ATP binding site and is capable for autophosphorylation (Csermely and Kahn, 1991). Upon binding to ATP, the Hsp90 dimer structure undergoes substantial changes so that from an open form, in which the N terminal domains are separated, it transforms into a form that contains an associated ring formed by the two N terminal domains.

Intracellular Functions of Hsp90

As a consequence of its own hydrophobic characteristics, Hsp90 binds positively charged or hydrophobic proteins. The client proteins of Hsp90, which can have widely different functions, include proteases, kinases and a group of nuclear hormone receptors including the glucocorticoid receptor, indicating a role for Hsp90 in differentiation and development (Pratt, 1997; Pratt and Toft, 1997). Apart from steroid receptor signalling, Hsp90 is known to play a role in the maturation and function of several tyrosine and serine/threonine kinases, such as V-Src, Wee-1, the cyclin dependent serine/threonine kinase Cdk4 and Raf and other enzymes, including nitric oxide synthase and calcineurin (Mayer and Bukau, 1999; Young et al., 2001; Pratt and Toft, 2003). It is most likely that the ability to interact with such a wide variety of substrates arises from similarities in the structural properties of these proteins rather than their cellular function. These proteins are very large, with a multi-domain structure and rely on co-chaperones such as Hsp90 to stabilize their structure while they undergo conformational changes as a consequence of the interactions with their own substrates. Due to variability in its substrates, Hsp90 plays a role in a number of cellular processes. Hsp90 interaction is required for the function of a series of oncogenic tyrosine kinases, so that inhibition of Hsp90 can disrupt this specific binding. Thus, geldanamycin, a specific Hsp90 inhibitor, is a promising candidate for anti-tumor therapy. Geldanamycin and another Hsp90 inhibitor 17-allylamino-17-demethoxy-geldanamycin (17-AAG) alter the Hsp90 complex with Raf-1 protein, which plays a significant role in MAPK pathway. Without the stabilizing interaction with Hsp90, client proteins are directed towards

the proteasome for degradation (Waza et al., 2006a). This leads to a decrease in Raf-1 levels and a disruption of the MAPK-mediated altered transcription factor signalling pathway (Bertorelli et al., 1998). Thus, Hsp90 may be an effective target for some cancer strategies (Pearl and Prodromou, 2000).

It is widely accepted that in most cases the de novo folding of proteins does not require Hsp90. However, a very restricted number of eukaryotic proteins are temporarily stabilized by Hsp90. These are molecules that have large hydrophobic surfaces that are important for binding to other functionally important substrates (Nathan et al., 1997). Hsp90 plays an important role in promoting disaggregation and refolding of denatured proteins and also has been shown to suppress the aggregation of unstable proteins (Miyata and Yahara, 1992). It binds to partially re-natured forms of proteins, maintaining their "folding-competent" state, so that they can be successfully refolded by other chaperones such as Hsc70 (Freeman and Morimoto, 1996).

Hsp90 and microtubular proteins have been shown to co-localize in a number of cell types (Fostinis et al., 1992; Liang and Macrae, 1997). Binding of Hsp90 to microtubules may explain their role in receptor trafficking, as translocation of proteins to organelles is usually mediated by the cellular microtubule-system. Hsp90 links cargo proteins to the dynein motor protein for retrograde transport along microtubules (Galigniana et al., 2004; Harrell et al., 2004). Hsp90 also contributes to the transport of some steroid hormones to the nucleus, a process that also requires some binding to the cytoskeletal system (Csermely et al., 1998). Hsp90 may also be associated with intermediate filaments although other cytoskeletal counterparts, such as microfilaments do not bind to Hsp90 (Redmond et al., 1989).

More recently, significant antiapoptotic properties have been attributed to Hsp90. In an in vitro model of apoptosis, over-expression of Hsp90, but not Hsp27 or Hsp70, provided significant protection against apoptosis (Lee et al., 2001). Hsp90 can interfere with the apoptotic cascade at multiple levels. Thus, Hsp90 can bind to Apaf-1, inhibiting the activation of the apoptotic complex and caspase-9 (Pandey et al., 2000). Through the binding to its client protein, Akt, Hsp90 phosphorylates and inactivates ASK1 (Zhang et al., 2005), which mediates Fas/Fasligand mediated apoptosis downstream from Daxx activation. This pathway has been specifically described in motoneurons during SOD1 mediated toxicity in ALS (Raoul et al., 2002). In addition, Hsp90 also interferes with inflammatory pathways by promoting NFkappaB activation through its co-chaperone cdc37 and its client IKK that phosphorylates NF-kappaB (Chen et al., 2002; Arya et al., 2007).

Grp94 is the Hsp90 isoform that is present in the lumen of the endoplasmic reticulum (ER). Similarly to Hsp90, grp94 is also an ATP binding protein that associates with a number of other proteins, including protein kinases, actin filaments and calmodulin. It can also associate with other ER chaperones such as grp78 (Melnick et al., 1992). However, unlike most Hsps, grp94 is a glycoprotein. Although Grp94 is constitutively expressed in all cell types, its expression is increased in response to various forms of stress including low glucose levels, ischaemia, low extracellular pH and viral infections (Argon and Simen, 1999).

A common feature of these stress conditions is that they all involve the accumulation of misfolded proteins within the ER. A change in the pattern of glycosylation has also been described following environmental stress, which results in an increased resistance of grp94 protein against endoglycosidase digestion (Booth and Koch, 1989). In addition to altered glycosylation, stress also induces the translocation of grp94 from the ER to the Golgi apparatus (Feldweg and Srivastava, 1995).

Disribution of Hsp90 in Healthy and ALS Tissues

Hsp90 is constitutively expressed in most mammalian tissues and in the nervous system it accounts 1%–2% of the total protein content (Heikkila, 1993; Loones et al., 2000). Hsp90 is up-regulated in vivo in response to various forms of stress, such as heat shock and ischaemia (Quraishi and Brown, 1995; Gasbarrini et al., 1998). Since Hsp90 is expressed at relatively high levels in neurons, up-regulation in Hsp90 levels following exposure to stress mainly occurs in astroglia and microglia. For example in mice, excitotoxic insults result in an increase in grp94 expression in astroglia and Hsp90 expression in microglia (Jeon et al., 2004). Furthermore, ischemic stress causes a more intense up-regulation of the endoplasmic grp94 than the cytoplasmic Hsp90 (Jeon et al., 2004), whereas oxidative damage causes a down-regulation of grp4 in primary neurons (Paschen et al., 2001).

In ALS tissue, weak Hsp90 immunoreactivity has been observed in sporadic and familiar ALS spinal cord sections, with expression mainly localized to inclusions (Watanabe et al., 2001). In spinal cord tissue of different strains of SOD1 mice, Hsp90 levels have been reported to be either normal ($SOD1^{G93A}$ mice; Kieran et al., 2004) or slightly elevated ($SOD1^{G85R}$ strain; Liu et al., 2005) during the late stages of disease.

Therapeutic Potential of Hsp90 Manipulation in ALS

Therapeutic manipulation of Hsp90 has been widely employed in cancer therapy, where inhibition of Hsp90 results in modifications in the cell cycle and selective degradation of Hsp90 client proteins that induce apoptosis in cancer cells (for review see Xiao et al., 2006; Cullinan and Whitesell, 2006). However, acting via an alternative mechanism, an Hsp90 targeted therapy may also be effective in the treatment of neurodegenerative diseases, particularly if aggregation of an Hsp90 client protein is involved. For example, in Kennedy's Disease (KD) a trinucleotide repeat expansion in the androgen receptor (AR) results in nuclear inclusions of the mutant AR with expanded polyQ in motoneurons and this eventually results in their death. In models of KD, treatment with Hsp90 inhibitors such as geranylgeranyl acetone (GGA), geldanamycin and 17-AAG have shown to be successful in reducing aggregation of the mutant androgen receptor by reducing the total amount of mutant AR (Katsuno et al., 2005; Waza et al., 2006a, b). However, this effect of Hsp90 inhibiton is not due to an enhanced heat shock response, but is instead the result of preventing Hsp90 from interacting with its disease-causing

client protein (Neckers, 2002; Zaarur et al., 2006; Waza et al., 2006b). Indeed, in some neurodegenerative diseases such as the tauopathies, it has been shown that Hsp90 maintains the mutant but not wild-type Tau protein (Luo et al., 2007). Inhibition of Hsp90 in cellular and mouse models of tauopathies leads to a reduction of the pathogenic activity of these proteins and results in elimination of aggregated Tau. Thus, in this model Hsp90 plays a crucial role in maintaining and facilitating the degenerative phenotype by binding to the aberrant protein. Thus, inhibition of Hsp90 provides a common platform for the development of therapies aimed at both cancer as well as neurodegenerative diseases (Luo et al., 2007).

An alternative hypothesis to explain the neuroprotective effects of Hsp90 inhibition involves the mechanism of activation HSF-1. It is possible that when HSF-1 activity is suppressed, an Hsp70–Hsp90 complex sequesters HSF-1. Interaction of geldanamycin with Hsp90 frees HSF-1 from the binding of Hsp70–Hsp90 complex, enabling HSF-1 activation and translocation into the nucleus where it binds to the HSE element of heat shock genes (Ali et al., 1998; Kim et al., 1999; Lu et al., 2002), whereas GGA preferentially binds to the C-terminal of Hsp70 that binds and inhibits the activation of HSF-1 (Otaka et al., 2007). In this way, inhibition of Hsp90 can ultimately lead to an up-regulation of other Hsps, such as Hsp40, Hsp70 as well as Hsp90. If this mechanism of action could be successfully harnessed in ALS, it may reduce inclusion formation in motoneurons and astroglia. Indeed, in mSOD1 expressing primary spinal cord neurons, 17-AAG results in a robust up-regulation of multiple Hsps, such as Hsp70 and Hsp40 and a reduction in inclusion formation (Batulan et al., 2006). Arimoclomol (also known as BRX-220) is a co-inducer of Hsps that has been shown to protective to motoneurons against injury-induced and mSOD1-induced cell death. Arimoclomol also up-regulates the expression of multiple Hsps in motoneurons and astroglia under stress conditions (Kalmar et al., 2002b; Kieran et al., 2004). Thus, it is possible that Hsp90 up-regulation may be beneficial in ALS but is likely to be most effective when working in conjunction with the induction of other Hsps, such as Hsp70 and Hsp40.

HSP60 OR GROEL HOMOLOGUES RESIDING IN THE CYTOSOL AND MITOCHONDRIA

Members of this Hsp family are referred to by a variety of names, most commonly by the term chaperonin, although the terminology depends on when and in which organisms these Hsps were first described. In *Escherichia coli*, a 60 kDa protein and a 10 kDa protein that form the GroE complex were first described and named GroEL and GroES, respectively. All members of the Hsp60 family are large oligomeric ring-shaped proteins, consisting of 14 subunits arranged in two heptameric rings (for reviews see Sigler et al., 1998; Thirumalai and Lorimer, 2001). They contain a large hydrophobic central cavity in which non-native proteins can bind. In the mouth region of the central cavity is the ATP binding site (Fink, 1999). The chaperone function of Hsp60 is regulated by Hsp10, which binds to Hsp60 and regulates substrate binding and ATPase activity. In the presence of ADP, two Hsp10

molecules bind to one Hsp60 molecule. It is likely that the GroE family assists in the correct folding of newly synthesized proteins within the cytosol, but at a later stage in the folding process than members of the Hsp70 family. The GroE family tend to bind to partially folded intermediates, preventing their aggregation. In addition, the GroE complex folds proteins that are imported from the cytosol into the mitochondria (Welch, 1990). Although Hsp60 and Hsp70 proteins have very similar biochemical characteristics, their physiological functions are not interchangeable and they appear to act sequentially in a common pathway to facilitate the folding and assembly of proteins.

Distribution and Function of Hsp60 in Healthy Tissues

Constitutive expression of Hsp60 has been described in both neural and non-neural tissues (D'Souza and Brown, 1998) with most Hsp60 family members located in mitochondria. However, more recently it has been discovered that a homologue of the mitochondrial chaperonins resides in the cytosol (Gupta and Knowlton, 2002; Kirchhoff et al., 2002). It appears that of the total cellular Hsp60 content, around 80% is present in mitochondria and the remaining 20% resides in the cytosol (Soltys and Gupta, 1996) in almost all tissue types, apart from cardiac and skeletal muscle where cytoplasmic Hsp60 is even more abundant (Gupta and Knowlton, 2005). Exposure to stress, such as ischemia or heat shock, results in Hsp60 up-regulation (Naylor et al., 1996; Izaki et al., 2001).

Since the mitochondrial genome encodes only a handful of proteins, a great deal of protein transport takes place through the mitochondrial membranes, shuttling essential proteins through membranes that function in the mitochondria but are encoded by the nucleus. Because these proteins can be bulky, when passing a membrane they need to first unfold and then at their destination, they need to refold into their original conformations. Mitochondrial chaperones assist this process of refolding. The requirement for this refolding is greater during mitochondrial biogenesis and mitochondrial molecular chaperones are therefore thought to play a critical role in mitochondrial biogenesis (for review see Voos and Rottgers, 2002; Deocaris et al., 2006).

Different members of the Hsp60 family have opposing roles in the process of apoptosis. The cytosolic Hsp60 binds to pro-apoptotic Bax and Bcl-XL thereby preventing activation of the apoptotic cascade (Lin et al., 2001; Kirchhoff et al., 2002; Shan et al., 2003; Gupta and Knowlton, 2005). On the other hand, the mitochondrial Hsp60/Hsp10 complex binds to procaspase-3 and enhances its protease sensitivity, thereby promoting apoptosis (Samali et al., 1999).

Implications for a Role of Hsp60 in ALS

To date there have been a limited number of studies investigating the role of Hsp60 in the progression of ALS. In other neuropathological conditions there is evidence

that shows an up-regulation in Hsp60 expression in astroglia and oligodendrocytes. However, Hsp60 is not up-regulated in motoneurons in ALS (Martin et al., 1993). In spite of conflicting evidence for the involvement of Hsp60 members in the process of apoptosis, it appears that increased Hsp60 expression is protective against β-Amyloid toxicity (Veereshwarayya et al., 2006) although it has been only shown to be protective in conjunction with increased synthesis of other Hsps, such as Hsp70 and Hsp90 (Veereshwarayya et al., 2006).

THE HSP105/110 FAMILY OF STRESS PROTEINS

All members of this Hsp family possess an ATPase activity that is essential for them to exert their physiological action. The Hsp105/110 family of proteins were first described as homodimers co-precipitating with muscle actin (Koyasu et al., 1986). Members of this family also form homo-oligomers in the presence of adenine nucleotides. The heat-inducible members of this family are also known as Clp-s in prokaryotic cells and they are essential for survival following heat stress or exposure to ethanol or arsenite. Eucaryotic Hsp105/110 proteins contain two nucleotide-binding domains called NBD1 and NBD2 (Parsell et al., 1991; Schirmer et al., 1996). NBD1 is primarily responsible for the ATPase activity of this Hsp, whereas mutations in the NBD2 domain inhibit the oligomerisation of Hsp104, indicating its role in oligomer formation (Schirmer et al., 1998). Hsp104 has been described in yeast, whereas Hsp110 is the mammalian homologue (Parsell et al., 1991). Hsp105 alpha and Hsp105 beta are members of the same mammalian Hsp105/110 family.

The Hsp 105/110 family of proteins do not function to prevent the formation of aggregates but instead they mediate the re-solubilization of heat-inactivated proteins from insoluble aggregates (Parsell et al., 1994; Glover and Tkach, 2001). However, for effective protein refolding, Hsp104 requires a coordinated action with Hsp40 and Hsp70 (Glover and Lindquist, 1998). More recently it has been discovered that the nature of this cooperation between Hsps is such that Hsp110 acts as a co-chaperone of Hsp70, synergizing with Hsp40 in accelerating nucleotide exchange and thus, the ATPase (and folding) activity of Hsp70 (Dragovic et al., 2006; Shaner and Morano, 2007). On the other hand, whilst Hsp105 can strongly suppress aggregation, it can also inhibit the ATPase activity of Hsp70 (Yamagishi et al., 2003, 2004). This Hsc70-regulatory function of Hsp105 is regulated by its phorphorylation levels. Thus, suppression of Hsc70 mediated protein folding is abolished when Hsp105 alpha is phosphorylated (Ishihara, 2003).

Since Hsp105 levels are elevated in a number of malignant cells, it has been assumed that this family of Hsps can also play a role in the regulation of the cell cycle and apoptosis. Indeed, silencing Hsp105 induces caspase-mediated apoptosis (Hosaka et al., 2006). Hsp110 is also induced during developmental cell death (Evrard et al., 2000). It is thought that Hsp105/110 members negatively regulate the apoptotic pathway by inhibiting the translocation of the proapoptotic protein,

Bax into mitochondria and by inhibiting the activation of C-JUN kinase (Yamagishi et al., 2006; Hatayama et al., 2001).

DISTRIBUTION OF THE HSP100 FAMILY OF STRESS PROTEINS: IMPLICATIONS FOR ALS AND OTHER NEURODEGENERATIVE DISEASES

Members of the Hsp105/110 family are constitutively expressed in the developing and adult nervous system, in the cytoplasm and nuclei of most neurons and glial cells (Satoh et al., 1998; Easton et al., 2000; Hylander et al., 2000; Evrard et al., 1999, 2000). Stress conditions such as heat stress and ischemia all induce Hsp110 expression in neurons and glia (Gauley and Heikkila, 2006; Gashegu et al., 2007). During embryonic development, Hsp110 expression increases just prior to caspase activation, suggesting that Hsp110 may play a proapoptotic role during embryogenesis (Evrard et al., 2000; Gashegu et al., 2007). However, increased Hsp110 levels have been shown to be protective against proapoptotic stimuli in the adult nervous system (Hatayama et al., 2001; Yagita et al., 2001; Yamagishi et al., 2006).

As would be expected from their ability to re-solubilize protein aggregates, members of the Hsp105/110 family are excellent therapeutic targets for diseases that involve toxic aggregates. Thus, effective delivery of Hsp105/110 into cells with toxic aggregates could successfully reduce inclusions even at late stages of the disease. However, to date there is limited data demonstrating the effects of Hsp105/110 in models of neurodegeneration. In a cellular model of KD, overexpression of Hsp105 alpha efficiently reduced the formation of mutant androgen receptor aggregates (Ishihara et al., 2003). In a model of Huntington's disease, also caused by expanded polyglutamine tract, lentiviral delivery of Hsp104 successfully reduced aggregate formation when delivered in conjunction with an Hsp27 lentivirus (Perrin et al., 2007). In the SOD1 mouse model of ALS, mSOD1 has been shown to bind to Hsp105 and Hsp105 levels decrease with disease progression. This finding once again supports the possibility that depletion of Hsps is a disease-causing event in ALS that results in the accumulation of toxic aggregates (Okado-Matsumoto and Fridovich, 2002; Yamashita et al., 2007). Recent results have also shown that overexpression of Hsp105 in a cellular model of ALS dramatically reduced aggregate formation (Yamashita et al., 2007). However, it is yet to be seen if increased expression of Hsp105 can successfully ameliorate disease signs in in vivo models of ALS.

HOW CAN THE HEAT SHOCK RESPONSE BE MANIPULATED AND UTILIZED FOR THE TREATMENT OF ALS?

It is clear from this discussion of what is currently understood about the mechansism by which Hsps function, that the highest levels of neuroprotection are more likely to be achieved when several members of the heat shock response are recruited,

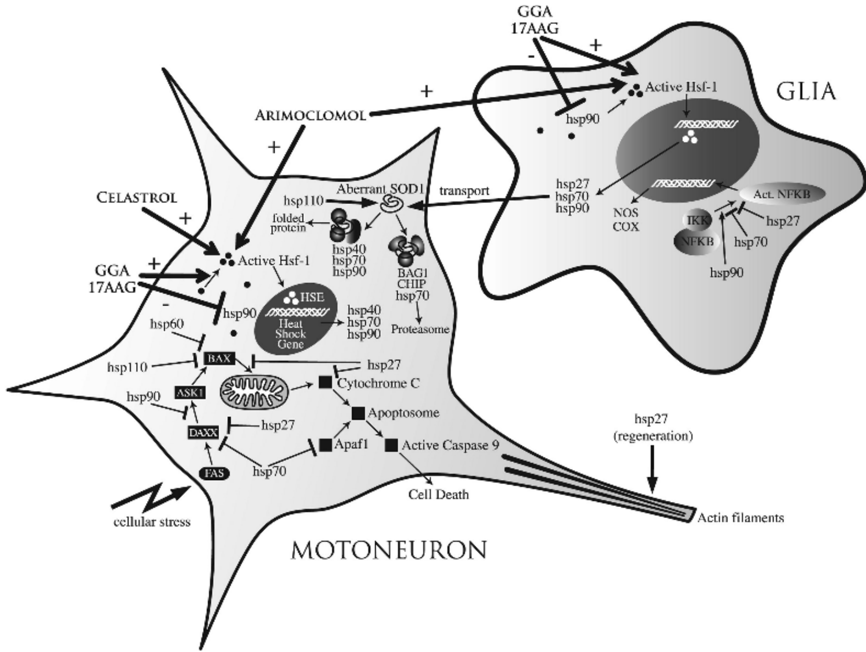

Figure 1. Heat shock proteins play an important role in the survival of motoneurons under normal conditions and during cellular stress in ALS pathology. The Figure illustrates some of the cellular events that can be modified in the CNS in motoneurons and glial cells by hsps under normal conditions and following exposure to stress. Motoneurons have an intrinsic deficit in the ability to activate the heat shock response and therefore do not up-regulate the expression of Hsps such as Hsp70. However, under normal conditions, surrounding glial cells, primarily astroglia, synthesize and release Hsps that can then be utilized by motoneurons. The Figure also summarizes the intracellular effects of Hsps. In glial cells, such as astroglia and microglia, Hsp70 acts as a negative regulator of NFkappaB activation, thereby reducing neuroinflammation, preventing the destructive process that would otherwise involve the over-production of inflammatory mediators such as Nitric Oxide Synthase (NOS) and Cycloxygenase-2 (COX-2). When Hsp levels are sufficiently high in motoneurons, they are capable of keeping aberrantly folded proteins in solution or, with the help of ubiquitinating co-chaperones, they can direct these mis-folded proteins to the proteasome for degradation. Hsps can also directly interact with different members of the apoptotic pathway thereby promoting cell survival. The Figure also indicates the cellular targets of those pharmacological compounds that have been shown to act on Hsps and the heat shock response. For example it can be seen that geranylgeranyl acetone (GGA) blocks hsp90, indirectly initiating the activation of Hsf-1, whereas arimoclomol and celastrol acts directly on the activation and trimerization of Hsf-1 (arrows) resulting in increased expression of a number of Hsps

and in the case of ALS, in multiple cell types (see Figure 1). Since all the main Hsp family members and their co-chaperones play a unique role in the machinery of the heat shock response, co-up-regulation of a number of Hsps is likely to be more successful at protecting cells than over-expression of individual Hsps (Kobayashi et al., 2000; Takeuchi et al., 2002; Patel et al., 2005; Batulan

et al., 2006). It is therefore imperative that the balance between the levels of intracellular hsps and their co-chaperones must be optimal if Hsps are to exert their protein folding and survival promoting effects. Furthermore, the state of phosphorylation (Hsp27 and Hsp105), oligomerization (Hsp27) and association with other Hsps (Hsp70 and Hsp90) must also be favourable. Thus, up-regulation of Hsps in conjunction with their assistant co-chaperones is likely to be a key feature of any therapy that aims to optimally harness the HSR for the treatment of diseases such as ALS. Furthermore, Hsps cannot exert their intracellular functions without assistance from co-chaperones which modify their function. Some co-chaperones, such as Hop, (Hsp70–Hsp90 Organizing Protein), Hip (Hsp70-Interacting Protein) and members of the Hsp40/DnaJ Hsps family, promote client binding and ATPase activity and are involved in the chaperoning and refolding function of Hsps (for reviews see Frydman and Hohfeld, 1997; Fan et al., 2004; Mayer and Bukau, 2005). Other co-chaperones, such as Bag-1 (Bcl-e Associated Athanogen-1) and CHIP, (Carboxy terminus of Hsc70 Interacting Protein) when associated with Hsp70, inhibit its ATPase activity (for reviews see McClellan and Frydman, 2001; Alberti et al., 2003). So far, the relevance of these co-chaperones in ALS has only been investigated in the case of CHIP. CHIP interacts with other proteins through its 3 tetratricopeptide (TPR) motives at its N terminal region and also possesses an E3 ubiqitin ligase activity in its C terminal U box domain as well as a chaperone activity (Ballinger et al., 1999; Murata et al., 2003; Rosser et al., 2007). This co-chaperone appears to be crucial in defining the function of the specific Hsp it is interacting with. Thus, it can inhibit the ATPase activity of Hsc70 and by ubiquitinating its client proteins, direct them towards the proteasome for degradation (Connell et al., 2001; Marques et al., 2006). Even more importantly, CHIP can initiate the heat shock response (HSR) by activating HSF-1 (Dai et al., 2003) or alternatively, after attenuation of HSF-1 activation, it can turn down the HSR. The latter occurs when the level of misfolded proteins is depleted, when CHIP ubiquitinates Hsc70 and directs it towards the proteasome (Qian et al., 2006). Up-regulation of CHIP has been described in several stress models, including α-synuclein induced toxicity, oxidative damage and proteasomal stress (Shin et al., 2005; Dikshit and Jana, 2007). CHIP also associates with mSOD1 in an in vitro model of ALS and over-expression of CHIP can reduce mSOD1 levels and its associated cytotoxicity (Choi et al., 2004). However, it appears that mSOD1 is not a substrate of CHIP, although the mSOD1-Hsc70 complex is (Urushitani et al., 2004). CHIP over-expression reduces aggregates in a cellular model of KD as well as Tau-induced aggregation (Sahara et al., 2005; Adachi et al., 2007). Over-expression of a chimeric protein containing CHIP and another ubiquitin ligase, Dorfin, also results in protection against mSOD1-mediated toxicity in neural cells (Ishigaki et al., 2007). Thus, it appears that at least in the case of CHIP, co-chaperones play a critical role in the regulation of the HSR and could therefore be more efficiently targeted in the treatment of protein misfolding diseases than Hsp70 and Hsp90 family members.

In view of the complexity of Hsp function, it is not surprising that most studies that have tested the effects of Hsp over-expression in transgenic ALS mouse models have been disappointing (Liu et al., 2005; Krishnan et al., 2006). Figure 1 summarises some of the cellular events that can be modified in motoneurons and glial cells by hsps. It is clear from this simplified representation of the complexity of Hsp interactions, that targeted up-regulation of a single member of this delicate system is unlikely to provide resistance to insults if its partner co-chaperones are not also targeted (Manzerra and Brown, 1992; Batulan et al., 2003; Taylor et al., 2007). Genetic manipulation of HSF-1 may overcome the problem of a disturbance in the balance between Hsps. Indeed, transfection of motoneurons with a constitutively active HSF-1 has overcome the intrinsic deficits in the ability of motoneurons to activate the HSR, resulting in an increase in the synthesis of multiple Hsps (Batulan et al., 2003, 2006). Transfection with activated HSF-1 is more effective in protecting motoneurons than transfection with Hsp25 or Hsp70 alone (Batulan et al., 2006). Under physiological conditions, motoneurons rely on surrounding cells such as astroglia, not only for trophic and metabolic support, but also for a supply of essential chaperones. It has been shown that astroglia are capable of releasing Hsp70 and that exogenous Hsp70 is taken up and utilized by motoneurons (Guzhova et al., 2001; Robinson et al., 2005). Therefore, for ALS it may be a more successful strategy to target spinal cord astroglia, which are naturally more responsive to stress and may transmit this increased resistance to motoneurons. Alternatively, exogenous application of a mixture of Hsps could deliver suitable amounts of protective Hsps to motoneurons. However, delivering proteins and peptides is always difficult in vivo due to the high risk of degradation by proteases before reaching their target and the problems of passing through the blood brain barrier. Small molecules that activate the general heat shock response, are easy to administer and are likely to cross the blood brain barrier could overcome this hurdle. The small hydroxylamine derivatives, Bimoclomol and Arimoclomol have been developed and act as co-inducers of the HSR in conjunction with other stressors. These compounds recruit and enhance the HSR, but only once it has been activated (Vigh et al., 1997; Kalmar et al., 2002b). The mechanism of action of these molecules is to act on the activation of HSF-1 itself, prolonging the activation of HSF-1 and thereby achieving an enhanced HSR (Hargitai et al., 2003; Kieran et al., 2004). Following promising results in a preclinical animal study, in which treatment with Arimoclomol successfully increased the lifespan and improved muscle performance of SOD1 mice (Kieran et al., 2004), this compound is now in a phase 2 clinical trial for the treatment of ALS. Celastrol, an herbal drug that has been shown to also cause activation of HSF-1 by hyperphosphorylation, has also successfully increased the lifespan of SOD1 mice (Westerheide et al., 2004; Kiaei et al., 2005). Due to the failure of a number of promising candidates for the treatment of ALS, the development of an effective disease-modifying therapy is a priority to all those who work in this field. It remains to be seen whether well designed strategies based upon manipulation of the HSR will be successful

in a clinical setting. However, in view of the multifactorial nature of ALS pathogenesis, it is likely that an effective therapy will involve targeting of a number of mechanisms. Thus, a combination of a number of drugs or drug-like agents is likely to be required if a successful therapy for the treatment of ALS is to be developed.

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CHAPTER 5

THE ROLE OF CHAPERONES AND CO-CHAPERONES IN RETINAL DEGENERATIVE DISEASES

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Abstract: Molecular chaperones play an important role in normal retina function and are implicated in several forms of retinal dystrophy. The retina is a specialized part of the CNS that presents a fascinating paradigm to investigate molecular chaperone function. Mutations in several photoreceptor proteins lead to protein misfolding mediated neurodegeneration. The best characterized of these are mutations in the molecular light sensor, rhodopsin, which cause autosomal dominant retinitis pigmentosa. Rhodopsin biogenesis may require chaperones and rhodopsin misfolding involves molecular chaperones in quality control and the cellular response to protein aggregation. Furthermore, the specialization of components of chaperone machinery to photoreceptor specific roles has been revealed by the identification of mutations in putative chaperone proteins that cause inherited retinal dysfunction and degeneration. These putative chaperones are involved in several important cellular pathways and further illuminate the essential and diverse roles of molecular chaperones in the nervous system

Keywords: Retina; degeneration; molecular chaperone; rhodopsin; RP2; AIPL1

INTRODUCTION

The neural sensory retina is a highly specialized part of the CNS. Diseases that affect the retina can be inherited or acquired. Inherited retinal dystrophies are characterized by a great degree of phenotypic and genetic heterogeneity with over 120 different disease genes implicated (RetNet, http://www.sph.uth.tmc.edu/RetNet/). Inherited retinal dystrophies can be Mendelian traits with dominant, recessive or X-linked inheritance. In addition, some retinal dystrophies are multifactorial; dependent on a subtle interplay between a number of genes or the genetic background and

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environment. Retinal dystrophies can affect many different cell types in the retina. In this chapter, we will focus on the role of molecular chaperones in the light sensitive photoreceptors including the role of chaperones in response to protein misfolding disease and mutations in putative chaperones.

MUTATIONS IN RHODOPSIN CAUSE RETINITIS PIGMENTOSA

Retinitis pigmentosa (RP) is a genetically heterogenous group of diseases, that are caused by a diverse number of genes with different pathology causing pathways but which converge in their symptoms and in the manner in which the retina is affected. Patients present with night blindness and then start to lose peripheral vision, as the rod photoreceptor cells dysfunction and die followed by cone photoreceptor cell death. The disease then progresses towards the centre of the retina, leading to characteristic tunnel vision and eventually blindness.

RP is a very diverse disease with non-syndromic types comprising 65% of cases, syndromic or systemic RP is 25% and other unknown types of RP represent the remaining 10% (Daiger et al., 2007). RP presents multiple modes of inheritance: 15%–20% of all cases are autosomal dominant (ADRP), 20%–25% are autosomal recessive (arRP), 10%–15% are X-linked (XLRP), and the remaining 40%–55% cannot be classified genetically (Wang et al., 2005). The rod cell light sensitive photopigment, rhodopsin, is mutated in approximately 30% of cases of ADRP, (Wang et al., 2005) making it one of the most common causes of RP. Rhodopsin is comprised of the rod opsin protein and the chromophore 11-cis-retinal.

Over 140 mutations have been identified in rod opsin (RetNet, http://www.sph.uth.tmc.edu/RetNet/). These mutations have been classified according to their cellular or biochemical properties (reviewed by Stojanovic and Hwa, 2002; Mendes et al., 2005). Of particular interest to this chapter are the class II mutants that lead to rod opsin protein misfolding. Class II is the most common class of rod opsin mutations and includes P23H, which is also the most common cause of ADRP in North America. Dryja et al. (1990) first identified the P23H mutation, the proline at position 23 is highly conserved among vertebrate and invertebrate opsins and in other G-protein coupled receptors, for example beta-2-adrenergic receptor. Similarly, many other residues affected by class II mutations are conserved and reside in the intradiscal and transmembrane domains that are critical to the correct folding of the protein.

CLASS II ROD OPSIN PROTEIN MISFOLDING AND AGGREGATION

A wealth of data from animal models and cell biological studies has shown that class II rod opsin mutations shift the folding equilibrium away from the native state and towards folding intermediates that have a propensity to misfold and aggregate. In animal models, wild-type rhodopsin is almost entirely restricted to the outer segment, a specialized rod photo-sensing organelle. In contrast, class II rod opsin mutants show abnormal accumulation in the photoreceptor outer nuclear layer, outer

plexiform layer and are also detected in inner and outer segments (Roof et al., 1994; Frederick et al., 2001). In cultured cells, wild-type opsin translocated to the plasma membrane, whereas class II mutants were retained in the ER, and could not be reconstituted with 11-cis-retinal suggesting protein misfolding (Sung et al., 1991, 1993; Kaushal and Khorana, 1994). The misfolded class II rod opsin is degraded by the ubiquitin-proteasome system or can aggregate into cytosolic aggregates (Illing et al., 2002; Saliba et al., 2002). These rod opsin aggregates can coalesce into large inclusions with the properties of an aggresome similar to those described for several polytopic and monotopic integral membrane proteins (Johnston et al., 1998).

In addition to gain of function mechanisms, misfolded opsin can act as a dominant negative to affect the processing and fate of the wild type protein. Studies on Drosophila Rh1 (Kurada et al. 1998) and mammalian rod opsin in cells (Saliba et al., 2002; Rajan and Kopito, 2005), suggested that misfolded rod opsin had a dominant effect on the wild-type protein. When both wild-type and mutant rod opsin were present, as in patients heterozygous for ADRP, P23H was found to affect the processing of wild-type opsin, causing the wild type protein to aggregate and form inclusions (Saliba et al., 2002). Furthermore, the mutant and wild-type opsins appeared to form high molecular weight, detergent insoluble complexes, in which the two proteins were in close (<70Å) proximity (Rajan and Kopito, 2005).

Rod opsin misfolding and aggregation has many similar features to the protein misfolding and aggregation observed in other neurodegenerative diseases discussed in chapters within this book and also involves molecular chaperones. For example, the P23H-opsin inclusions recruit ubiquitin and HSP70 chaperones (Saliba et al., 2002). Nevertheless, the basis of toxicity of class II rhodopsin mutations remain to be elucidated but it is likely that similar gain of function mechanisms apply as in other neurodegenerations (see Mendes et al., 2005 for more detail). Therefore, it is important that we consider the role of chaperones in opsin biogenesis and their role in opsin quality control and degradation.

ROD OPSIN INTERACTS WITH MULTIPLE CHAPERONES DURING BIOGENESIS

The biogenesis and quality control of multi-spanning membrane proteins occurs at the endoplasmic reticulum (ER). Certain steps in this pathway and the potential involvement of molecular chaperones are shown schematically in Figure 1. The rod opsin signal sequence binds to signal recognition particle (Audigier et al., 1987) directing the ribosome and the growing polypeptide to the ER membrane. This signal sequence is not cleaved (Friedlander and Blobel, 1985) and opsin inserts in the ER co-translationally (Kanner et al., 2002). The rod opsin transmembrane domains are thought to undergo active and passive displacement from the Sec61 translocon into the lipid bilayer (Ismail et al., 2006). PAT-10, a transmembrane specific chaperone, associates with the TM1 of rod opsin until the entire polypeptide chain has been translocated, either facilitating insertion or modulating the assembly of individual transmembrane domains (Meacock et al., 2002).

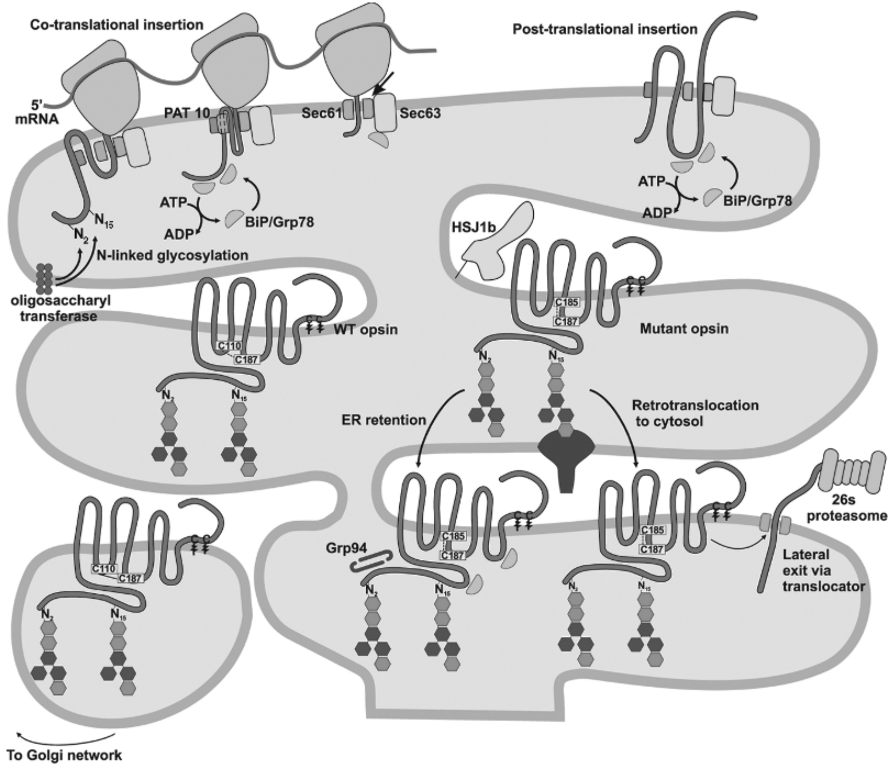

Figure 1. The role of chaperones in rod opsin insertion and processing in the ER. Rod opsin does not encode a cleaved amino-terminal signal sequence. The ER translocon is thought to align with the large ribosomal subunit to facilitate entry and prevent flow of ions from the ER. The ER HSP70, BiP, located in the luminal side, assists with protein insertion by a 'ratchet mechanism' coupled to ATP hydrolysis. The DnaJ protein Sec63 interacts with BiP through its J domain and modulates BiP function (Misselwitz et al., 1998). Properly folded WT rod opsin, with the correct disulphide linkage (Hwa et al., 1999) will translocate to the Golgi for further processing. Mutant opsin, with the wrong disulphide bond (Hwa et al., 1999) is retained in the ER by resident chaperone such as Grp94 (Anukanth and Khorana, 1994). The misfolded rod opsin may also interact with HSJ1b in the cytoplasm and a lectin chaperone in the ER lumen. Mutant opsin is degraded by the proteasome in the cytoplasm following ERAD (Illing et al., 2002; Saliba et al., 2002)

Upon insertion into the ER membrane the N-terminal intradiscal domain of rod opsin is N-glycosylated at Asn_2 and Asn_{15} by the oligosaccharyl transferase enzyme (Plantner et al., 1980; Kean, 1999). Glycans are known to be required for the association with lectin chaperones that can either assist in polypeptide folding or ER-associated degradation (ERAD) of a terminally misfolded protein. Glycan chains are required for efficient ERAD of misfolded rod opsin (Saliba et al., 2002). These data imply a role for lectin chaperones such as calnexin, calreticulin or EDEM in opsin quality control, but the specific lectin chaperones that regulate opsin ERAD

remain to be identified. Other chaperones may also regulate opsin quality control. For example, Anukanth and Khorana (1994) showed that mutant opsins were found in a complex with the HSP70 and HSP90 chaperones of the ER, namely Grp78 (BiP), and Grp94.

Specific lectins for the maturation and processing of mammalian rod opsin have not been identified. Calnexin, however, is required for the maturation of the *Drosophila* rod opsin protein, Rh1, a homologue of mammalian rod opsin (Rosenbaum et al., 2006). Flies with premature stop codons in their *cnx* gene displayed reduced levels of *cnx* transcript with no detectable calnexin protein. These mutations severely reduced Rh1 protein levels, suggesting that Rh1 degradation was taking place. Furthermore, *cnx* mutant flies displayed age-related retinal degeneration as shown by reduced translocation of Rh1 to rhabdomeres (*Drosophila* photoreceptors) 1–6 and reduced rhabdomere size. Calnexin also formed a stable complex with Rh1, consistent with its role as molecular chaperone (Rosenbaum et al., 2006). There may be, however, significant differences between Rh1 and mammalian rod opsin in this respect. Renthal et al. (1973) showed that an intact carbohydrate unit does not appear to be essential for the chromophoric properties of rhodopsin or for its regeneration. Kaushal et al. (1994) showed that treatment of wild-type opsin with the glycosylation inhibitor, tunicamycin, resulted in non-glycosylated opsin which was normally transported to the cell surface and formed the characteristic rhodopsin chromophore with 11-cis-retinal. On the other hand, treatment of P23H opsin expressing cells with tunicamycin led to P23H accumulation in the ER, suggesting that the glycans are required for mutant rod opsin ERAD (Saliba et al., 2002). Other proteins that undergo ERAD (Wiertz et al., 1996a, b; Petäjä-Repo et al., 2001; Gong et al., 2005) have been shown to dislocate to the cytosol where they are deglycosylated by cytosolic N-glycanases, suggesting that the carbohydrates are recognized by lectins that mediate and process the misfolded polypeptides for degradation by the proteasome. The actual targeting into ERAD of misfolded opsin is probably mediated by ER chaperones that may not interact with normal folding intermediates. This is the case for the cystic fibrosis transmembrane conductance regulator (CFTR) where HSP90 is required for the folding of its large cytoplasmic domains but HSP70 and HSP40 are required for targeting it for degradation (Loo et al., 1998; Youker et al., 2004; Zhang et al., 2006).

Other evidence suggests that Rh1 and mammalian opsin may have different chaperone requirements. NinaA is an ER resident integral membrane peptidyl-prolyl isomerase (PPI) chaperone that is required for the maturation of Rh1 rhodopsin. NinaA mutant flies have very little Rh1 opsin in their rhabdomeres, with most of Rh1 immunoreactivity associated with ER membranes (Colley et al., 1991; Stamnes et al., 1991; Baker and Zuker, 1994). These findings suggest that NinaA activity is required for the transport of Rh1 from the ER. Furthermore examination of ninaA mutants revealed reduced levels of rhodopsin and large accumulations of rough ER membranes (Colley et al., 1991). In contrast, heterologous expression of bovine rod opsin in *Drosophila* resulted in correctly folded and processed rod opsin even in the absence of NinaA (Ahmad et al., 2006). The reasons for this difference in

chaperone requirement remains to be elucidated but may help in clarifying the role of chaperones in mammalian opsin biogenesis.

OTHER POTENTIAL CHAPERONE OPSIN INTERACTIONS

The retina is enriched in chaperones and several could be involved in rod opsin biogenesis or the response to rod opsin misfolding. For example, almost 20 different crystallin genes have been identified in the retina (Xi et al., 2003). The crystallins can be divided into two major families, α and βγ, comprise a group of structural proteins normally found to be associated with the lens and the α-crystallins are members of the small HSP family of molecular chaperones. In the retina α-crystallin binds specifically to post-Golgi membranes in frog photoreceptors and therefore, could be involved in the transportation of newly synthesized rhodopsin, suggesting that these chaperones might participate in the renewal of the photoreceptor outer segment (Deretic et al., 1994).

In addition, specialized DnaJ (HSP40) proteins are expressed at relatively high levels in the retina compared to other tissues. HSJ1a and HSJ1b are neuronal DnaJ proteins that are present in all layers of the neural retina and have distinct staining patterns with HSJ1b found to be enriched in the site of rhodopsin production (Chapple and Cheetham, 2003). Heterologous expression in cell culture showed that the HSJ1b isoform increased the incidence of WT and P23H rod opsin inclusions, and increased the retention of the WT protein in the ER. This was not observed with HSJ1a or the C321S HSJ1b prenylation null mutant (Chapple and Cheetham, 2003). These DnaJ-like proteins act as neuronal shuttling factors for the sorting of chaperone clients to the proteasome (Westhoff et al., 2005). HSJ1 proteins colocalized with rod opsin in inclusions, suggesting that they may be part of the opsin degradation pathway. The full significance of the interaction of HSJ1 proteins with rod opsin remains to be determined, however, what is clear is that the retina has specialized chaperones that are essential for normal vision.

PUTATIVE CHAPERONES AS RETINAL DISEASE GENES

In the last 10 years the expanding list of retinal disease genes has identified several putative chaperone proteins as being essential either of the development or maintenance of a functional retina. In contrast to ADRP, all of these diseases are caused by loss of protein function highlighting that a lack of chaperone function can be critical in the retina.

RP2

RP2 was one of the first RP loci to be genetically mapped (Bhattacharya et al., 1984). *RP2* was shown to cause X-linked RP (XLRP), which tends to be the most severe form of RP in terms of age of onset and disease progression. Schwahn et al. (1998) positionally cloned the gene responsible for XLRP at the *RP2* locus and named

the gene *RP2* and gene product RP2 (Schwahn et al., 1998). Since then, a number of studies have identified a wide range of mutations in *RP2*, most of which are predicted to result in a truncated protein.

There is no obvious correlation between RP2 expression and disease pathogenesis. RP2 mRNA is present in all foetal and adult mouse tissues examined with the strongest level in the testes along with two additional shorter transcripts (Schwahn et al., 1998). The RP2 protein is also widely expressed in human tissues and does not appear to be enriched in retina (Chapple et al., 2000). As patients with *RP2* mutations appear to have only retinal pathology without any other organ involvement, it is still not clear why the loss of RP2 leads specifically to RP.

Initially, the strongest clue to the function of RP2 was its homology to the tubulin folding cofactor, cofactor C (TBCC). TBCC was identified as a post-CCT chaperone of the β-tubulin folding pathway (Tian et al., 1996) and RP2 was its first described homologue (Schwahn et al., 1998). RP2 is a 350 amino acid protein and the homology to TBCC extends over 151 amino acids from position 42 to 192 with 30% identity. Most of the reported *RP2* missense mutations correspond to this region and lie within the residues conserved with TBCC. TBCC forms a complex with other tubulin folding cofactors (TBCD, TBCE) and quasi-native α and β tubulin intermediates, after the tubulin subunits have been released from the cytosolic chaperonin (CCT). This process results in the production of the native tubulin heterodimer (Tian et al., 1996). In addition, TBCC with TBCD and TBCE stimulate the GTPase activity of native tubulin (Tian et al., 1999). This process is regulated by ADP ribosylation factor-like 2 (Arl2) (Bhamidipati et al., 2000; Shern et al., 2003). In vitro studies demonstrated that RP2, in conjunction with TBCD, could partly substitute for TBCC function as a tubulin-GTPase activating protein (GAP) (Bartolini et al., 2002). In the same study it was demonstrated that a pathogenic mutation in the conserved arginine residue, R118H, abolished the tubulin GAP activity in both RP2 and cofactor C, suggesting that this residue could be an ‘arginine finger’, which triggers the tubulin GAP activity. However, RP2 was not able to replace cofactor C in the tubulin heterodimerization reaction (Bartolini et al., 2002). Similarly, RP2 does not appear to be able to be substituted by TBCC, as it does not compensate for RP2 in rods of XLRP patients (Grayson et al., 2002).

The difference between RP2 and TBCC is further highlighted by modifications with myristoylation and palmitoylation of the N-terminus of RP2, which result in the targeting of the protein to the plasma membrane in cultured cells (Chapple et al., 2000, 2002, 2003) and in cells throughout the retina (Grayson et al., 2002). Importantly, a pathogenic mutation, ΔS6, interferes with the membrane localization of the protein, highlighting the importance of this modification for the function of the protein (Chapple et al., 2000).

Another feature of RP2 is its interaction with ADP ribosylation factor-like 3 (Arl3). Arl3 interacts with RP2 in a GTP-dependent manner (Bartolini et al., 2002) and the affinity of RP2 to GDP bound Arl3 is 400-fold weaker than to GTP-bound Arl3, indicating that RP2 is a bona fide effector of Arl3 (Kuhnel et al., 2006). Myristoylation of RP2 also decreases this affinity, leading to the proposal that Arl3

might interact with unmodified RP2 in vivo and facilitate its targeting for this modification (Chapple et al., 2000; Bartolini et al., 2002; Kuhnel et al., 2006).

As yet, Arl3 has not been shown to cause retinal dystrophy in humans, but studies in mice deficient in Arl3, revealed that this protein is essential for photoreceptor and kidney development (Schrick et al., 2006). This phenotype is consistent with a failure of primary cilia. Arl3 is a ubiquitous microtubule-associated protein (MAP) that is present at high concentration in the connecting cilium of photoreceptors. The connecting cilium is a specialized sensory axoneme that extends from the cell body of the photoreceptor to form the outer segment. Protein traffic to the outer segment passes through this cilium. Many other proteins causing retinitis pigmentosa are localized to this structure. Among such proteins are RP1, a member of doublecortin family (Liu et al., 2004a) and retinitis pigmentosa GTPase regulator (RPGR), which is targeted to the cilium by the RPGR-interacting protein (RPGRIP) and causes another X-linked form of the disease (Roepman et al., 1996; Hong et al., 2001). Arl3 may have other cytoskeleton functions as knock out of Arl3 expression in HeLa cells causes failure of cytokinesis (Zhou et al., 2006).

Another important difference between TBCC and RP2 is the non-conserved C-terminus. The C-terminus of RP2 has structural homology with nucleoside diphosphate kinase 1 (NDK1). NDK catalyzes the phosphorylation of nucleoside diphosphates to triphosphates and has previously been reported to act as an Hsc70 cochaperone (Leung and Hightower, 1997). The NDK reaction process requires a covalent intermediate step, in which the active residue His122 is phosphorylated. Despite the general structure similarity of C-terminus of RP2 with NDK, RP2 lacks the kinase activity since the active histidine residue is not conserved in RP2 and is replaced with Phe325 with no other histidines nearby (Kuhnel et al., 2006). On the other hand, RP2 exhibits exonuclease activity, which is a property of NDK1 and NDK2, and may have some of the properties of a stress response protein as it translocates to the nucleus upon DNA damage by UV exposure (Yoon et al., 2006).

Recently, a trypanosome homologue of TBCC and RP2 was localized to the region around the basal body of the trypanosome flagellum and appeared to recruit carboxyl-tyrosinated α tubulin to the basal body. This protein, TbRP2, may act as a chaperone to monitor the quality of tubulin prior to cilia assembly (Stephan et al., 2007). Therefore, RP2 may cause XLRP through problems in the assembly and chaperoning of components of the primary cilia but this hypothesis requires further investigation and does not explain why the RP2 phenotype is restricted to the retina. The importance of specialized chaperones in cilia function, however, is now becoming clear through the identification of causative genes in cilia diseases or ‘ciliopathies’.

BBS PROTEINS

Bardet-Biedl syndrome (BBS) is a genetically heterogeneous disorder connected with the defects in primary cilia in several organs. This syndrome is clinically characterized by retinal degeneration (RP-like), obesity, polydactyly, renal malformation and dysfunction, cognitive impairment and hypogenitalism [OMIM 209900].

To date, twelve BBS genes have been identified. Protein products of genes causing BBS6, BBS10 and BBS12 belong to a novel branch of the type II chaperonin superfamily, but they are considered unlikely to assemble in a complex, similar to CCT (Stoetzel et al., 2007). These proteins are thought to perform cilia-related functions and are especially important for the photoreceptor connecting cilia. BBS6 protein was also shown to be a centrosomal component required for cytokinesis (Kim et al., 2005). Besides homology to CCT, BBS10 also has an ATP binding site indicating that it is a bona fide chaperonin (Stoetzel et al., 2006). Recently, another putative chaperone BBS12 connected with Bardet-Biedl syndrome was identified. It has high similarity to group II chaperonins but the ATP-binding region is not conserved, similar to the BBS6 protein (Stoetzel et al., 2007). Suppression of each family member in zebrafish yielded gastrulation-movement defects characteristic of other BBS morphants, whereas simultaneous suppression of all three chaperonin-like BBS proteins resulted in severely affected embryos, possibly hinting at partial functional redundancy within this protein family (Stoetzel et al., 2007). Thus, some of the proteins causing BBS have high homology to group II chaperonins, demonstrating the conservation of the classical chaperonin domain architecture. Meanwhile they have specific insertions, probably giving these proteins another chaperone function that is critical for ciliated cells.

AIPL1

Mutations in *AIPL1* cause the autosomal recessive disorder Leber congenital amaurosis (LCA: OMIM 604392). LCA is the most severe form of retinal dystrophy characterized by rod and cone dysfunction and blindness or severe visual impairment at birth (Sohocki et al., 2000). *AIPL1* is expressed only in the pineal gland and retinal photoreceptors. Within the retina, the expression pattern of AIPL1 protein coincides with the spatiotemporal differentiation and development of the rod and cone photoreceptors (van der Spuy et al., 2003). In the adult human retina, however, expression is restricted to the rods suggesting a developmental switch in AIPL1 function (van der Spuy et al., 2002). AIPL1 is named based on its similarity (49% amino acid identity) with the aryl hydrocarbon receptor (AhR) -interacting protein (AIP), which is also known as X-associated protein 2 (XAP2) or AhR-activated 9 (ARA9) (Kuzhandaivelu et al., 1996; Carver and Bradfield, 1997; Ma and Whitlock, 1997). These proteins share the common cochaperone tetratricopeptide repeat (TPR) motif that mediates protein:protein interactions. XAP2 and the immunophilins FKBP51/52 use their TPR domains to participate with the HSP70–HSP90 molecular chaperone machinery in the regulation of their respective cognate client proteins, the aryl hydrocarbon receptor (AhR) and the steroid hormone receptors. Furthermore, XAP2 may also facilitate preprotein transfer from Hsc70 to Tom20 and mediate preprotein mitochondrial import in a TPR-dependent manner, suggesting a more general co-chaperone role for XAP2 (Yano et al., 2003). In addition to the similarity of AIPL1

to XAP2, the conservation of a TPR domain in AIPL1 suggests that AIPL1 may be a member of the family of TPR co-chaperones.

The client proteins and partner chaperones of AIPL1 remain to be fully defined, however, a number of AIPL1-interacting proteins have been identified. Akey et al. (2002) reported that AIPL1 interacted with the 'NEDD8 ultimate buster' protein 1 (NUB1). NUB1 would appear to participate in the function of the proteasome via UBL and UBA domains. NUB1 and an alternatively spliced larger isoform, NUB1L, associate with two small, ubiquitin-like proteins, NEDD8 and FAT10 (Kamitani et al., 2001; Kito et al., 2001; Hipp et al., 2004). NUB1 can bind the S5a subunit of the 19 S proteasome activator and recruit these ubiquitin-like proteins and their conjugates for proteasomal degradation (Kamitani et al., 2001; Kito et al., 2001; Tanaka et al., 2003; Hipp et al., 2004; Tanji et al., 2005).

The role of NUB1 and NUB1L in proteasomal function is becoming clearer; however, the molecular function of AIPL1 with respect to NUB1 and proteasomal regulation is not fully understood. Interestingly, similar to the action of XAP2 with its client AhR, AIPL1 is able to modulate the nuclear translocation of NUB1 (van der Spuy and Cheetham, 2004). Furthermore, AIPL1 was able to behave in a chaperone-like manner to suppress the formation of inclusions arising from heterologous expression of GFP tagged fragments of NUB1 (van der Spuy and Cheetham, 2004). This effect appeared specific for NUB1, as AIPL1 had no effect on the formation of inclusions by unrelated, aggregation-prone proteins, including the polyQ disease associated Huntingtin-exon 1-Q103 and P23H rod opsin. Moreover, the AIPL1 homologue XAP2 was unable to interact with or modulate NUB1 nucleocytoplasmic distribution and had no effect on the formation of NUB1 fragment inclusions (van der Spuy and Cheetham, 2004). This suggested that whilst the similarity between AIPL1 and XAP2 correlates with a conserved function in the modulation of nuclear translocation, the specificity for their respective client proteins are distinct.

In addition to NUB1, it has also been shown that AIPL1 can interact with and enhance the post-translational processing of farnesylated proteins, including the HSP40/DnaJ protein HDJ2 (Ramamurthy et al., 2003). Recently, mouse models of LCA with either the complete or partial inactivation of AIPL1 expression have suggested that AIPL1 may also function as a potential chaperone for cGMP phosphodiesterase (PDE), an essential component of the visual phototransduction cascade (Dyer et al., 2004; Liu et al., 2004b; Ramamurthy et al., 2004). In these models, the levels of all three subunits of the cGMP PDE holoenzyme (α, β and γ) were reduced by a post-transcriptional mechanism before the onset of photoreceptor degeneration, suggesting that AIPL1 may be necessary for the biosynthesis, assembly or stabilization of PDE to proteasomal degradation. Therefore, AIPL1 functions may converge on PDE as a putative retina specific client, as AIPL1 may modulate PDE-α subunit farnesylation and regulate the protein targeting to the proteasome. Nevertheless, the repertoire of AIPL1 photoreceptor clients and the

co-operation of AIPL1 with other chaperone proteins that mediate its essential role in the retina remain to be determined.

CONCLUSIONS

The retina offers a unique opportunity to study molecular chaperone function in a highly specialized and accessible part of the CNS. Several retinal dystrophy genes that are aggregation prone are well characterized and the availability of good in vitro and in vivo models will allow the dissection of the mechanisms of cell death associated with these misfolding events and testing of therapies based on manipulating molecular chaperones. The importance of chaperones to retinal function is reiterated by the multiplicity of diseases caused by loss of specific chaperones. Studying these chaperones in more detail may enhance our understanding, not only of retinal function, but also the biology of chaperone networks.

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CHAPTER 6

NEUROPROTECTIVE FEATURES OF HSP90 INHIBITORS EXHIBITING ANTI-INFLAMMATORY ACTIONS: IMPLICATIONS FOR MULTIPLE SCLEROSIS

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Abstract: Multiple sclerosis is a T-cell mediated, autoimmune disease of the central nervous system, affecting over 400,000 people in the US. While the exact causes of MS remain to be elucidated, several anti-inflammatory treatments can reduce disease severity in MS as well as in its animal model experimental autoimmune encephalomyelitis (EAE). It has been known for many years that induction of a heat shock response (HSR), either by thermal or pharmacological means, can suppress inflammatory responses, and indeed induction of a HSR can reduce the incidence and severity of EAE. More recently, it was reported that drugs which bind to the nucleotide binding pocket of HS protein 90 (Hsp90) induce a HSR, which led to testing of several naturally occurring and synthetic Hsp90 inhibitors in EAE. In this article, we will review possible mechanisms by which Hsp90 inhibitors could provide benefit in EAE, and potentially in MS

Keywords: Hsp90; ansamycin; EAE; nitric oxide; T-cells; cytokines

Abbreviations: 17-AAG, 17-allylamino-17-demethoxygeldanamycin; CPD, client protein degradation; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EC, experimental compound; HSR, heat shock response; HSF, heat shock factor; IFN, interferon; IL, interleukin; IκB, inhibitor of kB; IKK, IκB kinase; LPS, lipopolysaccharide; MHC, major histocompatability class; MOG, myelin oligodendrocytes protein; NOS2, nitric oxide synthase type 2; NFκB, nuclear factor kB

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INTRODUCTION

MS is an autoimmune T-cell mediated disease, influenced by genetic background, geographic location, and having a possible viral involvement. There are over 400,000 people diagnosed with MS in the US, and an estimated 2.5 million worldwide. Symptoms may be mild, such as numbness in the limbs, or severe, involving paralysis or loss of vision. MS is classified into four main subtypes, Relapsing-Remitting (RRMS, characterized by partial or total recovery after attacks), secondary progressive MS (SPMS, a relapsing-remitting course which becomes steadily progressive), primary progressive MS (PPMS, characterized by a progressive course from onset), and progressive relapsing MS (PRMS, a progressive course from the outset, also characterized by acute attacks). Current treatments for MS are limited, and include use of the anti-inflammatory cytokine interferon – β (IFNβ); the synthetic repeating polypeptide glatiramer acetate, the immune suppressant mitoxantrone, and recently the monoclonal antibody natalizumab which blocks binding of leukocytes to endothelial cells. However, these interventions either show limited efficacy, can only be used for short periods, need to be injected, or have significant associated risks, thus warranting development of newer and safer drugs.

ANTI-INFLAMMATORY EFFECTS OF THE HSR

It has been known for many years that the Heat Shock Response (HSR) can protect cells and tissues from a variety of noxious stimuli, including thermal, chemical, and physical stress, possibly by facilitating re-naturation of denatured proteins or by chaperoning nascent proteins to specific subcellular locations. Observations that HSPs are induced during stroke, and that ischemic damage is reduced in transgenic mice constitutively expressing Hsp70 demonstrated that the HSR is neuroprotective (Sharp et al. 1999; Giffard and Yenari 2004). However, the HSR can also reduce cell and tissue damage by inhibiting pro-inflammatory gene expression (Jaattela et al. 1992; Hotchkiss et al. 1993; Marber et al. 1995; Simon et al. 1995). In addition, induction of a HSR can prevent expression of the inducible form of nitric oxide synthase (NOS2) whose production of NO contributes to neurological damage in various diseases and conditions (Boje 2004). We showed that thermal induction of the HSR reduced NOS2 in cultured astrocytes (Feinstein et al. 1996), a finding replicated in numerous cell types including hepatocytes (de Vera et al. 1996), islet cells (Scarim et al. 1998), smooth muscle cells (Joly et al. 1997) as well as in whole animals (Hauser et al. 1996).

There are several mechanisms by which the HSR could inhibit inflammatory gene expression. A common feature of inflammatory responses is activation of transcription factor NFκB, and the pathways leading to NFκB activation and de-activation are well known (Traenckner et al. 1995; Simeonidis et al. 1999). In brief, activation of NFκB involves degradation of an inhibitory IκB protein (IκBα being most common in glial cells) which normally maintains NFκB in the

cytoplasm. Inflammatory stimuli leading to activation of IκB kinases (IKKs) allows for phosphorylation of IκBs, ubiquination, degradation by the 26S proteosome, and release of NFκB. The rapid re-expression of IκBα restricts prolonged NFκB activation. The HSR prevents NFκB activation in diverse cell types and by various stimuli (Wong et al. 1997; Scarim et al. 1998). We described that the cytokine induced loss of IκBα in C6 cells was blocked by the HSR, suggesting inhibition of degradation or increase of synthesis (Feinstein et al. 1996, 1997). At the same time, Wong et al. showed that HS blocked cytokine-dependent IκBα loss, and later that HS or arsenite prevented IκBα loss due to incubation with TNFα (Wong et al. 1997). Both groups demonstrated that expression of inhibitory IκB proteins (IκBα and IκBβ) is increased by the HSR, most likely due to the presence of a HS element in their promoter regions (Thomas et al. 1998). More recent studies demonstrate that the HSR inhibits IKK activity (Curry et al. 1999; Kohn et al. 2002; Pittet et al. 2005), thus providing another means to prevent IκB phosphorylation and degradation. HSR effects may also be in part due to other activities, such as repression of gene expression by binding of the HSF-1 transcription factor to upstream regions of inflammatory transcription factor cytokine promoters (Cahill et al. 1996). Together, these studies demonstrate that the HSR blocks NFκB activation via increasing the levels of inhibitory IκB proteins, and thus helps to explain the broad spectrum of inflammatory responses it can suppress.

EFFECTS OF THE HSR IN AN ANIMAL MODEL OF MS

The demyelinating autoimmune disease experimental autoimmune encephalomyelitis (EAE) is an often-used animal model to study possible causes and therapeutic interventions for MS. Active immunization with myelin oligodendrocyte glycoprotein (MOG) or a 21 residue peptide (MOG 35–55) in C57BL6 mice yields a chronic monophasic disease that lasts for up to several months. The central steps leading to disease progression are well known and in general are very similar to those responsible for symptoms in MS, namely priming and proliferation of T-cells in lymph nodes and spleen, their migration into brain, release of pro-inflammatory Th1 type cytokines (including IFNγ and TNFα) in brain, induction of chemokines and of cell adhesion molecules on brain microvasculature, and production of reactive oxygen (RO) and nitrogen (RN) species from glial cells. Infiltration of an additional T-cells and macrophages augments expression of pathological cytokines, ROS, and RNS, and leads to demyelination and axonal damage. Numerous studies have shown that several treatments which reduce the symptoms of EAE also reduce activation of transcription factor NFκB, consistent with the idea that NFκB plays a critical role in EAE (Du et al. 2001; Heneka et al. 2001; Dasgupta et al. 2004). Studies from our lab and others have described various means to prevent NFκB activation, including treatment with anti-inflammatory drugs such as NSAIDs, tetracycline derivatives, steroids (Li et al. 2005; Stanislaus et al. 2005;), endogenous neurotransmitters such as noradrenaline (Feinstein et al. 2002), natural products such as green tea extracts (Aktas et al. 2004), and agonists of the peroxisome proliferator-activated receptors

(PPARs) (Feinstein 2003; Kielian and Drew 2003). Interestingly, most, if not all of these treatments can also induce a HSR.

We therefore tested if induction of a HSR would provide benefit in the EAE model, and showed that a brief period of hyperthermia (body temperature raised to 42°C for 20 minutes), given 2 days after the MOG immunization injection (and therefore before the appearance of clinical symptoms) prevented disease onset in the majority of mice, and the few animals which became ill showed mild, transient symptoms (Heneka et al. 2001). The HSR reduced T-cell infiltration into brain, cortical chemokine and NOS2 expression, and brain NFκB activation. Subsequently, we confirmed that hyperthermia decreased inflammation within the brain (Heneka et al. 2000), since inflammatory responses (NFκB activation; NOS2 expression) to intracortical injection of the robust inflammatory agent lipopolysaccharide (LPS) were blunted and IκB (as well as Hsp70) expression was increased following treatment. These findings raised the intriguing possibility that inducing a HSR could be of therapeutic benefit in MS; however since hyperthermia is not well tolerated in MS patients, an alternative paradigm was needed.

HSP90 AND ANSAMYCINS

The HSR can be induced by a wide array of metabolic insults including exposure to elevated temperatures, heavy metals, ionophores, amino acid analogs, and metabolic poisons. These stressors adversely affect protein conformation, and the intracellular accumulation of abnormally folded proteins initiates a HSR by activating heat shock factor (HSF-1). HSF-1 is present in unstressed cells as an inactive monomer, which rapidly trimerizes in response to metabolic stress (Morimoto 2002). Trimerization enables HSF-1 to bind to a consensus DNA sequence, the heat shock element (HSE), located within the promoter element of genes encoding stress proteins, and thereby induces rapid HSP transcription. However, a HSR can also be elicited by treating cells or tissue with drugs that bind to Hsp90, which is expressed at high levels in the cytoplasm under normal conditions, and is further induced after stress (Burrows et al. 2004; Kamal et al. 2004). Hsp90 normally forms a complex with HSF-1 (Zou et al. 1998) and upon inhibition of Hsp90, HSF-1 dissociates allowing induction of the HSR.

Mammalian cells contain four distinct members of the Hsp90 family, the major forms being cytosolic Hsp90a and Hsp90b which share 76% homology. Hsp90b is generally constitutively expressed, whereas Hsp90a is inducible. Hsp90 proteins contain a low affinity ATP/ADP binding pocket in the N-terminal domain, which exhibits low ATPase activity. When ATP is bound, Hsp90 forms a multi-protein complex which binds to and stabilizes various Hsp90 client proteins. When ADP is bound (or ATPase inhibited), Hsp90 adopts a conformation that destabilizes the complex, inducing dissociation of client proteins and targeting some of them for degradation by the 26S proteasome. In addition to the N-terminal ATP binding site, the presence of a second nucleotide site and an independent substrate binding site has been also demonstrated (Scheibel et al. 1999). This region mediates formation of

Hsp90 dimers, the presumed active conformation of the chaperone and regulates the ATPase activity of the N-terminal region. Moreover, drugs that bind the C-terminal ATP binding region have been found to interfere with the Hsp90 chaperone activity (Marcu and Neckers 2003).

An increasing number of proteins have been identified as Hsp90 client proteins (Burrows et al. 2004; Zhao and Wang 2004; Millson et al. 2005); including steroid hormone receptors (SHRs), protein kinases, and transcription factors. In some cases, such as that for the steroid hormone receptors, the released proteins are not degraded, but instead can dimerize and activate gene transcription (Bagatell et al. 2001). Amongst the Hsp90 client proteins some have a clear role in regulating inflammatory gene expression, including IKK IκB kinase (IKK) (Pittet et al. 2005), PPARs (Sumanasekera et al. 2003), and NOS2 (Yoshida and Xia 2003). Thus, Hsp90 inhibitors can exert their effects by inducing a HSR or by inducing client protein degradation (CPD).

HSP90 PROTEINS AND BRAIN

There is relatively little known about the role of Hsp90 in the brain, and even less regarding Hsp90 regulation of brain functions. Hsp90 is expressed ubiquitously throughout brain, with preferential localization to neurons (Quraishi et al. 1996; Jeon et al. 2004). Hsp90 has been shown to play a role in neurite extension (Ishimoto et al. 1998), neural survival (Lee et al. 2001; Jeon et al. 2004), cell migration (Sidera et al. 2004), and neurotransmitter release (Gerges et al. 2004). Hsp90 is relatively less abundant in glial cells (Itoh et al. 1993; Uryu et al. 2006) but increased can be readily increased after induction of an excitotoxic lesion in mouse brains (Jeon et al. 2004), and may contribute to mechanisms of glial cell protection and adaptation in response to damage. Several studies demonstrated that Hsp90 forms a complex with the two constitutive forms of NOS (NOS1 and NOS3), and regulates their stability and activity (Osawa et al. 2003). More recently, a similar interaction of Hsp90 with NOS2 was reported (Yoshida and Xia 2003), demonstrating that Hsp90 normally increases NOS2 activity, and that Hsp90 inhibitors reduce it. Thus, Hsp90 inhibitors may modulate brain inflammatory damage by directly reducing NOS2 activity.

HSP90 AND T-CELL FUNCTIONS

Little is known regarding Hsp90 interactions with T-cell signaling in either EAE and MS. However, Hsp90 client proteins may play an important role in T-cell activation and co-stimulation. Current work on T-cell activation demonstrates that two distinct signals are needed for activation; ligand presentation by an antigen presenting cell (APC) cell to the T-cell receptor (TCR; CD3); and activation of a co-stimulatory molecule on T-cells. Together, the 2 signals induce T-cell proliferation,

differentiation, and cytokine production. In the absence of co-stimulation, T-cells die or become refractory to antigen stimulation (Chitnis and Khoury 2003; Allen et al. 2005). There are several identified costimulator pairs, however some of the more well-characterized interactions include binding of the ligand B7-1/2 to its receptor CD28; and binding of CD154 (a member of the TNFα family) to its receptor CD40. These interactions lead to activation of anti-apoptotic proteins, activation of inflammatory transcription factors such as NFκB, increased cell survival, and cytokine production. The signaling pathways involved are not entirely worked out but activation of the PI3Kinase: AKT/PKB system has been implicated in some cases (Ward 1999). CD28 through PI3K augments NFκB activation and BclX expression (Wu et al. 2005), and increases IL2 expression (Okamoto et al. 2003; Sanchez-Lockhart et al. 2004). A primary target of PI3K is the prosurvival kinase AKT/PKB, and several studies show that AKT influences T-cell function and survival (Jones et al. 2002).

Likewise, certain costimulatory interactions reduce T-cell activation, including binding of B7 to CTLA-4 (a receptor that is closely related to CD28), and these are thought to terminate T-cell function. CTLA-4 ligation signaling is less well characterized than that of CD28, but may involve different downstream targets of PI3K. Modulation of costimulatory interactions either pharmacologically or genetically has profound effects on EAE disease (Sporici and Perrin 2001; Chitnis and Khoury 2003; Howard and Miller, 2004); thus costimulatory molecules are now recognized as therapeutic targets for disease intervention. In view of the fact that AKT is a well characterized Hsp90 client protein, and considering that lymphocytes use similar signal transduction pathways as tumor cells do, Hsp90 inhibitors could be potential immunosuppressant drugs. Indeed several reports (Schnaider et al. 1998, 2000; Yorgin et al. 2000) have shown that the Hsp90 inhibitor geldanamycin inhibits CD28 mediated T-cell activation and that degradation of the non-receptor tyrosine kinase $p56^{lck}$ seems to be one of the molecular mechanisms involved.

The above studies suggest that Hsp90 inhibitors could modulate T-cell function interfering with binding of Hsp90 to client proteins involved in T-cell signaling pathways (AKT, NFκB). However several reports have demonstrated a more specific role for Hsp90 in mediating immune functions. Hsp90 participates in the transport, trimming, and presenting of antigenic peptides to the MHC class I molecules to evoke T-cell immune responses (Binder et al. 2001; Chen and Androlewicz 2001). Recently a role for Hsp90 in the ability of antigen presenting human dendritic cell (DC) phenotype and function has been reported (Bae et al. 2007). The authors showed that Hsp90 inhibition significantly decreased cell surface expression of costimulatory (CD40, CD80, CD86), maturation (CD83), and MHC (HLA-A, B, C and HLA-DP, DQ, DR) markers in both immature DC and mature DCs, and decreased the ability of mature DC to present antigen to T-cells. Thus, Hsp90 may modulate not only T-cell signaling but the ability of antigen presenting cells to activate the T-cells themselves.

HSP90 INHIBITORS AND EAE

The discovery of naturally occurring Hsp90 inhibitors followed characterization of the bacterial product geldanamycin, which had been identified as a tyrosine kinase inhibitor with anti-tumorigenic properties (Whitesell et al. 1994), yet exerted effects at concentrations that did not inhibit oncogenic kinase activities. Geldanamycin was shown to form a complex with Hsp90, disrupting the Hsp90:Src Kinase complex needed for cellular transformation (Bagatell et al. 2001). As a consequence, geldanamycin and its derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) are in clinical trials for treatment of breast cancer (Neckers 2002; Ramanathan et al. 2005).

In view of the fact that ansamycins were known to induce a HSR, we tested the effects geldanamycin in MOG induced EAE (Murphy et al. 2002). Both geldanamycin, as well as its less toxic derivative 17-AAG induced a HSR in primary rat astrocytes as well as rat C6 glioma cells, and both dose-dependently reduced NOS2 expression and activity (with IC_{50} values in the low nanoMolar range). The ansamycins increased expression of the inhibitory IκBα protein, suggesting their effects were mediated, iun part by suppression of NFκB activation. In the MOG EAE model, treatment with geldanamycin (a single injection of 300 ng i.p., given 3 days after the MOG booster) reduced disease onset by over 50% Although results with geldanamycin were promising, its known side effects (hepatotoxicity (Supko et al. 1995)) limit its potential.

In subsequent study we more carefully evaluated the ability of 17-AAG to influence the course of EAE (Dello Russo et al. 2006). In primary glial cultures, 17-AAG dose-dependently reduced LPS-dependent expression and activity of NOS2, reduced IL-1β expression and release, increased inhibitory IκBα protein levels, and induced HSP70 expression. 17-AAG prevented disease onset when given to MOG immunized mice at an early time during disease progression, and importantly could reduce clinical symptoms when given during ongoing disease. The effects of 17-AAG were due in part to actions on T-cells, since T-cells derived from 17-AAG treated-mice showed a reduced response to immunogen restimulation, and 17-AAG reduced the ability of T-cells to produce IL-2 (necessary for T-cell proliferation). Treatment with 17-AAG also provided neuroprotection, as determined by silver staining for axonal damage. Taken together, treatment with 17-AAG appeared to exert numerous protective effects in the MOG EAE model, including direct anti-inflammatory effects on brain glial cells, inhibition of T-cell activation, and possibility direct neuroprotective effects, suggesting that similar treatments could be of value in treating MS patients.

HSR AND NEUROPROTECTION

There is existing evidence that the HSR can provide neuroprotection against a variety of stimuli and in a variety of neurological conditions, with most studies describing protection in stroke (Sharp et al. 1999; Yenari 2002; Giffard

and Yenari 2004). Evidence that Hsp90 inhibitors can provide neuroprotection come from studies that show geldanamycin inhibits glutamate-induced toxicity in mouse hippocampal cells (Xiao et al. 1999) and blocks caspase activation in stressed neurons (Lee et al. 2001). Upregulation of Hsp90 (and Hsp70) reduced motoneuron degeneration following peripheral nerve axotomy in neonatal rats (Kalmar et al. 2002) and Hsp90 promoted neurite outgrowth of dissociated neurons (Ishimoto et al. 1998). As described above, neuronal pathology, including axonal transection, occurs in MS and EAE, and may be due to a combination of TNFα cytotoxicity, perforin-mediated damage, free radicals, matrix metalloproteinases, or NO production (Coleman and Perry 2002; Bjartmar et al. 2003), and more recently axonal damage in EAE was reported to be due in part to excitotoxic effects of glutamate since AMPA/kainate receptor antagonists reduce EAE disease (Werner et al. 2000; Gilgun-Sherki et al. 2003). Induction of a HSR may therefore provide neuroprotection in EAE by reducing glutamate induced excitotoxicity (Rordorf et al. 1991; Snider and Choi 1996). Recently, neural damage in EAE was shown to involve activation of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), since blocking antibodies to TRAIL reduced clinical signs of EAE (Song et al. 2000) and since TRAIL deficient T-cells were less able to induce symptoms (Aktas et al. 2005). Since the HSR can block TRAIL-induced apoptosis in other cell types (Ozoren and El Deiry 2002), this may be another means by which Hsp90 inhibitors could provide neuroprotection in EAE and MS.

CHARACTERIZATION OF HIGH AFFINITY HSP90 INHIBITORS

In hopes to obtain Hsp90 inhibitors with improved bioavailability and reduced cytotoxic effects, several geldanamycin derivatized analogs have been synthesized (Le Brazidec et al. 2004). In addition, structural studies have shown that ansamycins including geldanamycin bind to the amino-terminal nucleotide pocket of Hsp90 and destabilize interactions with Hsp90 client proteins, and several panels of high affinity fully synthetic drugs have been designed based on the crystal structure of the Hsp90:17-AAG complex (Le Brazidec et al. 2004; Biamonte et al. 2006; Chiosis and Tao 2006; Chiosis et al. 2006; Kasibhatla et al. 2007). These newer Hsp90 inhibitors act at lower (nanoMolar) concentrations, have fewer known side effects, and have improved solubility properties compared to first generation ansamycins and thus may be of greater therapeutic value.

In collaboration with the Conforma Therapeutics Corporation (San Diego, CA), we carried out an initial screening of a panel of novel geldanamycin derivatives, hopes to identify drugs which could reduce inflammatory activation of glial cells, suppress T-cell activation, and potentially allow us to distinguish the relative importance of inducing a HSR versus client protein degradation (e.g. loss of AKT) in protective effects in EAE. We screened experimental compounds (ECs) for their effects on IFNγ production from rat splenic T-cells and on LPS-stimulated nitrite production from primary rat astrocytes (Figure 1). Several compounds (EC78, 119, 86, and 82) were observed to be more potent than 17-AAG (EC72) to reduce

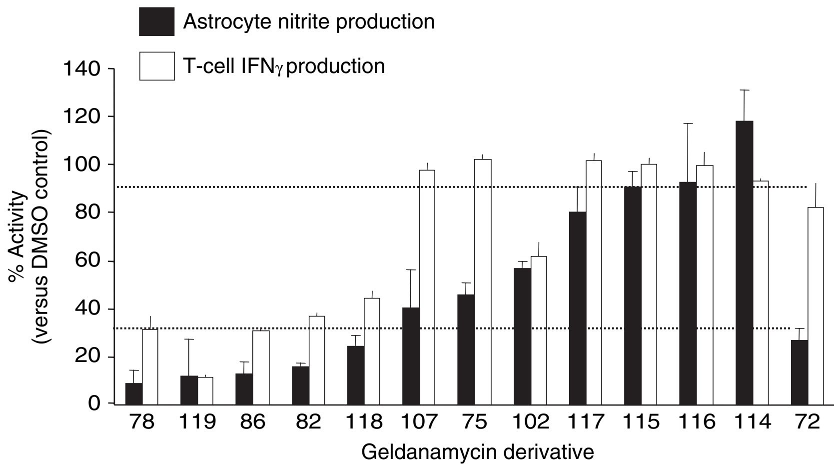

Figure 1. Anti-inflammatory effects of novel geldanamycin derivatives. Splenic rat T-cells were stimulated with Concanavalin A in the presence of 100 nM of the indicated geldanamycin derivative (the experimental identification numbers are given); IFNγ levels were measured by specific ELISA after 48 hr. Primary rat astrocytes were stimulated with a combination of IFNγ plus LPS to induce NOS2 expression in the presence of 100 nM drug, and nitrite levels were measured after 24 hr. The data is mean ± se of n=3 samples in each group and shown as the % response compared to vehicle (DMSO) controls

nitrite and IFNγ production. Moreover at the dose tested (100 nM), two drugs (EC107, 75) showed little effect on IFNγ production but >50% inhibition of nitrite production. Further studies with these selected drugs (those with highest potency and with differential effects on glial cells versus T-cells) are ongoing; as are studies using purine-derivatives which can selectively induce a HSR versus inducing client protein degradation.

CONCLUSIONS

Although the induction of a HSR and of HSPs is well known to provide neuroprotection, much less attention has been placed on the fact that induction of a HSR can have direct anti-inflammatory actions. Studies from our group and others demonstrate that inducing a HSR (either chemically or by use of Hsp90 inhibitors) can reduce a variety of pro-inflammatory molecules (including the NOS2 enzyme), and can suppress T-cell activation. The more recent findings that the Hsp90 inhibitors may be working via induction of a HSR as well as by inducing degradation of client proteins known to be involved in T-cell signaling or glial cell activation suggest that these drugs could have important effects on the progression of autoimmune diseases such as MS. In view of the fact that 17-AAG is already in phase II/III clinical trials, initial studies in MS seem feasible.

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CHAPTER 7

ROLE OF HSPB1 AND HSPB8 IN HEREDITARY PERIPHERAL NEUROPATHIES: BEYOND THE CHAPERONE FUNCTION

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Abstract: Within the last 10 years, mutations in genes encoding the small heat shock proteins (also called HspB) HspB1, HspB4, HspB5 and HspB8 have been associated with neurological and muscular disorders. In particular, HspB1 and HspB8 mutations result in hereditary peripheral neuropathies, which primarily affect motor and/or sensory peripheral neurons. Due to their extremely long axons, peripheral neurons are particularly dependent on an efficient vesicular trafficking and axonal transport, whose defects have been directly linked to the development of hereditary peripheral neuropathies. Genetic analyses generated new insights into the molecular pathways involved in hereditary peripheral neuropathies, which include not only vesicular trafficking but also protein quality control, protein degradation and RNA processing. This review summarizes the current understanding of HspB implication in motor diseases, starting with a general picture of HspB functions followed by a description of new emerging roles for HspB1 and HspB8 in axonal transport, protein sorting and degradation

Keywords: HspB; neuromuscular disorders; protein aggregation; protein degradation; macroautophagy; vesicular trafficking; axonal transport

HEREDITARY PERIPHERAL NEUROPATHIES AND MOTOR NEURON DISEASES: CLASSIFICATION, SYMPTOMS AND GENETIC HETEROGENEITY

Hereditary peripheral neuropathies are among the most common genetic disorders in humans and are characterized by a clinically and genetically heterogeneous profile. Due to their heterogeneous genetic profile (different mutations in the same

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gene can cause different phenotypes, as well as mutations in different genes can be associated with the same pathology), hereditary peripheral neuropathies are classified on the basis of the different clinical symptoms in conjunction with electrophysiological testing of peripheral nerves. Two main groups can be distinguished: the demyelinating neuropathies (e.g., Charcot-Marie-Tooth disease type 1) and the axonal neuropathies (e.g., Charcot-Marie-Tooth disease type 2 and distal hereditary motor neuropathy). In demyelinating neuropathies, the integrity of the myelin sheat that insulates the axon is compromised, thus resulting in reduced nerve conduction velocities and, eventually, in axonal transport defects. Demyelinating neuropathies primarily affect the Schwann cells, which tightly wrap the axons of peripheral neurons. In contrast, axonal neuropathies originate from damages that primarily affect the peripheral neuronal axon, without decreasing nerve conduction velocities. Moreover, on the basis of the type of neuron that is affected by the disease, two groups of inherited neuropathies can be distinguished: distal hereditary motor neuropathy (dHMN), a motor neuron disease and hereditary motor and sensory neuropathy (HMSN), characterized by damages to both sensory and motor neurons. Due to the selective degeneration of neurons of the peripheral nervous system, the classical clinical hallmarks of hereditary peripheral neuropathies are distal weakness of the lower limbs, decreased reflexes and, in the case of HMSN, sensory loss. Weakness of the upper limbs is also observed with the progression of the disease. Neuropathies with juvenile (dHMN type I and IV and dHMN-Jerash), early (dHMN type III) or late (e.g., dHMN type II and VII, CMT type 2F and 2L) onset have been described, with an age of onset between 2 and 20 years old for the early and juvenile forms and between 20 and 40 years old for the adult onset forms.

A common feature of peripheral neuropathies relies on the peculiar morphology of peripheral motor and/or sensory neurons, which are characterized by extremely long axon and need a high metabolic and energy supply. The length of the axon in the peripheral nervous system makes these cells particularly dependent on the efficient axonal transport of signaling molecules, trophic factors, cytoskeletal components and membraneous organelles. It is thus not surprising that among the proteins that are mutated in peripheral neuropathies are microtubule motors (Zhao et al., 2001), which are at the basis of axonal transport, elements of the intermediate filament network (Mersiyanova et al., 2000; Jordanova et al., 2003), elements of the vesicular trafficking system (Verhoeven et al., 2003) and proteins involved in RNA processing (Chen et al., 2004; Asaka et al., 2006). Alterations of intermediate filament network, axonal transport and protein degradation, as well as impairment of RNA processing, have all been documented in hereditary peripheral neuropathies. The genes associated with hereditary peripheral neuropathies and the putative functions of their products are listed in Table 1. Surprisingly, among the proteins that are mutated in peripheral neuropathies are also two members of the small heat shock protein family, HspB1 and HspB8, which are classified as molecular chaperones (Evgrafov et al., 2004; Irobi et al., 2004).

Table 1. Known genes mutated in hereditary peripheral neuropathies

Gene	Gene product	Disease	Proposed function
BSCL2	Bernelli-Seip congenital lipodystrophy 2 (1)	dHMN II	Integral membrane protein of endoplasmic reticulum
DCNT1	Dynactin (2, 3)	dHMN VI	microtubule binding, axonal transport
DNM2	Dynamin 2 (4)	DI-CMTB	endocytosis, endosomal trafficking, membrane fission, centromere organization
GARS	Glycyl-tRNA synthetase (5)	CMT2D	RNA processing
GDAP1	Ganglioside-induced differentiation protein 1 (6, 7)	CMT4A (CMT2H, CMT2K)	mitochondrial glutathione transferase
HspB1	HspB1 (8)	CMT2, dHMN	protein quality control, cytoskeleton stabilization
HspB8	HspB8 (9, 10)	CMT2L, dHMN II	protein quality control, macroautophagy-mediated protein degradation
IGHMBP2	Immunoglobulin mu-binding protein 2 (11)	SMARD1, dHMN VI, ALS4	RNA processing
KIF1B	Kinesin family member 1B (12)	CMT2A	axonal transport
LMNA	Lamin A/C (13, 14)	CMT2B1	nucleoskeleton, chromatin organization, gene regulation
MFN2	Mitofusin 2 (15)	CMT2A	mitochondrial membrane fusion
MPZ(P0)	Myelin protein zero (16–18)	CMT1B, CMT2I, CMT2J	myelin structural gene
NEFL	Neurofilament light (19, 20)	CMT2E	neuronal cytoskeleton
RAB7	Ras-associated protein RAB7 (21)	CMT2B	endosomal trafficking
SETX	Senataxin (22, 23)	dHMN	RNA processing
SIMPLE/LITAF	Small integral membrane protein of lysosome/late endosomes (24, 25)	CMT1C	putative E3 ubiquitin ligase of the RING finger motif containing E3 subfamily with function in protein degradation by the lysosomes

CMT: Charcot-Marie-Tooth disease; dHMN: distal hereditary motor neuropathy; SMARD1: Spinal muscular atrophy with respiratory distress, type 1; ALS4: Juvenile amyotrophic lateral sclerosis. Numbers in brackets correspond to the references. 1: Windpassinger et al., (2004); 2, 3: Puls et al., (2003); Munch et al., (2004); 4: Zuchner et al., (2005); 5: Antonellis et al., (2003); 6, 7: Baxter et al., (2002); Cuesta et al., (2002); 8: Evgrafov et al., (2004); 9, 10: Irobi et al., (2004); Tang et al., (2005); 11: Grohmann et al., (2001); 12: Zhao et al., (2001); 13, 14: De Sandre-Giovannoli et al., (2002); Goizet et al., (2004); 15: Zuchner et al., (2004); 16–18: Su et al., (1993); Marrosu et al., (1998); Senderek et al., (2000); 19, 20: Mersiyanova et al., (2000); Jordanova et al., (2003); 21: Verhoeven et al., (2003); 22, 23: Chen et al., (2004); Asaka et al., (2006); 24, 25: Street et al., (2003); Saifi et al., (2005)

HSPB: CLASSIFICATION AND KNOWN FUNCTIONS

The small Heat Shock Proteins family (sHSP), also called HspB in mammals, belongs to the superfamily of Heat Shock Proteins (HSP), which are ubiquitous proteins whose expression is induced by heat and other stresses (Lindquist, 1986). The mammalian HspB family comprises ten members called HspB1–HspB10 (see Table 2) (Fontaine et al., 2003; Kappe et al., 2003).

Two main types of functions have been attributed to HspB (as well as to sHSP from other organisms, including yeast and plant): the chaperone activity and the stabilization of the cytoskeleton.

A number of studies carried out in test tube have shown that HspB, like many other Heat Shock Proteins, function as molecular chaperones. Molecular chaperones are proteins that can recognize and bind to proteins in non-native conformations, whether due to genetic mutations or to protein-denaturing stress and they either assist in their refolding or in their targeting for degradation. On the basis of their ability to bind to misfolded proteins, a protective role for molecular chaperones in protein conformational disorders has been proposed (Stenoien et al., 1999; Cummings and Zoghbi, 2000; Jana et al., 2000; Cummings et al., 2001; Sittler et al., 2001; Bailey et al., 2002; Chavez Zobel et al., 2003; Carra et al., 2005). Accumulation of aggregated proteins is a pathological hallmark of many age-related (protein conformational) neurodegenerative disorders, including Alzheimer's, Parkinson's and Huntington's diseases, which are often referred to as brain amyloidosis. The neuroprotective role of the Hsp70 and Hsp40 molecular chaperones has been extensively demonstrated (Warrick et al., 1999; Kobayashi et al., 2000; Wyttenbach et al., 2000; Cummings et al., 2001; Auluck et al., 2002; Bailey et al., 2002; Bonini, 2002). On the contrary, the HspB proteins chaperone activity has been predominantly documented in vitro and in vivo evidence is rather indirect. For example, a neuroprotective role for HspB is strongly suggested by the observation that HspB1, HspB5 and HspB8 are up-regulated in brain amyloidosis, both in glial cells and neurons (Iwaki et al., 1989, 1992, 1993; Lowe et al., 1992a, b; Renkawek et al., 1992, 1994a, b, 1999; Shinohara et al., 1993; Dabir et al., 2004; Aquino et al., 1997; Wilhelmus et al., 2006a, b, c). Moreover, transient transfection of HspB8 in cultured cells facilitated the degradation of proteins containing polyglutamine extension, thereby reducing their accumulation as aggregates (Carra et al., 2005). HspB8 overexpression in culture cells also resulted in the solubilization of HspB5-R120G, a mutated aggregate-prone form of HspB5 (Chavez Zobel et al., 2003). Finally, HspB8 was shown to bind to amyloid-β proteins and to prevent their aggregation and toxicity (Wilhelmus et al., 2006b). On the basis of these findings it may be argued that loss of HspB chaperone function due to genetic mutation may contribute to the progression of neurological disorders, including hereditary peripheral neuropathies.

In contrast to the chaperone activity, that has mostly been demonstrated by in vitro studies, a number of in vivo evidence exists showing the ability of mammalian HspB to interact with and/or modulate the structure and dynamics of the cytoskeleton. Well documented is the role of HspB1, HspB4, HspB5 and HspB6 in the stabilization

Table 2. Nomenclature of the mammalian sHSP family members (HspB), tissue distribution and sHSP mutations associated with neurodegenerative disorders

Name	Alternative names	Tissue expression	Mutations	Disease
HspB1	Hsp27, Hsp25 (mouse) (1)	smooth, skeletal and cardiac muscles, liver, kidney, lung, stomach, colon, testis, lens, brain	R127W S135F R136W T151I P182L	dHMN CMT2 dHMN CMT2 dHMN
HspB2	MKBP	skeletal and cardiac muscles	not known	not known
HspB3	HspL27	smooth, skeletal and cardiac muscles	not known	not known
HspB4	αA-crystallin, CRYAA (2–6)	lens	R116C R49C W9X (G→A) R21L G98R	CC CC CC CC CC
HspB5	αB-crystallin, CRYAB (7–12)	smooth, skeletal and cardiac muscles, liver, kidney, lung, stomach, colon, testis, lens, brain	R120G 464delCT Q151X (C→T) 450delA (fram_ shift in codon 150) D140N P20S	DRM MM MM CC CC CC CC
HspB6	Hsp20, p20	smooth, skeletal and cardiac muscles, liver, kidney, lung, stomach, colon, brain	not known	not known
HspB7	cvHsp	skeletal and cardiac muscles	not known	not known
HspB8	Hsp22, H11, E2IG1 (13, 14)	smooth, skeletal and cardiac muscles, kidney, lung, stomach, colon, testis, brain	K141E K141N	dHMN II dHMN II, CMT2
HspB9	None	testis	not known	not known
HspB10	ODFP, ODF1	testis	not known	not known

MKBP: myotonic dystrophy protein kinase-binding protein; cvHsp: cardiovascular heat shock protein; ODFP: sperm outer dense fiber protein 1; dHMN: distal hereditary motor neuropathy; CMT2: Charcot-Marie-Tooth disease type 2; CC: congenital chataract; DRM: desmin-related myopathy; MM: myofibrillar myopathy; X = insertion of a translation stop codon. There is confusion in the literature concerning the name of the HspB proteins. Note that Drosophila Melanogaster Hsp27 is not related to the Homo Sapiens HspB1/Hsp27 and that Drosophila Melanogaster Hsp22, which is a mitochondrial protein, is not related to the Homo Sapiens HspB8/Hsp22. Numbers in brackets correspond to the references. 1: Evgrafov et al., (2004); 2–6: Litt et al., (1998); Pras et al., (2000); Mackay et al., (2003); Graw et al., (2006); Santhiya et al., (2006); 7–12: Vicart et al., (1998); Berry et al., (2001); Mackay et al., (2002); Selcen and Engel, (2003); Liu et al., (2006a, b); 13, 14: Irobi et al., (2004), Tang et al., (2005)

of microfilaments and intermediate filaments (Lavoie et al., 1993, 1995; Iwaki et al., 1994; Nicholl and Quinlan, 1994; Liang and MacRae, 1997; Perng et al., 1999; Dreiza et al., 2005). The effect of HspB on actin polymerization-depolymerization dynamics and on cytoskeleton stabilization depends on their phosphorylation state.

From the structural point of view, HspB are characterized by a low molecular weight, ranging from 15 to 40 kDa, by the presence in their C-terminus of a conserved sequence of 80–100 amino acids called the α-crystallin domain and by the ability to form dynamic high molecular weight structures (Kim et al., 1998; Van Montfort et al., 2001a, b). Exposure to stress can change the structural conformation of HspB, thus dramatically influencing their functional properties. For example, HspB1 mainly exits as high molecular weight oligomers (Lambert et al., 1999); however, in response to stress, HspB1 is phosphorylated. As a consequence of its phosphorylation, HspB1 oligomers dissociate. The dissociation of HspB1 oligomers is an essential step for recognizing and binding the non-native substrate (chaperone activity) and for interacting with and/or modulating the structure and dynamics of the cytoskeleton (Lavoie et al., 1993, 1995; Perng et al., 1999; During et al., 2007). A similar regulatory function has been shown for HspB1 and HspB5 at the level of the intermediate filaments. HspB1 and HspB5 directly interact with glial fibrillary acidic protein and vimentin intermediate filaments and protect them from stress-induced aggregation (Perng et al., 1999; Lee et al., 2005).

HSPB ARE MUTATED IN NEURODEGENERATIVE DISORDERS

Within the last 10 years, congenital cataract, neurological disorders and muscular disorders were shown to result from the mutations in genes encoding for four of the small heat shock proteins, *namely* HspB1, HspB4, HspB5 and HspB8 (Table 2) (Vicart et al., 1998; Pras et al., 2000; Berry et al., 2001; Mackay et al., 2002, 2003; Evgrafov et al., 2004; Irobi et al., 2004; Graw et al., 2006; Liu et al., 2006a, b; Santhiya et al., 2006). Two major consequences of protein mutations are the gain of toxic function and the loss of the chaperone activity, which both may participate in the disease progression. However, little is known concerning the physiological roles of the members of the HspB family, and hence, it can not be excluded that loss of specialized function of the HspB may also contribute to the development of the disease. The identification of HspB roles and activities becomes thus essential to better understand the association of HspB with neurodegenerative disorders.

Gain of Toxic Functions and Loss of HSPB Stability and Chaperone Activity: Role in Motor Diseases

Whereas mutations in HspB1, HspB4, HspB5 and HspB8 lead to different diseases, which affect diverse cell types and organs (eyes, muscles and peripheral nervous systems), all these mutations share the common property of leading to protein instability, aggregation and accumulation. The only exception is represented by the W9X missense mutation of HspB4, which adds a premature stop codon (Table 2). For example, the R116C mutation of HspB4, which contributes to the development of congenital cataract, results in the formation of a larger oligomer than normal and decreases its chaperone-like function (Shroff et al., 2000). The R120G mutation

of HspB5, which is associated with desmin-related myopathy, results in aggregation of both HspB5 and desmin filament (Vicart et al., 1998). In a similar way, mutations of HspB1 and HspB8, which cause peripheral neuropathies, also result in their aggregation (Evgrafov et al., 2004; Irobi et al., 2004; Kijima et al., 2005; Tang et al., 2005). These findings strongly suggest that perturbation of the HspB protein stability may significantly contribute to the disease progression. But how protein instability and aggregation can lead to the development of these neurological disorders? Both a gain of function and a loss of function mechanism have been described. The accumulation of mutated HspB may exert a negative effect due to the inherent toxicity of the aggregated species (gain of function). However, the aggregation of mutated HspB may also result in a decreased chaperone function, which would also contribute to the development of the disease (loss of function). The contribution of both mechanisms to the development of the disease has been demonstrated for HspB5 mutants. The R120G, as well as the 464delCT and the Q151X mutations in HspB5 are associated with Desmin related myopathies (often referred to as myofibrillar myopathies), which are inherited or sporadic skeletal myopathies associated with cardiomyopathy and peripheral neuropathy (Vicart et al., 1998; Selcen and Engel, 2003). Desmin-related myopathies are characterized by abnormal accumulation and aggregation of desmin, which is a key subunit of the intermediate filaments in cardiac, skeletal and smooth muscles and is essential for the structural integrity of muscle cells. Under physiological conditions, HspB5 directly interacts with the intermediate filament protein desmin and has a role in neurofilament network assembly (Bennardini et al., 1992; Djabali et al., 1997; Perng et al., 1999, 2004). The loss of HspB5 chaperone function at the level of desmin may thus contribute to muscular cell atrophy, as supported by the observation that HspB5-null mice, which however are also deficient in HspB2, develop a form of myopathy. However, the severity of the myopathy developed by the HspB5-null mice is milder than the one observed in a transgenic mouse model expressing the R120G mutant (Brady et al., 2001), thus suggesting that the loss of chaperone function may only partially explain the development of the disease. In parallel, mutated HspB5 disrupts the organization of desmin filaments both in culture cells and in a transgenic mouse model expressing the R120G mutant (Vicart et al., 1998; Wang et al., 2001; Perng et al., 2004). These results suggest that the progressive accumulation of mutated HspB5 and desmin in the form of inclusion bodies also contributes to muscle cells atrophy and death, due to the inherent toxicity of the aggregates (gain of function mechanism). In a similar way, overexpression of mutated HspB1, which is unstable and aggregates, but not overexpression of wild-type HspB1, destabilized the intermediate filament network and decreased the neuronal cells viability (Evgrafov et al., 2004). Aggregates containing both elements of the intermediate filaments and mutated HspB1 have been demonstrated in culture cells (Evgrafov et al., 2004). It may be argued that, by affecting HspB1 structural properties, the mutations promote the loss of HspB1 activity at the level of the cytoskeleton. The resulting disruption of the intermediate filament network may then weaken the structural framework of the cell, alter the axonal transport, and contribute to

motor neuron death. Interestingly, evidence demonstrating the crucial role of a proper cytoskeletal structure and function in neuronal survival comes from the finding that mutations of the neurofilament light chain gene (NEFL) are associated with the development of a specific form of peripheral neuropathy (Charcot-Marie-Tooth disease type 2E) (De Jonghe et al., 2001; Jordanova et al., 2003; Fabrizi et al., 2007). All together these data suggest that defects in the maintaining of the axonal cytoskeleton and transport, either resulting directly from mutation of the cytoskeletal elements (NEFL mutation), or indirectly from HspB1 and HspB5 mutations may dramatically contribute to the disease progression.

HSPB Form Hetero-Oligomers: Mutations of One Specific HSPB may Affect the Structural and Functional Properties of Other Members of the HSPB Family

Another important aspect that should be considered is the ability of HspB to interact with each-other. Both high molecular weight homo-oligomers and hetero-oligomers have been described. For example, HspB1 forms homotypic oligomers of ca. 24 subunits but can also form heterotypic oligomers with HspB4 and HspB6. Heterotypic complexes are also formed between HspB2 and HspB3 (Sugiyama et al., 2000). Finally, HspB1 and HspB8 have been reported to dynamically interact with each other (Benndorf et al., 2001; Sun et al., 2004). Altering the structural properties of one specific member of the HspB family may consequently disturb the structural and functional properties of other HspB proteins; this may also contribute to the progression of the disease. In line with this hypothesis, alteration of the HspB8 binding properties has been described for the mutated species K141E and K141N, which are associated with peripheral neuropathies. Both K141E and K141N HspB8 mutants interact more strongly with HspB1 than wild-type HspB8 (Irobi et al., 2004). Moreover, HspB8 mutants aggregate within the cells, thus sequestering HspB1 (Irobi et al., 2004). Sequestration of other members of the HspB family may have deleterious effect in cell types where their basal level is critical. In particular, HspB1 plays an essential role for sensory and motor neurons survival (Benn et al., 2002) and exerts antiapoptotic activity (Garrido et al., 2006). Sequestration of HspB1 into mutated HspB8 containing aggregates may also participate to compromise sensory and motor neurons viability. All together these results suggest that the imbalance in HspB1/HspB8 interaction, as well as the imbalance in their interaction with other members of the HspB family, may contribute to the disease progression (Irobi et al., 2004; Fontaine et al., 2006).

EMERGING ROLES FOR HSPB: IMPLICATION IN HEREDITARY PERIPHERAL NEUROPATHIES

The identification of the genes that are associated with hereditary peripheral neuropathy has started to enlighten the signaling pathways that are implicated in the disease progression (Table 1). Altered RNA processing, disruption of axonal

transport, alteration of the cytoskeleton stability, defects in protein quality control and deregulation of the macroautophagy proteolytic process have been reported in different kind of neuromuscular disorders. As previously mentioned, mutations of HspB1, similarly to mutations of HspB5, affect their chaperone function at the level of the intermediate filaments, thus contributing to the development of the disease. However, new specialized functions of HspB1 and HspB8 in intracellular trafficking and protein degradation are emerging. It may thus not be excluded that alteration of more specialized functions of the HspB may also participate to the hereditary peripheral neuropathy progression. Their potential contribution to neuromuscular disorders development will be discussed in detail.

AUTOPHAGIC VACUOLES ACCUMULATION IN NEURODEGENERATIVE DISORDERS

A number of genes involved in protein sorting and degradation have been described in hereditary peripheral neuropathies. These include Rab7, DNM2, LITAF/SIMPLE, MTMR2 and MTMR13 (Bolino et al., 2000; Azzedine et al., 2003; Street et al., 2003; Verhoeven et al., 2003; Saifi et al., 2005; Zuchner et al., 2005). MTMR2 and MTMR13 interact together and are involved in the regulation of endocytosis, vesicular protein sorting and degradation. Rab7 belongs to the superfamily of small GTPase; it plays a role in the late endocytic pathway and lysosome biogenesis and participates in the final maturation of late autophagic vacuoles (Jager et al., 2004). LITAF/SIMPLE has been colocalized with LAMP-1 (lysosome-associated membrane protein-1) to perinuclear lysosomes and late endosomes (Moriwaki et al., 2001; Street et al., 2003) and may play a prominent role in the degradation of proteins essential for peripheral nerve survival. This raises the question whether improper protein degradation may participate in the pathogenesis of CMT1A. Indeed, reduced degradation of mutated PMP22 and its progressive aggregation have been proposed as key factors in the pathogenesis of CMT1A. Using a mouse model of CMT1A, it has been shown that mutated PMP22 impairs the proteasome function and is mainly targeted to the macroautophagy for degradation (Fortun et al., 2006).

Macroautophagy is a nonselective bulk degradation process that is induced under starvation and involves the engulfment and digestion of portions of the cytoplasm in double-membraned vesicles called the autophagic vacuoles or autophagosomes. The autophagic vacuoles acquire proteolytic capacity after fusion with lysosomes/endosomes to form the autophagolysosomes (Ohsumi, 2001; Shintani and Klionsky, 2004). A growing body of evidence suggests a major role for macroautophagy in the degradation of protein microaggregates associated with conformational diseases (Bjorkoy et al., 2005; Iwata et al., 2005a, b). In particular, using animal models of neurodegenerative disorders, stimulation of macroautophagy has been shown to facilitate the clearance of misfolded proteins, including aggregated PMP22 (Ravikumar et al., 2004; Berger et al., 2006; Fortun et al., 2007). These results, together with the recent finding that loss of macroautopahgy causes

neurodegeneration, strongly suggest that impairment in this proteolytic pathway may contribute to neuronal death (Hara et al., 2006; Komatsu et al., 2006).

In addition, deregulated autophagy has been reported in diseases affecting muscular cells. Accumulation of autophagic vacuoles is a hallmark of different types of myopathies (Nishino, 2003, 2006) and a malfunction of the lysosomes is linked to the Danon's disease, which is characterized by cardiomyopathy, myopathy and mental retardation (Nishino et al., 2000). It is not known whether the accumulation of autophagic vacuoles in myopathies results from their increased biogenesis or from their decreased fusion with the lysosomes. On one hand, it has been observed that treatment with chloroquine, a lysosomotropic agent that increases the pH of lysosomes and abrogates their proteolytic properties, induces in cell culture and rat a myopathy with accumulation of vacuoles. This supports the hypothesis that an impairment in macroautophagy, rather than its stimulation, may be at the basis of the disease progression (Kumamoto et al., 1989). On the other hand, transcription of hATG5 and hATG12, two essential genes in macroautophagy, is elevated in inclusions body myositis, thus suggesting that an increased formation of autophagosomes may also contribute to the disease (Kumamoto et al., 2004).

All together these data clearly demonstrate that the macroautophagy/lysosomal pathway plays an essential role in both neuronal and muscular cell survival and that impairment of this proteolytic process may dramatically contribute to peripheral neuropathies and myopathies progression. To date no evidence directly linking HspB and the macroautophagy machinery exists. However, recent data from our laboratory suggest that HspB8 may stimulate autophagic vacuoles formation and may facilitate macrautophagy-mediated misfolded protein degradation.

EMERGING ROLE FOR HSPB8 IN AUTOPHAGIC VACUOLES FORMATION AND IN MACRAUTOPHAGY-MEDIATED MISFOLDED PROTEIN DEGRADATION

Recent results from our laboratory strongly suggest that HspB8 facilitates the macroautophagy-mediated degradation of an aggregation-prone protein containing a strecht of polyglutamine (Carra et al., 2005). In the cell, HspB8 forms a stable and stochiometric complex with Bag3, which is member of the BAG family (Bag1–6). All Bag proteins are co-chaperones of Hsc70/Hsp70 and bind to the antiapoptotic protein Bcl2 (Takayama and Reed, 2001). Upon transient transfection of HspB8 and Bag3 we observed an accelerated degradation of the mutated polyglutamine protein huntingtin and induction of autophagic vacuoles formation (Carra and Landry, unpublished). In cell culture, HspB8 and Bag3 partially co-localized with GFP-LC3-positive autophagic vacuoles, thus suggesting that the HspB8/Bag3 chaperone complex may cooperate with the macroautophagy machinery to facilitate the degradation of misfolded proteins. Moreover, we observed no change in the capacity of either the K141E or the K141N HspB8 mutants to bind Bag3. However, in the slowly developing disease, the progressive aggregation of the unstable HspB8 mutants may result in a decrease of the HspB8/Bag3 chaperone complex ability to stimulate

the autophagic vacuoles formation. This would in turn promote the accumulation of aggregated proteins and would amplify the toxic cascade of events leading to cell death. Furthermore, the aggregation of the K141E and K141N mutants may sequester the co-chaperone Bag3. The reduced availability of Bag3, which interacts with Bcl2 and exerts antiapoptotic activity, may also participate in the progression of the disease (Lee et al., 1999; Liao et al., 2001; Romano et al., 2003a, b; Bonelli et al., 2004).

Our findings that the HspB8/Bag3 chaperone complex is able to stimulate macroautophagy and to participate in the protein quality control are in line with the observation that impairment of autophagosome maturation and autophagic vacuoles accumulation have been shown in a number of protein conformational diseases as well as in Charcot-Marie-Tooth disease and different kinds of myopathies. It also supports the idea that defects in misfolded proteins degradation contribute to the progression of these pathologies (Laforet et al., 2004; Selcen et al., 2004; Shintani and Klionsky, 2004). Further studies are needed to determine the mechanism by which HspB8 and Bag3 modulate autophagic vacuoles formation and to better elucidate the implication of this specialized function in the progression of hereditary peripheral neuropathy.

AXONAL TRANSPORT DISRUPTION AS A COMMON CAUSE OF NEURODEGENERATION

Neurons are specialized cells with a complex architecture that includes an elaborate dendritic branching and the axon, a long projection that extends from the cell body to the synaptic terminal and that provides a physical conduit for the transport of essential cellular components. The transport of cargoes as diverse as synaptic vesicle precursors, growth factors, protein complexes, cytoskeletal elements, endosomes and other membraneous organelles is coordinated by specific molecular motors moving along the microtubules and relies on an intact microtubular network. It is thus not surprising that disruption of the axonal transport has been directly linked to neurodenegeration and that a number of genes that are involved in axonal transport are mutated in motor neuron diseases. For example, mutations in proteins that regulate dynein-mediated transport have been found in motor neuron diseases including Amyotrophic Lateral Sclerosis and distal hereditary motor neuropathy (McEntagart et al., 2001; Puls et al., 2003; Munch et al., 2004). Mutations in the motor proteins dynactin (McEntagart et al., 2001; Puls et al., 2003; Munch et al., 2004) and Kinesin family member 1B (Zhao et al., 2001) and mutation in the neurofilament light chain protein (Mersiyanova et al., 2000) are associated with hereditary peripheral neuropathies (Table 1). Moreover, alteration of axonal transport may indirectly affect proteolytic processes like the macroautophagy. Macroautophagy relies on the intact microtubule-mediated transport, as disruption of the microtubules blocks the fusion of the endosomes/lysosomes with the autophagic vacuoles and leads to aggregated proteins accumulation (Webb et al., 2004). This is further supported by the finding that mutations in dynein impair

the macroautophagy-mediated clearance of aggregate-prone proteins (Ravikumar et al., 2005). It is thus not surprising that defects in axonal transport are also involved in age-related neurodegenerative disorders, including Huntington's, Parkinson's and Alzheimer's diseases, which are characterized by the accumulation of aggregated proteins. All together these findings reinforce the theory that disruption of axonal transport may cause or dramatically contribute to neurodegeneration.

Interestingly, Ackerley et al. recently reported that by aggregating mutated HspB1 sequesters specific elements essential for the axonal transport, including the protein p150 dynactin, which is involved in retrograde axonal transport, and the neurofilament middle chain subunit, thus disrupting transport processes (Ackerley et al., 2006). The dynactin complex functions as a link between dynein, the major axonal retrograde motor, the microtubule network and the axonal transport of cargoes and vesicles. By sequestering p150 dynactin, overexpression of mutated HspB1 seemed to selectively disrupt retrograde axonal transport, without affecting the anterograde transport of specific cargoes, including mitochondria or synaptotagmin I (Ackerley et al., 2006). These findings suggest that HspB1 may play a role in axonal transport processes and that alteration of this specific function may also contribute to the development of neuromuscular disorders. Further studies will better elucidate HspB1 implication in the axonal transport of specific cargoes.

CONCLUSIONS AND PERSPECTIVES

The association of HspB mutations with neurological disorders suggests that they may play a prominent role in neuronal survival. A potential link between molecular chaperones, protein quality control and neurodegeneration is further suggested by their ability to protect cells against diverse stress, including proteotoxic stress and by the findings that HspB are upregulated in several age-related neurodegenerative disorders. However, a number of fundamental questions remain unanswered and HspB physiological functions are still largely unknown. Yet it remains unclear whether the mutations of HspB associated with hereditary peripheral neuropathies cause a loss of their chaperone function or give rise to dominant negative effects resulting in protein toxicity. Recent evidence points to the critical role of HspB in housekeeping functions, including cytoskeleton stability, axonal transport and misfolded protein degradation. This suggests that defects in specialized functions of HspB may participate in the progression of the disease. Further studies will be necessary to better elucidate HspB1 role in axonal transport and HspB8 function in macroautophagy and to establish their relevance to the disease progression.

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PART II

HEAT SHOCK PROTEINS AND NEUROPROTECTION

CHAPTER 8

HEAT SHOCK PROTEINS HSP70 AND HSP27 AND NEURAL CELLULAR PROTECTION

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Abstract: Heat shock proteins (Hsps) are expressed throughout the body. In the brain, the 27 kDa Hsp, Hsp27, and the 71–72 kDa Hsp, Hsp70, are expressed and can be protective against many stressors. Following cellular stress and injury including inflammation, ischemia, and seizures, Hsp27 and Hsp70 are induced to high levels and can protect cells from further injury and death. Hsps are also protective against neurodegenerative disorders. Huntington's disease, Parkinson's disease, and Alzheimer's disease with dementia are characterized by protein aggregations and elevated levels of Hsp27 and Hsp70 are thought to protect neurons from the cytotoxic effects of protein aggregates. Hsp27 and Hsp70 protect cells from the damaging effects of stressors by interrupting apoptotic and cell death pathways, decreasing inflammation, slowing and decreasing protein aggregation, decreasing oxidative damage, and maintaining cell homeostasis.

Experimentally, the induction of Hsp27 and Hsp70 in neurons and glia is usually through stress, or altered gene expression. If levels of Hsp27 or Hsp70 can be increased to higher levels by drug or supplement administration, increased levels of Hsps may be a therapy for neurological injuries and aliments such as Huntington's disease and Parkinson's disease. This chapter will outline some the neuroprotective effects of Hsp27 and Hsp70

Keywords: Hsp27, Hsp70, neuroprotection, kainic acid seizure, ischemia, inflammation, neurodegenerative diseases

HEAT SHOCK PROTEINS

Heat shock proteins (Hsps) are a family of highly conserved proteins that are involved in cell survival, normal cellular homeostasis, and metabolism. Hsps can

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be rapidly induced in response to cell stress and Hsps protect cells from heat and a variety of stressors. The characteristic function of Hsps is the ability to act as a molecular chaperone by assisting in the folding, transportation, stabilization, and assembly of proteins. The Hsps are categorized according to their molecular weight and form 6 families: the 110 kDa Hsps, the 90 kDa Hsps, the 70 kDa Hsps, the 60 kDa Hsps, and the small Hsps ranging from 15 to 30 kDa like Hsp27 and ubiquitin.

Hsp27

One of the major small Hsps is Hsp27, also referred to as Hsp25 in the mouse. The gene encoding Hsp27 is *HSPB1*, and is regulated by heat shock factors (HSF) that bind to the heat shock element (HSE) in the promoter region of Hsp genes to initiate transcription. Hsp27 is evolutionarily conserved and can be found in all organisms from eukaryotes to bacteria. Hsp27 is considered to be neuroprotective and there are four main mechanisms by which Hsp27 exerts this protection: molecular chaperoning, interference with cell death pathways, antioxidant activities, and stabilization of the cytoskeleton.

Hsp70

Hsp70 is highly inducible following stress. Hsp70 has a molecular weight of 71 kDa in rats and 72 kDa in humans and consequently is also known as Hsp71 in rats and Hsp72 in humans. The constitutive form of the 70 kDa Hsp is known as Hsc70 or Hsp73 because the molecular weight is 73 kDa. Hsp70 and Hsc70 share more than 90% sequence identity and are thought to have the same main functional characteristics, acting as molecular chaperones and facilitating nascent protein translocation into cellular organelles.

NORMAL HSP EXPRESSION

Hsp27, Hsc70, and Hsp70 are all expressed at varying levels during development. Hsc70 is constitutively expressed in regions of the brain, but is not highly inducible. However, Hsp70 is generally at low levels in the brain but is highly inducible following a stressor. Hsp27 is both constitutively expressed in the CNS and is highly inducible following a stressor.

Hsp Expression During Embryonic Development

Hsp27 is found in low levels during embryonic development. On embryonic day E9.5, the early neural fold stage, Hsp27 is present in rats (Walsh et al., 1997), and Hsp27 can be detected throughout embryonic development in mice (Gernold et al., 1993). The constitutively expressed mouse Hsc70 is present in the preimplantation embryo and is present as early as a two cell stage (Bensaude

et al., 1983). By the blastocyst stage, mouse Hsp70 can be induced as a result of stressors but is not normally found except in very small amounts (Hahnel et al., 1986; Walsh et al., 1997). Hsc70 is also present at high levels during closure of the neural tube, and during differentiation and proliferation of the neuroectoderm (Walsh et al., 1989, 1997). Constitutive levels of Hsps are important for the proper neural development of embryos. Hsc70 levels are normally high in the embryo, however, if Hsp70 levels are induced, formation of the neuroectoderm is abnormal (Walsh et al., 1989).

Hsp genes have heat shock elements (HSE) that are activated by the binding of heat shock factors (HSF) to the HSE to regulate Hsps gene expression. There are three HSF's in mammals, HSF1, HSF2 and HSF3. HSF2 is involved in normal development of the CNS. During embryonic development HSF2 is located in the nucleus and is highly expressed in the neural tube (Akerfelt et al., 2007). *Hsf2* inactivation studies have demonstrated increased embryonic lethality and embryonic brain abnormalities including collapsing of ventricle systems and hemorrhages in the cerebrum (Wang et al., 2003).

Hsp Expression Post-Natally

Hsc70 is constitutively present in the brain of rats from post-natal day 1 to adulthood (Currie et al., 1983). Hsc70 is localized to Purkinje neurons in the cerebellum, and in neuronal cytoplasm and dendrites (D'Souza and Brown, 1998). Hsp70 levels in the rat are increased in the cerebral hemispheres by post-natal day 20 and remain increased during adulthood compared to postnatal day 1 rats. Although Hsp70 levels increase in the rat brain postnatally, detectable levels are low (D'Souza and Brown, 1998).

While very little constitutive expression of Hsp27 is seen the cerebral cortex, constitutive expression of Hsp27 is found in regions of the nervous system including, some neurons of the brainstem and spinal cord (Figure 1) (Plumier et al., 1997a), neurons in the dorsal root ganglion (Costigan et al., 1998), and in some glial cells in the ependymal layer of the lateral ventricle, corpus callosum, septum, and internal capsule (Sanz et al., 2001).

As rats age, basal levels of Hsp70 remain constant compared to younger control rats. However the ability of Hsp70 to be induced to high levels following cell stress is decreased as rats age (Blake et al., 1991; Pardue et al., 1992). This decrease in inducible Hsp70 is thought to leave the brain more vulnerable to stressors.

INDUCTION OF HSPS

Cellular stress can result in induction of the heat shock response. The heat shock response is a global response to noxious stimuli that is generally thought to result in cellular protection by regulating cell homeostasis. The heat shock response is characterized by activation of HSF1, an increase in transcription, translation, and levels of heat shock proteins Hsp27 and Hsp70, and a corresponding increase in

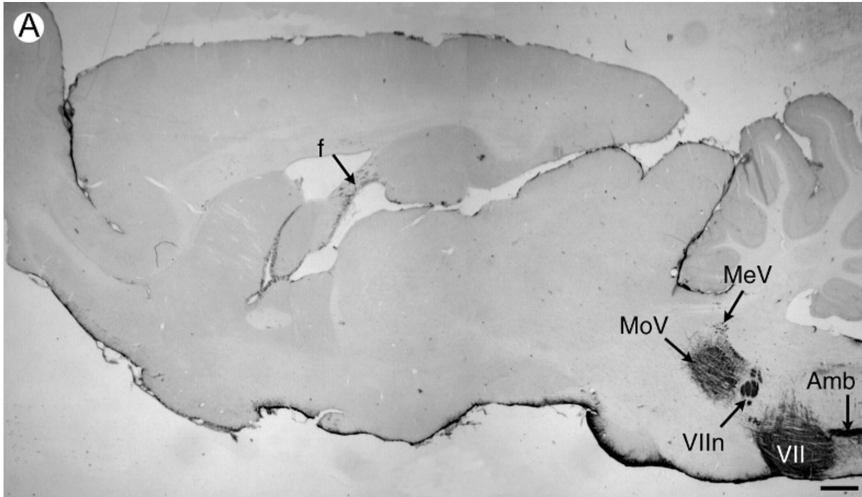

Figure 1. Constitutive levels of Hsp27 in the adult rat brain. Sagittal section, strong immunoreactivity in the brain stem with minimal immunoreactivity in the forebrain. Scale bar = 1 mm. Amb, nucleus ambiguous; f, fornix/fimbria; MoV, trigeminal motor nucleus; MeV, mesencephalic nucleus of the trigeminal nerve; VII, facial nucleua; VIIn, facial nerve root. The image was first published in Plumier et al., J. Comp. Neurol. 1997; 384, 409–428 and is reprinted here with permission

cellular protection. Hsp27 and Hsp70 have repeatedly been linked to neuroprotection following a noxious stimuli or a stressor and are induced to high levels following a variety of stressors.

Hyperthermia

Hyperthermia or heat shock is an increase in body temperature of mammals or basal temperature of cells by approximately 3°C–5°C for 15–60 min. A common heat shock protocol for rats is to increase internal body temperature to 42°C–42.5°C for 15 min (normal body temperature is approximately 38°C), however, various durations of high body temperature are used. Hyperthermia can induce the heat shock response and induce high levels of Hsp70 and Hsp27 in the brain and in various other tissues of the body including the heart (Currie and White, 1981; Krueger-Naug et al., 2000).

Hsp70 is induced to high levels in the brain following hyperthermia. Glial cells have high levels of Hsp70 and Hsp70 mRNA following a temperature elevation (Brown, 1990). Induction of Hsp70 is also present in the choroid plexus, and microvasculature following hyperthermia. Hsp27 levels are increased in the cerebral cortex, hippocampus, cerebellum, brainstem, choroid plexus, and the lateral septum and ependyma around the lateral ventricles (Figure 2) following hyperthermia. Hsp27 is localized to glia (Figure 3) and some neurons with peak levels occurring at approximately 24 hr following heat shock (Krueger-Naug et al., 2000).

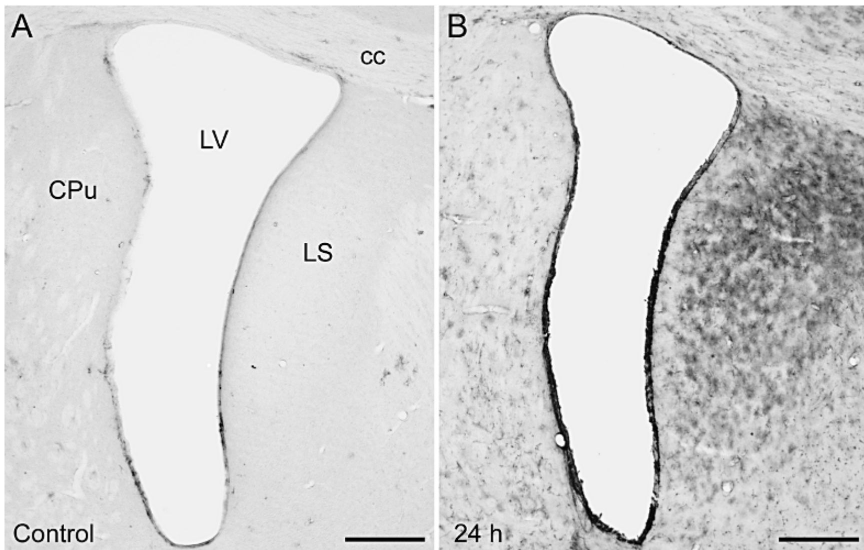

Figure 2. Hsp27 levels in the ependyma and lateral septum in **A**, the control rat brain; and in **B**, 24 hr after hyperthermia. Scale bar = 400 μm. CPu, caudate putamen; LV, lateral ventricle; LS, lateral septum. The images were first published in Krueger-Naug et al., J. Comp. Neurol. 2000; 428, 495–510 and are reprinted here with permission

Seizure Activity

Kainic acid is used to model limbic seizure activity. Kainic acid is an analogue of glutamic acid and acts on glutamatergic neurons resulting in hyperstimulation. Eventually hippocampal neurons die as a result of kainic acid administration. Kainic acid increases Hsp27 mRNA predominately in glia, however, it also increases Hsp70

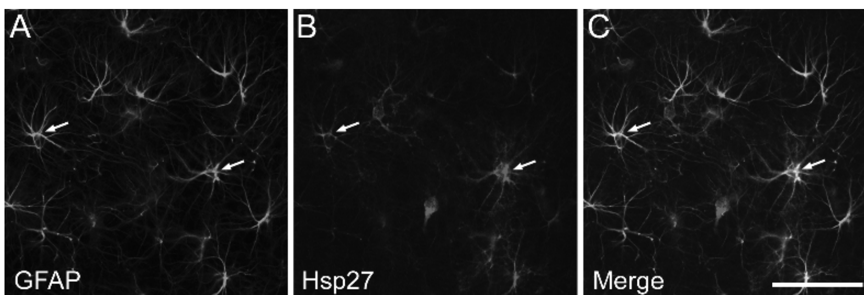

Figure 3. Confocal micrographs of the dentate gyrus 24 hr following hyperthermia showing immunoreactivity in **A**, for glial fibrillary acidic protein (GFAP); in **B** for Hsp27; and in **C**, double labeled cells are identified by arrows. Scale bar = 50 μm. The images were first published in Krueger-Naug et al., J. Comp. Neurol. 2000; 428, 495–510 and are reprinted here with permission

mRNA in neurons. Kainic acid administration results in widespread increased levels of Hsp27 mRNA but also an increase in Hsp70 mRNA levels in the hippocampus, amygdala, parietal cortex, piriform cortex, and distinct nuclei of the thalamus (Armstrong et al., 1996; Krueger et al., 1999; Akbar et al., 2001). Three hours following kainic acid administration, an increase in Hsp70 mRNA is detected and by 12 hr Hsp70 mRNA levels begin to decrease, but 24 hr post kainic acid administration Hsp70 mRNA is still detectable in some regions of the brain (Armstrong et al., 1996; Krueger et al., 1999).

Increasing levels of kainic acid can increase the intensity of seizures. Interestingly, increased amounts of kainic acid and seizure intensity resulted in a corresponding increase in Hsp70 protein levels (Gass et al., 1995). After kainic acid induced seizures, Hsp70 was localized in neurons of the limbic system, striatum, thalamus and cortex including the hippocampus (Figure 4) (Gass et al., 1995; Armstrong et al., 1996; Krueger et al., 1999). In addition, Hsp27 levels were markedly increased in astrocytes in the parietal cortex, piriform cortex and the hippocampus following kainic acid induced seizures (Plumier et al., 1996; Kato et al., 1999).

Ischemia

Following a reduction of blood flow to the brain, there is an increase of Hsps in the brain (Nowak, 1985). Hsp70 levels are increased in neurons in regions of the brain including the hippocampus, striatum, and entorhinal cortex (Vass et al., 1998). Peak levels of Hsp70 following ischemia occurred approximately 24–48 hr after ischemic injury (Vass et al., 1998; Currie et al., 2000), and levels of neuronal Hsp70 mRNA increase and peak following ischemic injury approximately 8 hr after a stroke (Abe et al., 1991). In addition to Hsp70, the small heat shock protein Hsp27 is induced following ischemia injury. Different than Hsp70 localization, Hsp27 levels are increased primarily in glial cells following cerebral ischemia (Kato et al., 1994). Hsp27 levels are distributed widely in the brain and induced levels are longer lasting than the transient expression of Hsp70. Hsp27 positive glial cells increased after 24 hr and were positive until 14 days after the ischemic injury (Kato et al., 1995; Currie et al., 2000). Hsp27 mRNA levels peak later in the brain than Hsp70 mRNA levels, approximately 24 hr after ischemic treatment Hsp27 mRNA levels are at their highest (Higashi et al., 1994). Both Hsp70 and Hsp27 are induced in the brain following ischemic injury, but the localization and time course is different.

Other Stressors

Hsp70 and Hsp27 are induced in the brain by a variety of cell stressors in addition to seizure activity, ischemia, and hyperthermia. Interestingly, extreme exercise can induce heat shock protein levels. Following 180 min of exercise there is an increase in Hsp70 cerebral serum levels in human male participants (Lancaster et al., 2004). The application of potassium chloride to the cortex induces spreading depression and is associated with increases in astrocytic Hsp27 levels (Plumier et al., 1997b).

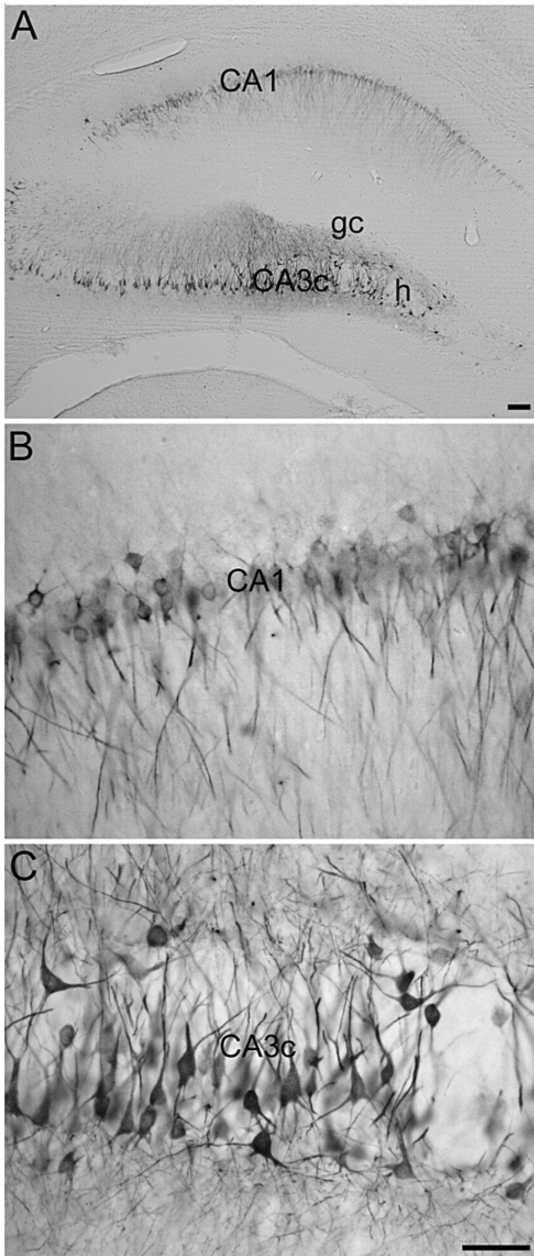

Figure 4. Hsp70 immunoreactivity in the hippocampus 24 hr after kainic acid-induced seizure activity. Hsp70 neuronal immunoreactivity in **A**, in CA1, pyramidal layer of the CA3, and hilus regions of the hippocampus. Hsp70 immunoreactivity in **B**, in the CA1 region of the hippocampus. Hsp70 immunoreactivity in **C**, in the CA3c region of the hippocampus. Scale bars = 50 μm. The images were first published in Krueger et al., Brain Res. Mol. Brain Res. 1999; 71, 265–278 and are reprinted here with permission

Sodium arsenite can induce both Hsp27 and Hsp70 (Fauconneau et al., 2002), and ethanol can increase levels of Hsp70 (Holownia et al., 1995).

NEUROPROTECTION THROUGH HSP70 AND HSP27

The physiological importance of increases in Hsps during cell stress is the subsequent cellular protection that is associated with the Hsps. Hsp70 and Hsp27 can be protective when induced to high levels before and during an injury. A mild stress to induce Hsps can protect cells from a subsequent severe stressor. Hsp70 and Hsp27 can protect cells from severe environmental strain by acting as molecular chaperones assisting in the proper folding, transportation, stabilization and assembly of proteins, disrupting pro-inflammatory and cell death pathways, and blocking protein aggregation. Many studies have used heat shock preconditioning, transgenic models, and RNAi to investigate the role of Hsp70 and Hsp27 in neuroprotection (Plumier et al., 1997c; Magrane et al., 2005; Dodge et al., 2006).

Hsps Reduce Stroke-Like Injury

Transgenic overexpression of rat Hsp70 in mice decreases neuronal cell death and decreases infarct volume in the brain after 24 hr occlusion of the middle cerebral artery (Rajdev et al., 2000). Even forced expression of Hsp70 starting 2 hr after middle cerebral artery occlusion attenuates infarct size (Hoehn et al., 2001; Giffard et al., 2004). While transgenic overexpression of human Hsp70 in mice had no effect on infarct volume in the brain after 24 hr middle cerebral artery occlusion, the hippocampus in these mice was spared from neuronal cell death (Figure 5) (Plumier et al., 1997c). Hsp70 protects cells from ischemia reperfusion injury by inhibiting cell death pathways and interacting with proteins involved in apoptosis and necrosis.

Apoptosis-inducing factor (AIF), the mitochondrial protein involved in apoptosis, is important in the caspase-independent cell death pathway. Overexpression of Hsp70 can sequester AIF and reduce ischemic brain injury in neonatal mice (Matsumori et al., 2005). The release of cytochrome c from the mitochondria and the mitochondrial pathway of apoptosis can be disrupted by Hsp70 through decreased cleavage of caspase 9 and binding to the apoptotic protease activating factor 1 (Apaf-1) (components of the apoptosome) (Beere et al., 2000; Matsumori et al., 2006). Neonatal mice transgenically overexpressing Hsp70 have reduced cytosolic levels of cytochrome c compared to control mice after an ischemia reperfusion injury, suggesting a reduction in cytochrome c release from the mitochondria as a result of Hsp70 (Matsumori et al., 2006). In addition there is a decrease in cleaved caspase 9 and an increase in binding of Hsp70 to the Apaf-1 in neonatal rats overexpressing Hsp70 exposed to ischemia reperfusion compared to control (Matsumori et al., 2006). Thus, Hsp70 is protecting cells from ischemic injury by

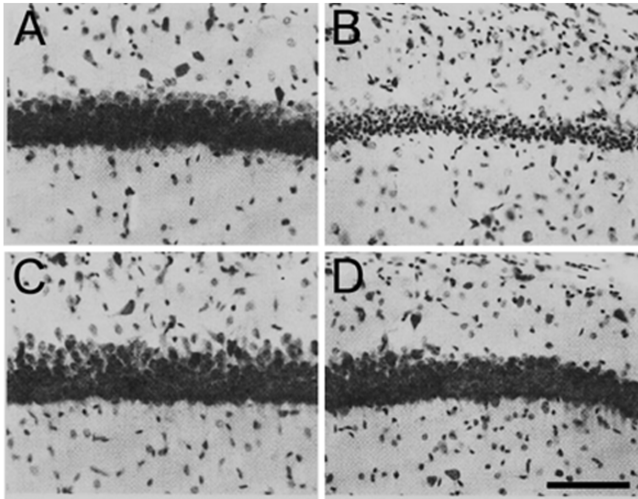

Figure 5. Hsp70 transgenic mice hippocampal CA1 neurons are resistant to ischemia induced neurotoxicity, 24 hr post middle cerebral artery occlusion. Brain sections stained with cresyl violet. **A**, on the contralateral side to the ischemic injury, non-transgenic mice showed no pyknotic cells in the CA1 region. **B**, ipsilateral to the injury, non-transgenic mice had many pyknotic cells in the CA1 region of the hippocampus. **C** and **D**, transgenic mice with overexpression of human Hsp70 had no pyknotic cells in the contralateral (**C**) or ipsilateral side of the ischemic injury (**D**). Scale bar = 100 μm. The images were first published in Plumier et al., Cell Stress Chaperones 1997; 2, 162–167 and is reprinted here with permission

interrupting apoptotic signaling pathways that are both caspase-independent and caspase-dependant.

Following global cerebral ischemia, the anti-apoptotic protein bcl-2 is increased in the hippocampus of rats overexpressing Hsp70 by a viral vector (Kelly et al., 2002). Hsp70 can increase cell survival and increase bcl-2 levels in other models of cell stress (Polla et al., 1996). The increase in bcl-2 protein levels in rats overexpressing Hsp70 following global cerebral ischemia may be an important factor in understanding the mechanism of neuroprotection by Hsp70.

Hsp27 also appears to be involved in neuroprotection following cerebral ischemia. When the brain is preconditioned with a sublethal cerebral ischemic insult there is an increase in Hsp27 levels and tolerance to subsequent lethal cerebral ischemic insults (Kato et al., 1994). This result has been corroborated by Currie et al. (2000), who showed that focal preconditioning produces significant and prolonged ischemic tolerance of the brain to subsequent permanent middle cerebral artery occlusion, and this is associated with elevated levels of Hsp27 and Hsp70. Hsp27 appears to be important in the protective effects of ischemic preconditioning (Valentim et al., 2001). Rats over expressing Hsp27 have a 44% reduction in lesion volume 24 hr after ischemia compared to controls. Both Hsp70 and Hsp27 are important in cell survival and neuroprotection during and after ischemic injury.

Hsps Suppress Seizure-Induced Cell Death

Hsps are neuroprotective against kainic acid induced seizures and cell death. Overexpression of Hsp27 and Hsp70 are associated with cell survival (Akbar et al., 2003; Kalwy et al., 2003; Tsuchiya et al., 2003). Overexpression of Hsp27 significantly reduces hippocampal cell death, seizure severity, and mortality in Hsp27 transgenic mice treated with kainic acid (Akbar et al., 2003). Caspase 3 mRNA, an early marker of the apoptotic cell death cycle, is increased in control mice administered kainic acid but the increase in caspase 3 was attenuated in transgenic mice overexpressing Hsp27 (Akbar et al., 2003). Hsp27 may be neuroprotective by regulating apoptosis of neurons.

Kainic acid induced seizures in Hsp70 overexpressing transgenic mice results in fewer TUNEL labeled cells than control mice and the mortality rate appeared to be lower in Hsp70 transgenic mice, but did not reach significance (Tsuchiya et al., 2003). Elevated levels of Hsp70 appear to play an important role in protecting the brain from kainic acid induced seizures.

Hsps Regulate Inflammation

Hsp27 and Hsp70 have anti-inflammatory functions decreasing levels of inflammatory factors and interrupting inflammatory pathways like nuclear factor-κB (NF-κB) in a variety of tissues and cells including the heart, vascular smooth muscle, macrophages, fibroblasts, and endothelial (Chen et al., 2004; Chen and Currie, 2006; Markovic and Stuhlmeier, 2006; Shi et al., 2006; Nakabe et al., 2007). Hsp27 and Hsp70 also have anti-inflammatory actions in the brain, but the action of Hsps on CNS inflammation is not well understood.

Glial cells heat shocked *in vitro* have a reduced inflammatory response, decreased NF-κB nuclear localization (suggesting decreased activation), and reduced nitric oxide synthase (NOS-2) in response to lipopolysaccharide (LPS) (Feinstein et al., 1996). Hsp70 is suggested to be mainly responsible for the anti-inflammatory effects in the glial cells. When glial cells overexpressing Hsp70 are subjected to LPS, the cell protection is similar to the heat shocked cells, suggesting that Hsp70 may partly mediate the anti-inflammatory effects of heat shock on glial cells (Feinstein et al., 1996).

In normal unstressed cells NF-κB is found in the cytoplasm bound to its inhibitor IκB. For NF-κB to become activated its inhibitor must be phosphorylated by IKK and NF-κB is then free to translocate to the nucleus (Ghosh et al., 1998). Recently it has been suggested that in the brain, Hsp70 binds to the NF-κB–IκB complex preventing IκB phosphorylation by IKK, therefore effectively blocking the pro-inflammatory NF-κB pathway (Zheng et al., 2008).

Hsps and Neurodegenerative Diseases

Heat shock proteins can be protective in neurodegenerative diseases that are characterized by protein aggregation or misfolding. Huntington's disease, Parkinson's

disease, and Alzheimer's disease with dementia are all examples of neurodegenerative diseases with protein aggregation or misfolding. Hsps appear to have a protective role in all of these diseases.

Huntington's disease (HD) is a neurodegenerative disease characterized by neuronal cell loss and intracellular protein aggregation. HD patients have a mutant form of the huntingtin gene that is defined by an expanded CAG repeat region in the gene encoding the huntingtin protein on chromosome 4. As a result HD patients have a mutant form of the HD protein such that there is an expanded glutamine (Q) repeat region (Bates, 2003). In HD, the accumulation of the huntingtin protein with polyQ repeats greater than 35–38 residues precipitate and form aggregates in the affected neurons. Hsps are protective against polyglutamine toxicity and decrease aggregation and cell death.

Hsp27 can have a protective role in different cell and animal models of HD. Wyttenbach et al., (2002) used a cell model of HD and demonstrated that Hsp27 can suppress polyQ mediated cell death without suppressing polyQ aggregation. Hsp27 decreased levels of reactive oxygen species (ROS) in the HD cells suggesting that the protective effects of Hsp27 on cell death may be related to ROS (Wyttenbach et al., 2002). Overexpression of Hsp27 or Hsp70 and Hsp40 in a cell model of HD with 72 CAG repeats inhibited mitochondrial membrane disruption, and the increase in ROS, and reduced the size of the inclusion bodies formed (Firdaus et al., 2006). Interestingly, overexpression of Hsp27 or Hsp70 and Hsp40 in a cell model of HD with 103 CAG repeats did not result in decreased inclusion size or oxidative damage (Firdaus et al., 2006).

Zourlidou et al. (2007) crossed a mouse model of HD, the R6/2 mouse, with mice overexpressing Hsp27. Interestingly, this double transgenic mouse did not improve phenotypically. There was no behavioural improvement, decrease in oxidative stress, or change in aggregate formation. However, the transgenic Hsp27 mouse and the double transgenic R6/2-Hsp27 mouse overexpressed Hsp27 but the R6/2-Hsp27 mouse did not have an *active* form of Hsp27. Zourlidou et al. (2007) suggested that the slow increase and accumulation of damaged protein may not have been detected by the cell stress response mechanisms, or induced tolerance.

Hsp70 is associated with reduced polyQ toxicity and aggregation in HD models. In a cell model of HD, cells with long polyQ repeats formed aggregates and died through apoptosis but when Hsp70 and Hsc70 levels were increased, apoptotic cell death was significantly reduced (Novoselova et al., 2005). Hsp70 and Hsc70 not only decreased apoptosis but also decreased the size and number of inclusions (Novoselova et al., 2005). An *in vivo* study crossed R6/2 mice with mice overexpressing Hsp70 to investigate whether the protective effects of Hsp70 seen *in vitro* HD models occurs in an animal model of HD as well. However, the number and size of inclusions, cell death, and behaviour did not differ in the double transgenic mice compared to the R6/2 mice (Hansson et al., 2003). One effect of Hsp70 overexpression in the R6/2 mice that was noted, was a delayed loss of body weight. Studies using R6/2 double transgenic mice expressing Hsp27 or Hsp70 have not shown the same protective effects that have been reported in *in vitro* studies with

high levels of Hsps. The effects of Hsps on HD aggregation may be more complicated than what the *in vitro* studies suggest, and it may be of benefit to conduct other *in vitro* studies in HD models other than R6/2.

Heat shock proteins are neuroprotective in neurodegenerative disorders characterized by Lewy bodies (LB), also known as LB diseases. LB are inclusions the contain α-synuclein as the main component. These LB are found in Parkinson's disease and Alzheimer's disease with dementia. Overexpression α-synuclein results in LB inclusions that are cytotoxic. Hsp27 decreases α-synuclein toxicity and is protective in LB diseases (Outeiro et al., 2006). Hsp70 is also protective against LB in different disease models and inhibits α-synuclein aggregation and toxicity (Auluck et al., 2002; Klucken et al., 2004; Huang et al., 2006).

DRUG AND SUPPLEMENT INDUCTION OF HSPS

Hsps have neuroprotective roles when they are induced to high levels. For Hsps to be induced, cells need to be subjected to a stressor or need to be modified for genetic overexpression of specific Hsps. If Hsps can be induced in an organism without subjecting cells or the whole organism to stress or modifying it genetically, then high levels of Hsps may be used as a therapy for neurological injuries or aliments. Geldanamycin, geranylgeranylacetone (GGA), and a blueberry rich diet all have neuroprotective effects and increase Hsp70 levels.

Geranylgeranylacetone

GGA was originally developed as an antiulcer drug in Japan but has recently been studied for its Hsp70 inducing properties. Oral administration of GGA induces Hsp70 in the brain, gastric mucosa, and the heart (Hirakawa et al., 1996; Ooie et al., 2001; Fujiki et al., 2003). Following oral administration, GGA increases Hsp70 levels in hippocampal neurons with peak levels of Hsp70 approximately 2 days after GGA administration (Fujiki et al., 2003).

The polyglutamine disease spinal and bulbar muscular atrophy (SBMA) is a motor neuron disease that is the result of an expansion of the GAG repeat in the androgen receptor gene. In a mouse model of SBMA, oral administration of GGA can increase Hsp70, Hsp90, Hsp105, and reduce the nuclear accumulation of the pathogenic androgen receptor protein (Katsuno et al., 2005). These results suggest that GGA actions may be through induction of Hsps and that GGA may be useful as a therapy for polyglutamine-mediated neurodegenerative diseases (Katsuno et al., 2005).

A single dose of GGA orally administered 48 hr before permanent left middle cerebral artery occlusion was neuroprotective against cerebral ischemic neuronal injury. Hsp70 levels were significantly increased and the cerebral infarct volume was significantly decreased in GGAtreated rats compared to control rats (Nagai et al., 2005). This study also suggests that the neuroprotective effects of GGA may be through the induction of high levels of Hsp70.

Administration of GGA before and 3 days following intracerebral hemorrhage increases Hsp70, decreases cell death, and improves functional recovery in rats (Sinn et al., 2007). When GGA was administered as a single dose prior to intracerebral hemorrhage induction, Hsp70 levels were not upregulated. Though there was an increase in Hsp70 following multiple days of GGA administration, there were also increases in the cerebral levels of eNOS, pSTAT3, and pAkt that are associated with preserved cerebral blood flow, neuronal regeneration, and cell survival, respectively. In addition, pSTAT3 and pAkt are associated with the activation of HSF-1. As well, there was a decrease in pro-inflammatory molecules in the brain of rats receiving multiple day GGA administration including IL-6, macrophage inflammatory protein-1 (MIP-1), and matrix metalloproteinase-9 (MMP-9) (Sinn et al., 2007).

The mechanism for Hsp70 induction through GGA has been suggested to be a result of GGA-induced induction of protein kinase C (PKC) (Uchida et al., 2006). One of the mediators of Hsp70 expression is thought to be PKC. GGA administration increases PKC levels and Hsp70 levels and this is associated with neuroprotection against cerebral infarction in rats. When chelerythrine, a specific PKC inhibitor, is administered before GGA and cerebral infarction, GGA failed to increase HSP70 levels and the neuroprotective effects of GGA against cerebral infarction were prevented (Uchida et al., 2006). This suggests that PKC promotes increased Hsp70 levels following GGA administration and that PKC and Hsp70 are important for the neuroprotective actions of GGA.

Geldanamycin

Geldanamycin is a Hsp90 inhibitor, but also increases Hsp70 levels (Shen et al., 2005). Geldanamycin is a benzoquinone ansamycin antibiotic that binds and inhibits Hsp90. Geldanamycin inhibits the association of Hsp90 with other proteins, including HSF-1 (Shen et al., 2005). Shen et al. (2005) suggested that if Hsp90 can no longer bind with HSF-1 when geldanamycin is administered, free HSF-1 can trimerize, translocate into the nucleus and bind with its promoter and induce Hsp70 expression.

Geldanamycin has demonstrated neuroprotective effects in a variety of brain injury models including 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity, i.e., a model of Parkinson's disease, cerebral ischemia, and huntingtin aggregate formation (Sittler et al., 2001; Lu et al., 2002; Shen et al., 2005). When mice are pretreated with geldanamycin, administered via intracerebral ventricular injection, 24 hr before MPTP injection there is a significant increase in Hsp70, dopamine, and tyrosine hydroxylase in the striatum (Shen et al., 2005). The increase in dopamine and tyrosine hydroxylase suggests that geldanamycin may be useful in research and treatment of Parkinson's disease.

When geldanamycin is administered to a cell model of Huntington's disease, Hsp70 and Hsp40 are significantly increased and there is a corresponding decrease in huntingtin protein aggregation (Sittler et al., 2001). When Huntington's cells were

transfected with Hsp70 and Hsp40, there was a significant decrease in huntingtin protein aggregation suggesting that the inhibition of huntingtin aggregation by geldanamycin is through increased levels of Hsp70 and Hsp40 (Sittler et al., 2001).

Pretreatment with geldanamycin before cerebral ischemic injury to rats is neuroprotective. Rats receiving geldanamycin before injury had a significant decrease in infarct volume, significant increases in Hsp70, Hsp27, and improved behavioural recovery (Lu et al., 2002). Hsp70 was localized to neurons and Hsp27 was localized to glia and arteries. Geldanamycin has neuroprotective effects against a variety of brain injuries, and may be useful for the treatment, prevention, or attenuation of disease.

Blueberry Diet Supplementation

Reductions in Hsp70 have been identified in aging, and this reduction may result in increased susceptibility to different stressors (Blake et al., 1991; Pardue et al., 1992). Interestingly, blueberry supplementation increased the hippocampal levels of Hsp70 induced by LPS in old rats (19–21 months) (Galli et al., 2006). Young rats (4–6 months) on a control diet had significantly higher levels of hippocampal Hsp70 induced by an in vitro LPS challenge than old rats on a control diet. Old rats on a blueberry supplemented diet had significantly higher Hsp70 levels induced by an in vitro LPS challenge than old rats on a control diet. There was no difference in LPS induced Hsp70 levels between old rats on a blueberry diet and young rats on a control diet (Galli et al., 2006). These results suggest that blueberry supplementation may assist in enhancing the Hsp70 response to stressors during aging.

Continued investigation into methods to induce Hsps in a non-invasive manner may be of benefit for treatment or prevention of a variety of injuries and diseases. Insulin may also be a method to induce Hsp70. Li et al. (2006) reported insulin induced Hsp70 and myocardial protection, and if this induction and protection is seen in the brain, insulin may be another method used to induced Hsp70. Still, geldanamycin, geranylgeranylacetone, and a blueberry supplemented diet are all promising methods to induce elevated expression of Hsps, non-invasively.

CONCLUSION

Hsp27 and Hsp70 are neuroprotective against a variety of cell stressors and neurodegenerative diseases. Induced cell type-specific expression of Hsp27 (glia) and Hsp70 (neurons) may be crucial for neuroprotection. Protecting the brain from stroke or degenerative disease may rely on induced expression specific Hsps in specific cell types. For example, high levels of Hsp27 in glia may buffer the neurotoxicity of degenerating neurons in stroke injury and protect adjacent neurons from excitotoxic cell death. While more investigation is needed to understand the neuroprotective mechanisms of Hsp27 and Hsp70, Hsps are likely to be valuable in treatment of neurodegenerative diseases and neurological insults. Most importantly, to take full advantage of the protective effects of Hsps, non-invasive, non-noxious and

non-stressful methods need to be identified to increase expression of each Hsp in the appropriate cell type.

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CHAPTER 9

MOLECULAR CHAPERONES AND PROTECTION IN ANIMAL AND CELLULAR MODELS OF ISCHEMIC STROKE

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Abstract: Chaperones, including heat-shock proteins (Hsps) Hsp70, Hsp40 and GroEL, has been shown to protect from both global and focal ischemia in vivo and cell culture models of ischemia/reperfusion injury in vitro. While the mechanism of protection in part reflects chaperone functions (i.e., preventing abnormal protein folding or aggregation), work from our laboratory and others has implicated additional mechanisms including direct interference with cell death pathways, modulation of inflammation, and preservation of mitochondrial function. In this chapter we will first briefly introduce the Hsps, then describe the animal and cellular models of cerebral ischemia in which effects of Hsps have been studied, including reviewing methods used to overexpress Hsps. We will focus on the protective effect of overexpressing different Hsps against ischemic stroke and elaborate the potential mechanisms involved. Despite a great deal of study, much remains to be learned about the multifaceted effects of Hsps in cerebral ischemia. The endogenous stress response remains a model of cell protection with promise for the development of novel therapies for ischemic brain injury

Keywords: Apoptosis; cell culture; cerebral ischemia; inflammation; heat shock proteins; neuroprotection

INTRODUCTION

Molecular chaperones are a functionally related group of proteins that assist protein folding in bacteria, plant, and animal cells under physiological and stress conditions. In addition to their role in protein folding, chaperones facilitate translocation of proteins across membranes, help assemble and disassemble protein complexes, help present substrates for degradation, and suppress protein aggregation (Hartl, 1996;

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Ohtsuka and Hata, 2000). An important subgroup of highly evolutionarily conserved chaperones is the ATP-dependent heat shock proteins (Hsps), which share the ability to recognize and bind nascent and unfolded proteins, preventing aggregation and facilitating correct protein folding (Beissinger and Buchner, 1998; Frydman, 2001). Chaperones are currently being considered for the potential treatment of diseases involving protein aggregation and misfolding from neurodegenerative diseases (Bonini, 2002) to cancer (Scott and Frydman, 2003). In this chapter, we will first briefly describe the families of Hsps, the animal and cellular models of cerebral ischemia, and ways in which Hsps have been overexpressed to study their effect in ischemic brain injury. Finally, we will summarize the mechanisms that have been investigated to explain the protective effects of Hsps against ischemic stroke.

HEAT SHOCK PROTEINS (Hsps)

One of the important functions of Hsps is chaperoning molecules within the cell. Hsps were first identified as proteins induced by stressful stimuli that induce protein denaturation or aggregation, and were found to help maintain cellular integrity and viability. Hsps consist of both stress inducible and constitutively expressed family members, as well as members that are associated with specific organelles. The Hsps are named by size class. The 70 kD family is perhaps the most extensively studied. Included in this family are Hsc70 (the constitutive form), Hsp70 (the inducible form), Hsp75 (also called Grp75/mortalin, a constitutively expressed glucose regulated protein largely localized to mitochondria), Hsp78 (also referred as Grp78, a constitutively expressed glucose regulated protein largely localized to the endoplasmic reticulum). Other HSP families include those of HSP90, HSP27 and HSP40. The HSP60 family members are chaperonins, having a distinct multimeric hatbox-like structure, but also facilitate folding in an ATP dependent manner.

In the nervous system, the Hsps are induced in a variety of pathologic states including cerebral ischemia, neurodegeneration diseases, epilepsy, and trauma. Overexpression of Hsps, such as Hsp70, Hsp60, and Hsp40, has been shown to provide protection from cerebral ischemia both in animal models of stroke and in cell culture models.

HSP70 Family

Structurally Hsp70 can be roughly divided into a 44 kDa amino-terminal adenosine triphosphate binding (ATP) ase domain, and a carboxyl-terminal region that contains the 18 kDa peptide or substrate-binding domain, followed by a 10 kDa stretch terminating in the highly conserved EEVD sequence (Wang et al., 1993; O'Brien et al., 1996; Ohno et al., 2004). Both a constitutive form, Hsc70, and the highly stress inducible Hsp70 are primarily cytosolic in localization. A large number of proteins are known to interact with different domains within Hsp70. Both nascent polypeptide chains and hydrophobic peptides and regions of proteins are substrates for the peptide-binding domain of Hsp70 (Wang et al., 1993; Erbse et al., 2004).

Hsp75/grp75/mortalin is a primarily mitochondrial resident protein involved in protein translocation into mitochondria (Kaul et al., 2007). It is not heat inducible. The majority of mitochondrial proteins are synthesized as precursor proteins on cytosolic ribosomes. After the amino-terminal part of a preprotein has reached the inner face of the inner membrane driven by the membrane potential, the subsequent translocation of the polypeptide chain is driven by the mitochondrial Hsp75. After the crucial interaction with Hsp75 during import, preproteins are released mostly as unfolded proteins (Manning-Krieg et al., 1991). Only then can subsequent folding and assembly reactions take place. Depending on their biochemical properties, the newly imported proteins can interact with different mitochondrial chaperones, most prominently Hsp60 or Hsp75.

HSP60 Family

The HSP60 family of chaperonins, also carries out ATP dependent folding, but has a different oligomeric structure (Lin and Rye, 2006). The E. coli homolog of HSP60 named GroEL assembles into a structure composed of two heptameric rings of GroEL subunits (57 kDa) to form a 14-subunit hollow cylinder and interacts with its cochaperone GroES which functions as the lid on the hatbox (Weissman et al., 1994). GroEL is known to fold eukaryotic proteins. The mutant GroEL-D87K contains a lysine substituted for an aspartic acid residue at position 87. This mutation blocks ATP binding, rendering it ATPase deficient and therefore deficient in protein folding (Frydman and Hartl, 1996; Thulasiraman et al., 1999). GroEL-D87K efficiently interacts with stress-denatured proteins in mammalian cells but since it is unable to release them in the presence of ATP (Weissman et al., 1994; Frydman and Hartl, 1996; Thulasiraman et al., 1999), it acts efficiently as a denatured protein trap.

HSP40 Family

The HSP40 family is Hsp70 cochaperones, characterized by J domains used to interact with Hsp70. Hdj-2, (human DnaJ-2), a member of the HSP40 family, is highly homologous to DnaJ from Escherichia coli. Hdj-2 targets Hsp70 to specific intracellular tasks and accelerates the Hsp70 ATPase activity (Minami et al., 1996; Gebauer et al., 1997; Tang et al., 1997).

ANIMAL AND CELLULAR MODELS OF CEREBRAL ISCHEMIA

Forebrain Ischemia

Forebrain ischemia leads to selective delayed loss of pyramidal neurons in the CA1 subregion of the hippocampus. This is the pattern of brain injury observed following cardiac arrest and resuscitation. It is induced in rats by occluding both carotid arteries and inducing hypotension (Smith et al., 1984; Ouyang et al., 2007).

Hypotension (to 50 mm Hg) is induced by removing blood into heparinized sterile tubing; recirculation is induced by reinfusing the shed blood and releasing the carotid artery clamps. Temperature is maintained by servocontrol and blood gases and glucose are monitored.

To evaluate outcome the brains are perfused with saline then ice-cold 4% phosphate buffered paraformaldehyde before removal for sectioning and immunohistochemical analysis and analysis of infact volume by cresyl violet staining.

Focal Cerebral Ischemia

Focal cerebral ischemia indicates temporary or permanent loss of blood flow to one region of the brain, which produces the type of brain injury observed in ischemic stroke. Focal cerebral ischemia is induced in rat and mouse models using the suture occlusion technique, as previously described (Sun et al., 2006). A suture is introduced via the external carotid artery and carefully advanced to occlude the middle cerebral artery (MCA) at its origin. The suture is removed to allow reperfusion. Sham-operated animals undergo an identical procedure, except that the suture is not inserted.

To evaluate outcome neurological score is assessed 24 hr after MCA occlusion and then the animals are anesthetized with isoflurane and decapitated. Brains are removed and sectioned coronally. Sections are incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline for 20 min at 37°C. To determine infarct volume at 24 hr by TTC staining, six slices per rat are analyzed by an observer blinded to the treatment condition, using the NIH Image program. To correct for the effects of cerebral edema and of differential shrinkage resulting from tissue processing, the infarction area in each section is calculated by subtracting the area of the healthy, uninfarcted (TTC-stained) tissue in the ipsilateral hemisphere from the area of the contralateral hemisphere as described previously (Swanson et al., 1990; Sun et al., 2006). Infarction volume is determined by summing the infarction areas of all sections and multiplying by the slice thickness. For immunohistochemistry, brains are perfused with saline then ice-cold 4% phosphate buffered paraformaldehyde prior to preparing coronal sections with a Vibratome and staining with antibodies.

Primary Cell Cultures, Slice Cultures, and Injury Paradigms

Central nervous system ischemia can be modeled in vitro by exposing cultures to ischemia- like conditions such as substrate and/or oxygen deprivation (Papadopoulos et al., 1996). Dissociated cultures of either single cell types or mixtures, such as neurons and astrocytes, can be prepared. Primary cortical neuronal cultures are prepared from fetal mice or rats as described previously (Dichter, 1978; Dugan et al., 1995). Primary astrocyte cultures are prepared from postnatal (days 1–3) mice or rats (Simonsen) (Papadopoulos et al., 1998).

Slice cultures occupy a place between isolated cell culture and intact brain, in that within a hippocampal slice many of the normal anatomic relationships are retained, and some of the normal neuronal circuitry is preserved. Slice cultures are prepared according to Stoppini et al.'s method (Stoppini et al., 1991), with some modifications (Ouyang et al., 2005). Hippocampi for organotypic cultures are isolated from 6-day-old mice then cut into 200-μm-thick transverse sections for culture on semiporous membrane inserts.

In vitro Injury paradigms For oxygen glucose deprivation (OGD) experiments, cultures are transferred to an anaerobic chamber and deprived of oxygen and glucose by changing the culture medium to a balanced salt solution (Papadopoulos et al., 1996). After the predetermined insult duration OGD is ended by adding glucose to the culture medium to a final concentration of 5.5 mM and returning the cultures to the normoxic incubator for recovery. Variations include isolated glucose deprivation, or addition of oxidants such as peroxide. Different labs use either buffer conditions of balanced salt solution similar to normal extracellular ionic conditions but lacking glucose, or choose to mimic the ionic changes observed in ischemic brain (Bondarenko and Chesler, 2001; Rytter et al., 2003). Hippocampal slice culture injury closely resembles the delayed selective loss of CA1 neurons observed in forebrain or global ischemia (Ouyang et al., 2005).

Assessment of injury Primary astrocyte, neuron, or astrocyte neuronal co-culture injury is estimated morphologically by phase-contrast light microscopy and quantitated by measuring the lactate dehydrogenase (LDH) released from lysed cells into the bathing medium (Koh and Choi, 1987). Alternatively cultures are stained with vital dyes and stained and total cells counted. In the case of neuronal-astrocyte mixed cultures the durations of OGD required to kill the two cell types are sufficiently different to allow selective killing of neurons with short durations of OGD. Cell death in slice cultures is detected with the fluorescent cell death marker propidium iodide (PI, 1 μg/mL). Cultures are examined with an inverted fluorescence microscope at excitation 510–550 nm and emission at 590 nm and above. In some cases slices are fixed and stained after sectioning to assess changes in protein expression or localization.

METHODS OF OVEREXPRESSION AND TRANSFECTION

While this chapter will focus on results obtained with overexpression of inducible Hsp70, it is important to note that in studies in which inducible Hsp70 expression was eliminated by disruption of the gene (Lee et al., 2001b) ischemic brain injury was exacerbated. Further, anti-sense inhibition of Hsp70 induction by heat abrogated the induction of neuroprotection (Sato et al., 1996).

Hsp70 Transgenic Mice

Three strains of Hsp70 overexpressing mice have been studied for response to cerebral ischemia, or used for isolation of brain cells to study injury in culture.

One strain (FVB/N background) overexpressing human inducible Hsp70 (Hsp72 Tg) from the human β-actin promoter was produced by the Radford and colleagues (Radford et al., 1996), while the second strain overexpressing rat inducible Hsp70 from the β-actin promoter was produced by the Dillman group in B6/SJL mice (Marber et al., 1995). A third strain also expressing human inducible Hsp70 from the human β-actin promoter was made in CBA/C57Bl6 mice (van der Weerd et al., 2005).

DNA Mediated Transfection

Stereotactic intracerebroventricular injection has been used in several studies for in vivo expression. DNA mixed with cationic lipid is stereotactically injected either intracerebroventricularly (Hecker et al., 2001) or into brain parenchyma, generally either into the hippocampus or striatum depending on the ischemia model, 24–72 hr prior to ischemia (Sun et al., 2006; Xu et al., 2006).

Viral Vector Infection

Several laboratories have used Herpes vectors (Kelly et al., 2002) or adenoviral vectors (Kelly et al., 2001a) to induce expression of inducible Hsp70 in animal models of cerebral ischemia and observed protection. These vectors have also been used in cell culture studies (Uney et al., 1993; Fink et al., 1997), as have retroviral vectors (Papadopoulos et al., 1996).

A further refinement is to use either conditional or cell type specific promoters with the gene to further dissect the role of Hsp70 at specific times or in specific cell types. In this regard studies have been conducted with neuron-specific promoters (Kelly et al., 2001b). The expression of mutants of inducible Hsp70 has also been used to dissect the regions of Hsp70 that are required for brain protection from focal ischemic injury (Sun et al., 2006).

PROTECTION AGAINST ISCHEMIA WITH HSP OVEREXPRESSION

To directly test protective capabilities of Hsps, cells can be made to selectively overexpress the protein, or expression can be blocked. This is accomplished using transgenic or knockout animal models, gene transfer, or antisense or RNA_i approaches. We will discuss results of studies using these techniques to understand the extent and mechanisms of Hsp70 protection from cerebral ischemia.

Inducible Hsp70 Overexpression Protects from Ischemia In Vivo and In Vitro

During normal conditions, the level of inducible Hsp70 in the brain is low. In times of stress such as ischemia, however, Hsp70 protein is elevated in cells stressed by, but surviving the ischemic insult (Nowak, 1990; Sharp et al., 1991; Planas

et al., 1997). Hsp70 induction has also been identified in cells after preconditioning with either brief ischemia or heat (Yenari et al., 1999). Work from several laboratories including ours has demonstrated that augmenting the levels of Hsp70 by either transgenic overexpression (Plumier et al., 1997; Rajdev et al., 2000; van der Weerd et al., 2005) or induced overexpression with either a Herpes or Adenoviral vector reduces brain injury from ischemia in rat stroke models of both global and focal ischemia (Yenari et al., 1998; Kelly et al., 2001a, c, 2002). Although in the majority of cases Hsp70 overexpression protects from cerebral ischemia, this result has not been uniformly found (Lee et al., 2001a; Badin et al., 2006). Varying degrees of protection may reflect the titration of Hsp70 against the insult; if Hsp70 expression is at low levels or if the insult is severe, protection may not be observed (Lee et al., 2001a).

Retroviral vectors were used to induce Hsp70 overexpression in murine astrocytes and provided significant protection from glucose deprivation, combined oxygen and glucose deprivation, and H_2O_2 exposure (Papadopoulos et al., 1996; Xu and Giffard, 1997). Overexpression of Hsp70 in cultured neurons is also associated with protection (Uney et al., 1993; Amin et al., 1996; Fink et al., 1997). Interestingly, we also found that overexpression of Hsp70 in astrocytes protected co-cultured wild-type neurons (Xu et al., 1999). These in vitro injury models mimic some of the types of injury involved in ischemic damage during stroke. They reveal several potential ways in which Hsp70 could provide protection. The ability of astrocytes to better protect neurons when only the astrocytes overexpress Hsp70 raises several intriguing issues about the ways in which astrocytes interact with neurons during and following ischemia, and may be due to better antioxidant support of neurons and/or even direct provision of Hsp70 by astrocytes to neurons (Guzhova et al., 2001). We have demonstrated recently that selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia in animal stroke models and after OGD in hippocampal organic slice cultures (Ouyang et al., 2007). This raises the question whether targeting Hsp70 overexpression to astrocytes in vivo would be sufficient to protect CA1 neurons.

Hdj-2 (Hsp40) Overexpression Reduces Ischemic Injury In Vitro

Hdj-2 has been shown to decrease injury in models of degenerative brain disease involving protein aggregation. Cotransfection of Hdj-2 with mutant ataxin-1 resulted in a significant reduction in aggregate formation (Stenoien et al., 1999). In a model of the polyglutamine disease Huntington's disease, overexpression of Hdj-2 suppressed aggregate formation, and was associated with decreased toxicity (Jana et al., 2000). We demonstrated that overexpression of Hdj-2 reduces ischemia-like injury in vitro (Qiao et al., 2003).

The J domain of HDJ-2 is the region of the protein responsible for mediating the binding of Hdj-2 to Hsp70 (Hartl, 1996). Hdj-2 binds to and stimulates the ATPase activity of Hsp70, thereby enhancing the chaperone function of Hsp70 (Hartl, 1996).

The fact that Hdj-2 is still able to suppress aggregation when the J domain is deleted suggests that this suppression shows independent of interactions with Hsp70 (Langley et al., 1999). It has been suggested that Hdj-2 alone can bind misfolded proteins, suppress aggregation, and facilitate delivery of misfolded polypeptides to the cellular machinery for proteolytic degradation (Stenoien et al., 1999). It has been speculated that the chaperone function of Hdj-2 in the brain may be one of the factors responsible for the relative resistance of brain cells to damage (Miller et al., 1990; Jana et al., 2000). Because Hsp70 levels are not increased in parallel with Hdj-2 following transfection (Qiao et al., 2003), the protection is likely to reflect the direct effects of Hdj-2.

Mitochondrial Hsp75 Overexpression

While many studies have been performed on the cytosolic inducible Hsp70, very little research has been conducted on the mitochondrial form, Hsp75/Grp75/mortalin in cerebral ischemia. It has been shown that Hsp75 is induced by glucose or amino acid deprivation (Heal and McGivan, 1997) and ischemia (Massa et al., 1995), suggesting that the induction of Hsp75 may represent a sensitive marker of stressed tissue. Recent unpublished studies from our laboratory demonstrate that Hsp75 transfected astrocyte cultures show increased survival after OGD compared to controls. Because Hsp75 is essential for mitochondria, and mitochondrial function and dysfunction are central to the cell's ability to survive ischemia, more attention needs to be paid to the role of Hsp75 in cerebral ischemia.

Bacterial Chaperonin Groel Reduces Ischemic Injury In Vitro and In Vivo

In addition to studies on the effects of eukaryotic chaperones on ischemic brain injury we have also studied the ability of the bacterial chaperonin GroEL to reduce injury in cultured astrocytes and in rats subjected to focal ischemia (Xu et al., 2006). Protection was observed in both cases.

Geldanamycin Induction of the Stress Response Protects from Ischemia

Geldanamycin (GA), a benzoquinone ansamycin, binds Hsp90 leading to the release and activation of HSF1 under nonstress conditions and prolongs the heat shock response (Ali et al., 1998). Prior work has demonstrated that GA disrupts Hsp90 function and induces expression of Hsp70 in mammalian cells (Zou et al., 1998; Xu et al., 2003). GA induced activation of HSF1, increases Hsp70 and Hsp25 expression, reduces brain infarct volume, and improves post-ischemic behavioral outcomes after focal ischemia (Lu et al., 2002). In a mouse hippocampal cell line, GA induced Hsp70 expression and prevented glutamate-induced DNA cleavage and oxidative toxicity (Xiao et al., 1999). GA has also been shown to activate a heat shock response, induce expression of Hsp70 and suppress protein aggregation in a

cell culture model of Huntington's disease (Sittler et al., 2001). We found that pre-treatment with geldanamycin increased protein levels of Hsp70 in primary astrocyte cultures and protected astrocytes from either necrotic or apoptotic injury induced by OGD deprivation (Xu et al., 2003). We further found that pre-treating mouse hippocampal organotypic slice cultures with GA prior to OGD induced Hsp70 and significantly reduced CA1 neuronal damage (Ouyang et al., 2005). At this point protection by geldanamycin may reflect higher levels of Hsp70, but may also be in part due to induction of other Hsps, especially Hsp25. Further work is needed to determine whether Hsp70 induction can account for all the protection seen with GA, or how much is due to other Hsps.

POTENTIAL MECHANISMS OF PROTECTION

Prevention of Protein Aggregation – Molecular Chaperone Activity

Unfolded or misfolded proteins have exposed hydrophobic segments that render them prone to aggregation. Protein aggregates are thought to be toxic to the cell (Taylor et al., 2002), so to avoid aggregation, abnormal proteins are either kept soluble by molecular chaperones or quickly degraded by the ubiquitin/proteasome system (Hershko and Ciechanover, 1998). Under pathological conditions, the level of abnormal proteins may exceed the ability of the cell to maintain them in a soluble form or degrade them, allowing aggregation to proceed (Cohen, 1999; Zoghbi and Orr, 2000). Protein aggregates can inhibit function of the proteosome, thus further limiting the cell's ability to dispose of the protein aggregates and interfering with the normal processing of certain short-lived proteins (Bence et al., 2001). Protein aggregates commonly contain ubiquitin immunoreactivity, suggesting that proteins targeted for degradation that fail to be degraded may end up in aggregates (Alves-Rodrigues et al., 1998). Protein aggregates are found in most chronic neurodegenerative diseases (Kakizuka, 1998; Taylor et al., 2002), in global and focal ischemia (Hu et al., 2000, 2001) as well as hypoglycemic coma (Ouyang and Hu, 2001).

These earlier studies in ischemia showed that ubiquitin immunoreactivity labeled protein aggregates associated with intracellular vesicles early after injury, and later associated with mitochondria, Golgi and certain regions of the plasmalemma (Hu et al., 2000). Follow up studies suggested a link between protein aggregation and the inability to restart protein synthesis in neurons that go on to die. While it is proposed that stress induced translational arrest be considered one component of the cellular stress response, it can progress from a reversible condition to irreversible arrest in cells destined to die, with extensive aggregation of the protein synthesis machinery, and modifications of stress granules that sequester inactive 48S preinitiation complexes and maintain translational arrest (DeGracia and Hu, 2007).

Overexpression of chaperones, including Hsp70 and Hsp40, reduces protein aggregation and cell injury in models of polyglutamine repeat diseases (States et al., 1996; Creagh and Cotter, 1999; Tsuchiya et al., 2003; Matsumori et al., 2005)

and in a model of familial amyotrophic lateral sclerosis (Barnes and Karin, 1997). In performing its chaperone activities, Hsp70 can interact with other heat shock proteins such as Hsp40. Hsp40 is a co-chaperone that itself can bind denatured protein and inhibit aggregation, but it can facilitate protein folding only in conjunction with Hsp70. We have demonstrated that overexpression of Hsp70 in hippocampal CA1 neurons reduces evidence of protein aggregation under conditions where neuronal survival is increased (Figure 1; (Giffard et al., 2004)). We have also demonstrated protection by the cochaperone Hdj-2 in vitro and demonstrated that this is associated with reduced protein aggregation identified by ubiquitin immunostaining (Qiao et al., 2003). Hdj-2 can prevent protein aggregate formation by itself, but can only facilitate protein folding in conjunction with Hsp70. We further found that the protective effect of pharmacological induction of Hsp70 in organotypic hippocampal slice cultures subjected to OGD is protective and associated with reduced protein aggregation (Ouyang et al., 2005).

Protein Folding Is Not Required for Protection

Studies with Hsp70 lacking the ATPase domain was previously shown to protect cells from heat (Li et al., 1992). The 18 kDa peptide-binding domain of Hsp70 alone is sufficient for high-affinity binding (Wang et al., 1993). However, deletion of the C-terminal EEVD residues can result in loss of substrate-binding activity, suggesting that correct folding of Hsp70 is important for stable substrate association (Freeman et al., 1995). An Hsp70 deletion mutant missing the ATPase domain was shown to interact physically with AIF and protect cells from death induced by serum withdrawal or staurosporine, whereas the peptide-binding domain deletion mutant of Hsp70 was not protective, suggesting that the carboxyl-terminal portion of Hsp70 is necessary and sufficient to neutralize AIF (Ravagnan et al., 2001).

To address the relative importance of protein folding activity vs. inhibition of aggregation in protection from ischemic injury we have begun structure–function studies of Hsp70 to identify which activities and which protein–protein interactions are most relevant to protection. We found that (see Figure 2), animals transfected with plasmids encoding wild type inducible Hsp70 (Hsp70-WT), the point mutant Hsp70-K71E (Rajapandi et al., 1998), or the deletion mutant lacking the ATPase domain, Hsp70-381–640, prior to 2 hr middle cerebral artery occlusion and 24 hr reperfusion had significantly better neurological scores and smaller infarcts than control animals transfected with LXSN (Sun et al., 2006). We found that astrocytes overexpressing Hsp70-WT, -K71E or -381–640 were all significantly protected from 28 hr glucose deprivation (Ouyang et al., 2006), or 4 hr combined oxygen–glucose deprivation and 24 hr reperfusion (Sun et al., 2006). In one case, Hsp70-K71E is a point mutation that abrogates ATP binding (Rajapandi et al., 1998), therefore rendering the full length molecule unable to facilitate folding, while the deletion mutant lacks the entire amino terminal portion of the molecule. Since both of these provide protection, the carboxyl-terminal portion of Hsp70 is sufficient; prevention of aggregation without facilitating folding reduces ischemic damage.

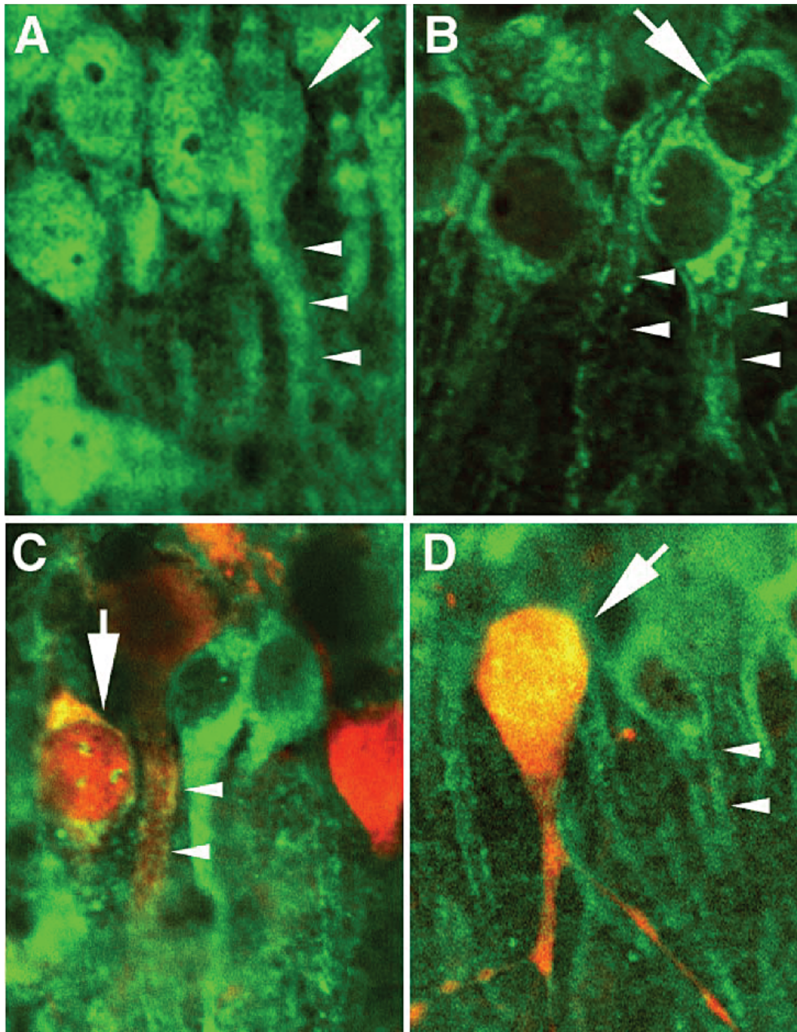

Figure 1. Ubiquitin staining patterns in CA1 neurons, 24·hr following 8·min of dense forebrain ischemia. Ubiquitin staining is color-coded green, β-gal staining is color-coded red. (**A**) A sham control animal not subjected to ischemia was examined 24·hr later. A diffuse pattern in the processes (arrowheads) with strong nuclear staining (arrow) is observed. (**B**) In an animal subjected to dense forebrain ischemia, the pattern has changed to a patchy pattern in processes (arrowheads) with little nuclear staining (arrow). (**C**) An animal subjected to ischemia following injection with Herpes vector encoding only β-gal and then colabeled for β-gal to identify a vector targeted cell shows the same pattern in the targeted neuron as in B and in neighboring untargeted neurons with loss of nuclear staining (arrow). (**D**) An animal injected with Hsp-72 vector shows a relatively maintained pattern of ubiquitin staining in the neuron that is overexpressing Hsp70; note colocalization of ubiquitin and ß-gal staining in the nucleus (arrow) identified by the yellow color. Reproduction from Giffard et al. (2004)

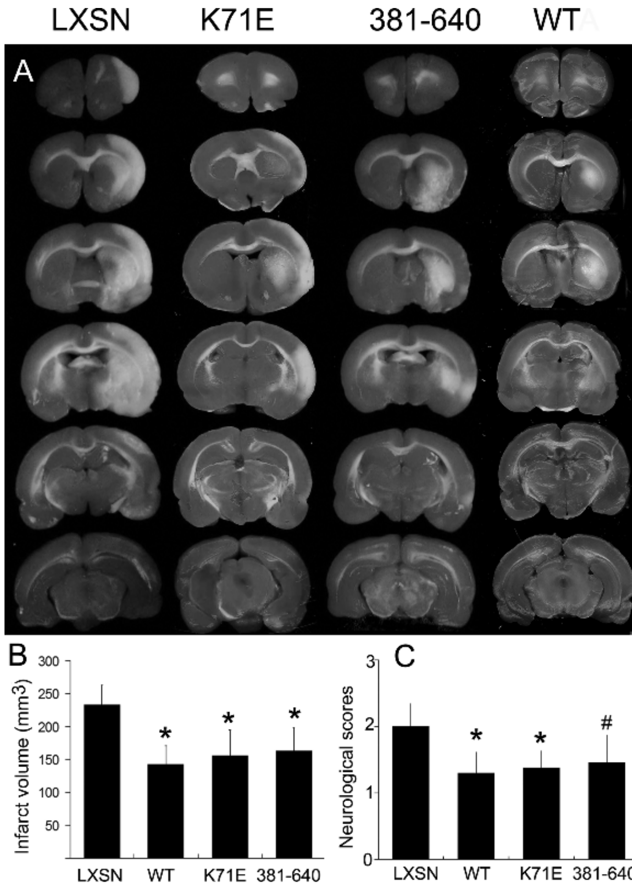

Figure 2. Heat shock protein 72 (Hsp70)-wild type (WT), -K71E, and -381–640 reduce infarct volume and improve neurological outcome after transient MCAO. Hsp70-WT, -K71E, -381–640, or vector LXSN was infused into the right lateral cerebral ventricle followed 24 hr later by MCAO. Infarct volume and neurological scores were assessed at 24 hr reperfusion after 2 hr MCAO. (**A**) Representative TTC-stained coronal brain sections showed a reduction in infarct size in Hsp70-WT-, -K71E- or -381–640-overexpressing rats compared with vector LXSN-treated rats. (**B**) Quantitative analysis of infarct volume showed that infarct volume was significantly decreased in either Hsp70-K71E- or -381–640-overexpressing rats ($n=8-10$ per group). (**C**) Overexpression of Hsp70-WT or mutants significantly improved neurological outcome ($n=10-13$ in each group). Lower values represent better neurological function. #$P<0.05$, $^{*}P<0.01$ relative to LXSN vector control (ANOVA and post hoc Newman–Keuls test). Reproduced from Sun et al. (2006)

Studies with GroEL Another approach to this question is to study the ability of the bacterial chaperonin GroEL to protect from ischemia. The bacterial chaperonin GroEL efficiently interacts with stress-denatured proteins when expressed in mammalian cells, but it is unable to bind newly translated polypeptides (Thulasiraman et al., 1999). GroEL is known to fold eukaryotic proteins

but is less likely to interact with the many co-chaperones and binding partners described for Hsp70 in eukaryotic cells. GroEL-D87K is a mutant with lysine substituted for aspartic acid at position 87. This mutation blocks ATP binding, rendering it ATPase deficient and therefore deficient in protein folding (Frydman and Hartl, 1996; Thulasiraman et al., 1999). GroEL-D87K (Weissman et al., 1994) is capable of binding stress-denatured proteins in mammalian cells but is relatively unable to release them, making it an efficient trap for unfolded proteins (Frydman and Hartl, 1996; Thulasiraman et al., 1999). We tested the bacterial chaperonin GroEL and the mutant GroEL-D87K in CHO cells, in primary cultures of astrocytes subjected to ischemia-like injury (Xu et al., 2006), and in rats to transient middle cerebral artery occlusion (MCAO) (Xu et al., 2006). Both GroEL and its mutant D87K protected eukaryotic cells from ischemia-like injury, and reduced infarct volume and improved neurological outcome after MCAO in rats. Protection against cerebral ischemia was associated with reduced protein aggregation (Xu et al., 2006). Marked neuroprotection by folding-deficient GroEL and Hsp70 demonstrates that inhibition of aggregation is sufficient to protect the brain from ischemia.

Reduction of Apoptosis

Many studies in cancer cells established an association between high levels of expression of Hsps, and Hsp70 in particular, with reduced ability to induce apoptosis. Apoptosis, or programmed cell death, is known to occur in pathological states either by activation of specific death receptors or internally via activation of the mitochondria dependent pathway starting with release of cytochrome c. Central to mitochondria-based apoptosis is the assembly of the apoptosome when procaspase-9 binds apoptosis protease activating factor-1 (Apaf-1) and becomes activated when cytochrome c is released from the mitochondria to the cytosol. This release of cytochrome c is blocked by the antiapoptotic protein, Bcl-2. Activated caspase-9 then activates effector caspases including caspase-3. Caspase-independent pathways have also been identified, one of which is apoptosis inducing factor (AIF) (Susin et al., 1999). When released from the mitochondria, AIF translocates to the nucleus, where apoptosis can occur in the absence of caspase activation. Like cytochrome c, AIF release can also be antagonized by Bcl-2 (Zhao et al., 2004). Other mediators of apoptosis include second mitochondria-derived activator of caspases (Smac)/direct inhibitor-of-apoptosis protein (IAP) – binding protein with low pI (Diablo), which is also released from the mitochondria and reverses apoptosome inhibition by IAPs (Yenari et al., 2005).

Hsps have been shown to interfere with apoptosis at multiple points in the death cascade. Overexpression of Hsp70 in lymphoid tumor cell lines inhibits apoptosis by blocking caspase activation and activity (Gabai et al., 1997; Mosser et al., 1997; Gabai et al., 1998). Hsp70 can inhibit caspase activation by interfering with Apaf-1 and prevent the recruitment of procaspase-9 to the apoptosome (Beere et al., 2000;

Saleh et al., 2000). How this occurs is not yet clear, but has been hypothesized to be due to a direct competition between Hsp70 and procaspase-9 for Apaf-1 binding (Beere et al., 2000; Saleh et al., 2000; Beere and Green, 2001).

However, Mosser et al. (1997) demonstrated that Hsp70 did not appear to interfere with caspase-3 processing, and others have not been able to demonstrate a direct interaction between Hsp70 and Apaf-1 (Steel et al., 2004). Other data suggest that Hsp70 interferes with apoptosis downstream of caspase activation (Jaattela et al., 1998). Yet other studies in tumor cell lines have shown that Hsp70 may block stress kinase (SAPK/JNK) activation (Mosser et al., 1997). Hsp70 also appears to prevent chromatin condensation independent of Apaf-1, and specifically interacts with AIF (Ravagnan et al., 2001). However, Hsp70 did not block Fas-mediated, receptor-activated apoptosis (Creagh and Cotter, 1999; Beere and Green, 2001), and has even been found to enhance apoptosis induced by TRAIL (Clemons and Anderson, 2006). Overall, these studies suggest that Hsp70 acts at multiple sites to confer protection from apoptosis, though there may be differences in mechanism with cell type and route of induction of apoptosis.

Recent studies indicate that Hsp70 can also prevent apoptosis from occurring in the brain. Cells with DNA fragmentation (detected by DNA nick end labeling) following focal cerebral ischemia rarely express Hsp70 protein (States et al., 1996), and transgenic mice overexpressing Hsp70 have fewer apoptotic cells, less DNA laddering, and reduced cytochrome c release (Tsuchiya et al., 2003). Consistent with observations in cell-free systems (Ravagnan et al., 2001), recent data in an in vivo model of neonatal hypoxia/ ischemia indicate that Hsp70 is not only protective, but also binds and sequesters AIF (Matsumori et al., 2005). Interestingly, even though Hsp70 overexpression prevented both cytochrome c and AIF translocation, it did not appear to have any effect on Smac/DIABLO, which remained in the mitochondria up to 24 hr after the hypoxic/ischemic insult, even when cytochrome c and AIF were already released. Hsp70 also appears to have upstream antiapoptotic effects as well. Following viral vector-mediated Hsp70 overexpression, increased levels of Bcl-2 protein were observed (Kelly et al., 2002). In this scenario, it is conceivable that Hsp70, by an as yet unknown mechanism, could increase Bcl-2 expression, which in turn could block cytochrome c and AIF release and effector caspase activation. Thus there is evidence for both direct and indirect anti-apoptotic actions of Hsp70.

Reduction of Cell Death by Necrosis

Several studies have also found that necrotic cell death is reduced by Hsp70 overexpression. This is especially relevant in the setting of ischemia where both necrotic and apoptotic death occur. These include protection by Hsp70 from cell death that is independent of caspase activation and in some cases not blocked by Bcl-2 (Nylandsted et al., 2000; Xu et al., 2003).

Anti-inflammatory Effects

Recent work has begun to look at the ability of Hsp70 to modulate inflammation in the setting of stroke (Yenari et al., 2005; Zheng et al., 2007). The inflammatory response is, in part, regulated by the transcription factor, nuclear factor kappa B (NFκB) (Barnes and Karin, 1997), whose activition leads to synthesis of pro-inflammatory downstream mediators. Normally in the cytosol bound to its inhibitor, IκB, NFκB is activated by stresses including ischemia, oxidative stress, and endotoxin exposure. This leads to activation of the IκB kinase (IKK), which phosphorylates IκB, allowing degradation by the proteasome. NFκB then translocates to the nucleus, binds its consensus sequences, and activates transcription. Inflammatory genes activated by NFκB NF include iNOS, the inflammatory cytokines TNF and IL-1, inducible cyclooxygenase (COX-2), chemokines, and adhesion molecules. NFκB also induces IκB, leadingto its own downregulation.

Others have shown that prior thermal stress leads to inhibition of the inflammatory response, and this inhibition was associated with increased levels of Hsp70 induction and decreased nuclear NFκB translocation (Guzhova et al., 1997; Heneka et al., 2000). It has been speculated that Hsp70 could interact with NFκB's inhibitor protein, IκB, and prevent IκB phosphorylation and NFκB dissociation (Feinstein et al., 1997). A recent study by Ran et al. (2004) showed that Hsp70 can interact with IKK, thereby preventing IκB's phosphorylation and activation. Another recent studied identified binding of Hsp70 to IκB and NFκB and suggested that Hsp70 could inhibit IκB phosphorylation when it was in the NFκB: IκB complex formation, this stabilizing the inhibited state of NFκB (Zheng et al., 2007).

Several studies demonstrate the anti-inflammatory effects of Hsp70 in disease settings. Prior induction of Hsp70 decreases the release of inflammatory mediators in a porcine model of recurrent endotoxemia (Klosterhalfen et al., 1996), protects against TNF-induced lethal inflammatory shock (Van Molle et al., 2002), and attenuates the cardiopulmonary bypass-induced inflammatory response (Hayashi et al., 2002). Overexpression of Hsp70 inhibits bacterial endotoxin (LPS, lipopolysaccharide) – induced production of cytokines (Ding et al., 2001) and ameliorates experimental acute respiratory distress syndrome (Weiss et al., 2002). Induction of Hsp70 by heat stress in peripheral leukocytes has also been shown to reduce NADPH oxidase (responsible for inflammatory cell generation of superoxide) and increase superoxide dismutase, an endogenous antioxidant (Polla et al., 1995). Conversely, inhibition of Hsp70 expression by antisense Hsp70 partially reverses such anti-inflammatory functions (Schroeder et al., 1999; Bhagat et al., 2000; Ding et al., 2001). These anti-inflammatory properties of Hsp70 are thought to be due, in part, to inhibition of NFκB (Feinstein et al., 1996; Heneka et al., 2000; Hayashi et al., 2002; Meng and Harken, 2002). Feinstein and colleagues (Feinstein et al., 1996) showed that glial cells exposed to heat shock or transfected with Hsp70 followed by stimulation with LPS experienced less nuclear NFκB translocation and consequently less inducible nitric oxide synthase expression, an NFκB -regulated gene. Meng and Harken (2002) reported that Hsp70 induction in monocytes or during myocardial ischemia led to reduced upregulation

of yet another NFκB-regulated gene, the inflammatory cytokine TNF-α. Similarly, in a model of brain inflammation, heat stress led to less microglial/macrophage activation and NFκB activity (Heneka et al., 2000). Whether Hsp70 plays an anti-inflammatory role in brain ischemia has not been extensively studied. Soriano and colleagues documented early Hsp70 expression in microglia following experimental stroke (Soriano et al., 1994), and matrix metalloproteinase-9 mRNA and protein was reduced in cultured Hsp70-overexpressing astrocytes subjected to OGD (Lee et al., 2004).

Recent work confirms an anti-inflammatory phenotype in Hsp70Tg mice following brain ischemia. When brain sections were examined for microglia/monocytes and MHC class II antigen, Hsp70 transgenic mice had fewer activated microglia compared to wild-type mice, and fewer cells showed NFκB translocated to the nucleus (Zheng et al., 2007). Furthermore, Hsp70 overexpression in inflammatory cells is also protective. Using cocultures of astrocytes and microglia, it is possible to mix and match transgenic and wild-type cells. When we cultured transgenic or wild-type microglia with wild-type astrocytes, and exposed them to hydrogen peroxide, we found that cultures containing Hsp70 transgenic microglia suffered less injury following hydrogen peroxide exposure than wild-type microglia cultured with wild-type astrocytes (Zambrano, 2004). These data suggest that Hsp70 in inflammatory cells reduces their cytotoxicity. However, the mechanism of this observed protection is unknown. It is likely that Hsp70 prevents inflammatory cell generation of one or more cytotoxic mediators. Work by others suggests that, following inflammatory cell stimulation by bacterial endotoxin, substances such as nitric oxide (Feinstein et al., 1996) and inflammatory cytokines (Ding et al., 2001) are reduced in the presence of Hsp70. Whether this is also the case for ischemic and ischemia-like insults is still unknown.

Mitochondrial Protection

Mitochondria are of primary importance in both apoptotic and necrotic brain cell death. Mitochondrial changes that contribute to cell death include alterations in mitochondrial respiratory function, production of reactive oxygen species, changes in mitochondrial membrane potential, and the release of regulatory and signaling molecules from the intermembrane space. Our studies have demonstrated neuroprotective effects of Hsp70 overexpression in astrocytes which extended to better preservation of mitochondrial function (Ouyang et al., 2006). Protection was associated with better maintenance of mitochondrial membrane potential and decreased formation of reactive oxygen species after a period of glucose deprivation (Figure 3). Astrocytes overexpressing Hsp70 also better maintained state IV respiration (resting state- reflecting the rate of leakage of protons across the inner mitochondrial membrane into the matrix) compared to control cells (Ouyang et al., 2006). Preliminary results from our laboratory suugest Hsp75 may have similar effects in astrocytes subjected to glucose deprivation (unpublished observations).

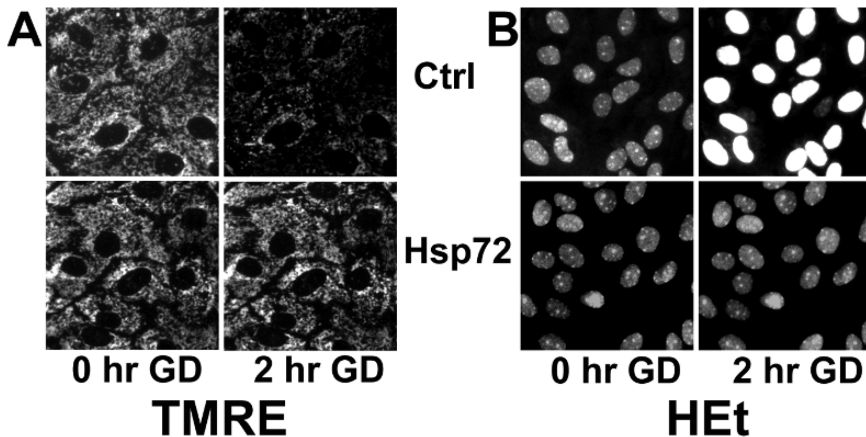

Figure 3. Overexpressing Hsp70 stabilize mitochondrial membrane potential (MMP) and reduce reactive oxygen species (ROS) production with glucose deprivation (GD). (**A**) MMP was measured using TMRE, a potentiometric fluorescent dye that incorporates into mitochondria in a MMP dependent manner. Photomicrograph of TMRE fluorescence image demonstrates punctate perinuclear fluorescence representing mitochondria. After 2 hr of GD, the TMRE intensity representing MMP in the control (Ctrl) group decreases significantly compared to 0 hr GD group (*upper panel*). Hsp70 protect this drop in the MMP (*lower panel*). (**B**) Oxygen radical production was monitored using the ROS-sensitive fluorescent dye hydroethidine (HEt). The increase of ROS in control group (*upper panel*) is reduced in Hsp70 treated cells (*lower panel*)

CONCLUSIONS

An increasing body of research demonstrates the ability of several different chaperones to protect the brain from ischemic injury. There is evidence for several different mechanisms, including reduction of protein aggregation, preservation of mitochondrial function, inhibition of both necrotic and apoptotic cell death, and reduction of inflammation. In some cases these mechanisms may overlap or reinforce each other. Despite this growing understanding, additional work will be required to understand which mechanisms are most important in reducing specific stresses, and in identifying useful strategies to harness the protective abilities of chaperones to reduce the morbidity and mortality associated with cerebral ischemia in the clinical setting. The burden on society of ischemic brain injury is increasing as the population ages, and currently treatment options are limited. Induction of endogenous chaperones or pharmacologic imitation of some of their activities may provide one line of treatment in the future. Understanding which functions of chaperones are most protective can direct development of novel therapies.

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CHAPTER 10

STRATEGIES FOR CONFERRING NEUROPROTECTION AND COUNTERING THE HIGH THRESHOLD FOR INDUCTION OF THE STRESS RESPONSE IN MOTOR NEURONS

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Abstract: The higher threshold for stress-induced upregulation of heat shock genes in certain populations of neurons, including motor neurons, has implications for their preferential vulnerability to disease and poses challenges for therapeutic intervention. One approach for identifying compounds that are effective in motor neurons involves understanding the mechanisms of heat shock gene regulation and hypothesis-driven therapeutic design. Central to stress-induced activation of heat shock genes is the transcription factor Hsf1, which must be released from Hsp90 complexes, translocate to the nucleus, bind to heat shock elements, and become activated. Most known inducers and co-inducers of the heat shock response promote one or more of these steps, but not all compounds are effective in motor neurons. However, other elements in the promoters of heat shock genes and heat shock transcription factors also contribute to constitutive and stress-induced regulation of heat shock genes and are potential therapeutic targets. Another approach is to screen chemical libraries using a test system that expresses motor neuronal properties, with positive hits being validated *in vivo*. The most effective therapies will be those that upregulate multiple chaperones and co-chaperones that enable refolding and degradation in addition to sequestering misfolded proteins

Keywords: Heat shock transcription factors; *hsp* promoter; Hsp networks; astrocytes; screening; motor neuron disease

INTRODUCTION

Heat shock proteins (Hsp) and the constitutively expressed heat shock cognate proteins (Hscs) play a pivotal role in maintaining cellular homeostasis, both in terms

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of general housekeeping functions and in protecting cells from environmental and disease-associated stresses. Underlying this homeostatic function is their action as chaperones to facilitate proteins adopting functional conformations and to prevent misfolded proteins from aggregating and interacting inappropriately with other partners. Misfolding can result from abnormal amino acid sequences, due to genetic mutation or errors in protein translation, or from post-translational modifications. In addition, the physicochemical conditions within the cell, such as pH, can influence the conformations adopted by polypeptides. Key to the ability of cells to defend themselves against proteotoxic stress is the inducible expression of heat shock genes to ensure adequate levels of Hsc/Hsp to chaperone damaged, misfolded proteins [See Figure 1 for overview].

Aberrant protein conformation is a common theme among neurodegenerative diseases. Thus, Hsp are thought to play an important role in delaying their manifestation and to serve as targets for therapeutic intervention with a broad spectrum of activity regardless of the initiating pathogenic factors. However, populations of cells differ in their ability to upregulate expression of heat shock genes (*hsp*) in response to stress. Many types of neurons, including motor neurons, have a particularly high threshold. This has implications both for the relative vulnerability of these cells to various disease-related insults and for effective strategies to upregulate this network therapeutically. In this chapter, our current understanding of stress response mechanisms in motor neurons and the sensitivity of motor neurons to known activators of these pathways are reviewed from the perspective of potential strategies for activation of heat shock genes and implications for therapeutic screening and preclinical evaluation.

HIGH THRESHOLD FOR STRESS-INDUCED ACTIVATION OF *HSP* IN MOTOR NEURONS

Motor neurons, primary target cells in diseases such as amyotrophic lateral sclerosis (ALS), spinal bulbar muscular atrophy (SBMA, Kennedy's disease), Charcot-Marie-Tooth disease type 2 and toxic neuropathies, have a high threshold for inducing a heat shock response in response to hyperthermia or other stressors both *in vivo* (Manzerra and Brown, 1992, 1996; Kalmar et al., 2002a; Batulan et al., 2003; Tidwell et al., 2004) and in primary culture (Batulan et al., 2003), although expression of the stress-inducible Hsp, Hsp70, has been documented under particular circumstances. Hsp70 was detected in spinal motor neurons following experimental ischemia (Sakurai et al., 1996, 1997, 1998; Motoyoshi et al., 2001); under some conditions of hyperthermia (Xia et al., 1998); in a subset of motor neurons at the symptomatic stage in SOD1^{G93A} transgenic mice, an animal model of familial ALS (Vlug et al., 2005), and transiently in motor neurons of NFL$^{-/-}$ mice (McLean et al., 2005). In spinal motor neurons of end-stage sporadic ALS, some cytoplasmic inclusions are Hsp70-immunoreactive (Batulan et al., 2003). Although the induction of Hsp70 and its detection may depend upon factors such as species, experimental conditions and methods of detection, induction of Hsp in motor neurons may be

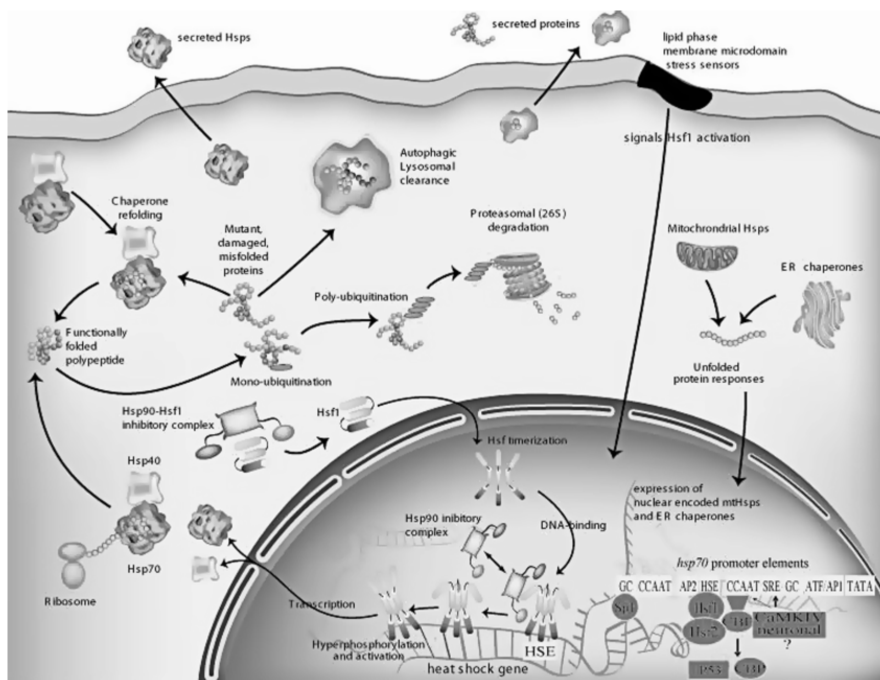

Figure 1. Function and control of chaperone networks. During polypeptide synthesis, Hsp act as chaperones to prevent inappropriate protein–protein interactions and assist nascent proteins in taking on and maintaining their functional conformation. Translational errors, genetic mutation or post-translational modifications can promote misfolding of proteins, leading to inappropriate interactions, altered solubility and aggregation. Hsp prevent aggregation by sequestering the proteins to be refolded (by Hsp70, Hsp40 and other co-chaperones in an ATP-dependent process) or facilitating their degradation by proteasomes or autophagic/lysosomal pathways. Hsp are also secreted to chaperone proteins in the extracellular space or for uptake into adjacent cells. Chaperones in mitochondria (mtHsp) and endoplasmic reticulum (ER) also function in protein quality control; stress provokes unfolded protein responses by these organelles, providing signals for transcription of nuclear encoded stress response genes. Cells that maintain a balance between the levels of Hsp, protein degrading pathways and the substrate load will be better positioned to defend themselves. Heat shock gene transcription is regulated by a negative feedback system whereby the major transcription factor controlling heat shock gene transcription, Hsf1, is maintained in an inactive state by binding to an Hsp90-chaperone complex. When misfolded proteins compete for these chaperones, Hsf1 is freed to translocate to the nucleus and bind as a trimer to heat shock elements (HSE) of heat shock genes. Hsf2 can also trimerize with Hsf1 and facilitate transcription. Binding of Hsf1 to DNA is not sufficient to initiate transcription. Rather subsequent steps are required including phosphorylation and dissociation of inhibitory proteins, including nuclear Hsp90 complexes. Changes in structure and fluidity of membrane microdomains can also act as stress sensors signaling upregulation of Hsp. Other transcription factors and regulatory elements influence expression of *hsp* genes, as illustrated in the lower right quadrant for the promoter of the human inducible *hsp70* gene, and could be therapeutic targets (For references see text)

too little and/or too late. The general age-associated decline in the heat shock response could further compromise motor neuronal function (Proctor et al., 2005). One approach to designing ways to lower the threshold for the heat shock response in motor neurons is to understand how *hsp* transactivation is inhibited. Another approach is to screen libraries of compounds in cultured cells that exhibit this motor neuronal property.

THE CENTRAL ROLE OF HEAT SHOCK TRANSCRIPTION FACTORS IN STRESS-INDUCED EXPRESSION OF *HSP*

Hsf1 is the major transcription factor controlling stress-induced expression of Hsp in mammalian cells (Morimoto, 1998; Voellmy, 2004). The most widely accepted mechanism of Hsf1 activation is a chaperone-mediated model whereby the diversion of Hsp by misfolded proteins releases a series of inhibitory influences that tightly regulate Hsf1 activity under basal conditions (Voellmy, 2004). Under homeostatic conditions, Hsf1 is sequestered as an inactive monomer in the cytoplasm by a multi-chaperone complex that includes Hsp90, the co-chaperone p23 and an immunophilin (Zou et al., 1998; Pratt and Toft, 2003). HSC/HSP70 and Hsp40 participate by forming intermediate complexes with Hsp90-client proteins, which then are linked to Hsp90 in the ADP-bound state by the cochaperone HOP. Maturation of the complex requires binding of ATP to Hsp90, followed by the addition of p23, which locks the complex in its ATP-bound state and displaces Hsc/Hsp70/Hsp40 and HOP (Panaretou et al., 2002; Pratt and Toft, 2003). Competition for these Hsc/Hsp by misfolded proteins disrupts formation of Hsp90-client complexes (Zou et al., 1998) with the consequence that Hsf1 molecules are freed to translocate to the nucleus and bind as trimers to heat shock elements (HSE) upstream of *hsp* genes (Morimoto, 1998; Voellmy, 2004). DNA binding is necessary, but not sufficient for Hsf1-mediated transactivation (Jurivich et al., 1992; Zuo et al., 1995). Mechanisms for activation of the transcriptional complex include hyperphosphorylation of Hsf1 (Xia and Voellmy, 1997) and removal of a nuclear Hsp90 inhibitory complex that interacts with Hsf1 trimers in the nucleus (Guo et al., 2001). For other factors influencing Hsf1 activation and *hsp70* transcription (e.g., CHIP, Ral-binding protein 1, chromatin structure, etc), the reader is referred to recent review articles (Voellmy, 2004; Tonkiss and Calderwood, 2005).

Mice and cultured cells lacking Hsf1 are sensitive to stress and fail to mount a heat shock response (McMillan et al., 1998; Xiao et al., 1999); however, Hsf1 another member of the heat shock transcription factor family important in neuronal development (Kallio et al., 2002; Chang et al., 2006), may play a role in stress-induced *hsp* expression depending on circumstances. Both Hsf1 and Hsf2 bind to the same promoter elements, but may be activated preferentially by different stressors, Hsf2 being more responsive to hemin, amino acid analogues and proteasome inhibitors and Hsf1 to thermal stress (Mathew et al., 2001; Trinklein et al., 2004). Hsf2 can heterotrimerize with Hsf1 and enhance Hsf1-dependent transcription of *hsp* and other stress-responsive genes (Sistonen et al., 1994; He et al., 2003; Bjork

and Sistonen, 2006; Loison et al., 2006; Ostling et al., 2007). The level of Hsf2 is regulated by proteasomal degradation (Mathew et al., 1998). Levels are low under normal conditions but upon proteasomal inhibition, Hsf2 accumulates and translocates to the nucleus. In cultured motor neurons, treatment with proteasome inhibitors does result in nuclear accumulation of Hsf2 and expression of Hsp70 (Batulan et al., 2003), but whether the relationship is a causal or just temporal remains to be determined.

Can Hsf2 compensate for lack of Hsf1 or its activation under conditions when proteasomes are overloaded? The degree of proteasomal inhibition required to induce Hsp70 in motor neurons of spinal cord cultures is about 35% under acute conditions (Aarons and Durham, unpublished data); however, Hsp70 was not upregulated in motor neurons of $SOD1^{G93A}$ mice, despite a 50% decrease in proteasome activity in lumbar spinal cord. These results do not definitely answer the question, but would indicate that Hsf2 is not a major activator of *hsp* expression under moderate conditions of stress that do not significantly impair protein degradation.

ROLE OF HSF1 IN THE HIGH THRESHOLD FOR INDUCTION OF THE HEAT SHOCK RESPONSE IN MOTOR NEURONS AND OTHER NEURONAL POPULATIONS

Low Level Expression of Hsf1

In cells that have a competent heat shock response, the concentration of Hsf1 limits the magnitude of Hsp induction (Wu, 1995; Rieger et al., 2005). Failure of cultured hippocampal neurons to mount a heat shock response to hyperthermia has been associated with the lack of expression of Hsf1 (Marcuccilli et al., 1996; Kaarniranta et al., 2002). Although these neurons express Hsf2, this heat shock factor is not particularly sensitive to hyperthermia (Mathew et al., 2001; Trinklein et al., 2004). In cultured motor neurons, lack of Hsf1 does not account for the impaired heat shock response because some Hsf1 was detected by immunolabeling and increasing expression by gene transfer did not confer a heat shock response despite nuclear localization (Batulan et al., 2003, 2006). Therefore, upregulation of Hsf1 would not be an effective therapeutic strategy and could even be counterproductive. *Hsf1* gene transfer actually prevented expression of Hsp70 induced by other means in motor neurons (Taylor et al., 2007a, b). In addition, Hsf1 can also repress transcription of other genes (Xie et al., 2003), which could have deleterious long term consequences.

Impaired Activation of Hsf1

Despite the futility of increasing $Hsf1^{wt}$ in motor neurons, expression of a constitutively active form of Hsf1 ($Hsf1^{act}$) did result in high level expression of Hsp70 and Hsp40, even in the absence of additional stressor (Batulan et al., 2003). The

construct lacks amino acids 202–316, the region implicated in activation of DNA-bound Hsf1 (Wagstaff et al., 1998). Its effectiveness in motor neurons points to lack of Hsf1 activation as the cause of the high threshold for stress-induced expression of *hsp* genes (Batulan et al., 2003). This hypothesis is supported by the ineffectiveness of nonsteroidal anti-inflammatory agents (NSAIDS) in facilitating a heat shock response in motor neurons of dissociated spinal cord cultures despite augmenting expression of Hsp70 in adjacent astrocytes (Batulan et al., 2005). NSAIDs increase the amount of Hsf1 bound to HSE in response to stress, but do not promote activation of the transcriptional complex (Jurivich et al., 1992; Lee et al., 1995; Wang et al., 2006). However, a contradiction is that Hsp90 inhibitors including geldanamycin, 17-allylamino-17-demethoxygeldanamycin (17-AAG), novobiocin and radicicol that disrupt Hsp90 complexes, and thus should release Hsf1 for trimerization and DNA-binding, did induce robust expression of Hsp70 and Hsp40 in motor neurons (Batulan et al., 2006). If the high threshold for expression of heat shock genes were due simply to impaired activation of transcription factor complexes regardless of the amount of Hsf1 bound to HSE, disruption of cytoplasmic Hsp90-Hsf1 complexes should not have promoted *hsp* gene expression. A potential explanation for this apparent contradiction is the report of Voellmy's group that other Hsp90 multichaperone complexes in the nucleus interact with trimeric Hsf1 to repress transcriptional competence (Guo et al., 2001) and that displacement of these Hsp90-p23-Fkbp52 complexes by death domain-associated protein (Daxx) relieved the inhibition of Hsf1 activation (Boellmann et al., 2004). These Hsp90 complexes bind to the region of Hsf1 that is deleted in the constitutively active form, $Hsf1^{act}$ (Guo et al., 2001). Thus, it is possible that geldanamycin and its analogues enabled *hsp* gene transcription by disrupting both cytoplasmic and nuclear Hsp90 complexes. However, attempts to lower the threshold for the heat shock response in motor neurons by over-expressing Daxx were unsuccessful (Taylor et al., 2007b).

Another potential mechanism to promote turnover of Hsp90 complexes is over-expression of Aha1, which competes for Hsp90 with p23, the co-chaperone that stabilizes the ATP-bound, mature complex (Mayer et al., 2002; Panaretou et al., 2002). However, Aha1 gene transfer also failed to enable the heat shock response in motor neurons (Taylor et al., 2007b). Thus, despite their effectiveness of chemical inhibitors of Hsp90-client complexes in inducing expression of multiple Hsp, the operant mechanism in motor neurons remains unclear.

Activation of Hsf1 is associated with hyperphosphorylation (Voellmy, 2004). The presence of key phosphorylation sites within the deleted domain of $Hsf1^{act}$ prompted an investigation of their role in the high threshold for activation of the heat shock response in motor neurons. However, this could not be linked to altered phosphorylation of Hsf1 by several kinases previously shown to positively or negatively affect activation of Hsf1 in other cells (PKC, GSK3β, ERK1, CaMKIIα) (Taylor et al., 2007a). If the same mechanisms operate *in vivo*, manipulation of these individual pathways is not likely to have therapeutic benefit through *hsp* gene expression.

Other Promoter Elements Controlling *hsp* Expression

Our study manipulating multiple phosphorylation pathways in motor neurons uncovered a novel activator of Hsp70 expression in motor neurons. A constitutively active form of CaMKIV (CaMKIV^{act}) induced robust expression of Hsp70 as well as a reporter driven by the inducible *hsp70* promoter (Taylor et al., 2007a). Interestingly, CaMKIV^{act} did not induce Hsp70 expression in mouse embryonic fibroblasts, suggesting a neuron-specific mechanism. CaMKIV is a monomeric, 60 kDa serine/threonine kinase that can move between nuclear and cytoplasmic compartments, is best known for phosphorylating transcription factors (Anderson and Kane, 1998), and is expressed in motor neurons (Taylor et al., 2007a).

CaMKIV^{act}-induced expression of Hsp70 in motor neurons was not accompanied by upregulation of Hsp40 and did not require application of an additional stress, as would be expected from a classical HSE-mediated stress response (Taylor et al., 2007a). In addition to HSE, the promoter of the human inducible *hsp70* gene contains multiple regulatory elements including GC (Sp1), purine-rich, CCAAT, SRE, ATF/AP1-like, and TATA elements (Wu et al., 1986; Williams and Morimoto, 1990). CaMKIV^{act} could enhance either CCAAT- and SRE-mediated transcription (Miranti et al., 1995; Yukawa et al., 1998); however, the failure of CaMKIV^{act} to coordinately induce Hsp40 in motor neurons points to *hsp70* transcription being SRE-mediated because the *hsp40* promoter lacks this element, but does contain two CCAAT motifs (Hata and Ohtsuka, 1998).

CCAAT motifs also play an important role in stress responses in neuronal cells. Induction of Hsp70 occurred in the absence of Hsf1 activation when mutant huntingtin or ataxin-1 was expressed in cultured cerebellar granule cells, but not in cortical neurons (Tagawa et al., 2007). Expression of *hsp70* in granule cells was enhanced by CCAAT box binding factor (CBF), but was repressed by p53. That granule cells express p53 at remarkably lower levels than cortical neurons, suggests an inverse relationship between p53 expression in a particular cell type and inducibility of *hsp70* gene transcription. Other studies have demonstrated the importance of CCAAT boxes in expression of Hsp, including facilitating HSE-mediated transactivation. Binding of a NFY-CBF activating complex to CCAAT elements contributes to constitutive and stress-induced expression of hsp70 and hsp40 (Agoff et al., 1993; Imbriano et al., 2001) and is inhibited by p53. Whether p53 inhibits *hsp* transactivation in motor neurons remains to be determined. Regardless, knocking down p53 did not alter the disease phenotype of SOD1^{G93A} transgenic mice, but effect on Hsp expression was not evaluated (Kuntz et al., 2000).

INFLUENCE OF INSULIN/INSULIN-LIKE GROWTH FACTOR RECEPTORS AND DOWNSTREAM PATHWAYS ON *HSP* EXPRESSION

Pathways downstream of the insulin/insulin-like growth factor receptor (IGFR in mammals; DAF-2 in invertebrates) promote cell survival and longevity, but can also contribute to decreased stress resistance and shortened lifespan (see

also chapter authored by Dillin). In *C. elegans*, *Drosophila* and mice, blocking one arm of this pathway promotes longevity and resistance to proteotoxicity and protein aggregation (Longo and Fabrizio, 2002). Stimulation of DAF-2 results in sequential activation of PI3 kinase and Akt, which in turn phosphorylates the forkhead transcription factor, DAF-16 (FOXO in mammals), preventing its translocation to the nucleus. Mutating or blocking upstream components of the signaling pathway relieves this inhibition. The protective effect is also dependent upon Hsf1 and Hsp, but the mechanism is not certain (Hsu et al., 2003; Walker and Lithgow, 2003; Morley and Morimoto, 2004). Cohen et al. have proposed that Hsf1 and DAF-16 represent two DAF-2-regulated pathways with opposing, but complementary, anti-proteotoxic activities (Cohen et al., 2006). According to their model, Hsf1-induced chaperones provide the machinery to rapidly disaggregate small aggregates and facilitate degradation. When small aggregates accumulate because of chaperone-overload, DAF-16-mediated mechanisms would promote recruitment into larger, less toxic aggregates. With stimulation of IGFR, both pathways could be inhibited, particularly DAF-16. Blocking DAF-2 could stimulate both Hsf1 and DAF-16 pathways, minimizing toxicity through multiple mechanisms. However, other studies show Hsp70 to be positively regulated by Akt (Kim et al., 2005).

In mammals, ubiquitin ligases are also upregulated by the DAF-2 homolog, FOXO, which would promote proteasome-mediated degradation, but overstimulation of this pathway leads to cachexia (muscle wasting) because of upregulation of the ubiquitin ligase, atrogen-1 and a general decrease in protein synthesis mediated through an alternate Akt-regulated pathway (McKinnell and Rudnicki, 2004; Sandri et al., 2004). Thus, suppression of the IGFR/PI3 kinase/Akt and activation of FOXO could be beneficial in terms of dealing with aggregation-prone proteins, but detrimental if over-activated in the long term. FOXO includes subfamilies of transcription factors that could differentially affect gene transcription when activated by stress, including expression of proapoptotic factors such as fas ligand and bim (Fukunaga et al., 2005). Just as blocking IGFR pathways can produce contradictory effects depending on the circumstances, so can stimulation of IGFR pathways, either promoting neuronal survival or decreasing stress resistance and shortening lifespan.

The overall picture is that altering insulin/IGF pathways can have paradoxical consequences depending on the context, cell type and degree of activation (Tang, 2006). Neuronal survival pathways are considered viable therapeutic targets against neurodegeneration, including IGF-1 in motor neuron disease. It is not known how activation of IGFR impacts on expression or activation of Hsf1 in motor neurons. In addition to PI3 kinase, IGF-1 also activates MAP kinases that negatively regulate Hsf1 activation (Chu et al., 1996, 1998; He et al., 1998). However, inhibiting GSK3β or Erk failed to restore the heat shock response in cultured motor neurons (Taylor et al., 2007a). The contribution of IGFR-pathways to cell survival and expression of Hsp is complex in mammals, to say the least.

MITOCHONDRIAL HSP

A key regulator of lifespan in *Drosphila melanogaster* is the mitochondrial sHsp, Hsp22. Overexpression of Hsp22 extended lifespan by over 30% and increased resistance to oxidative stress (Morrow et al., 2004b). Interestingly, expression of Hsp22 in motor neurons was key to both longevity and maintenance of locomotor activity. The involvement of mitochondrial dysfunction in aging and in motor neuron diseases, suggests mitochondrial Hsp (e.g., mammalian Hsp60, Hsp10, mtHsp70) as a target for therapeutic intervention (Morrow et al., 2004a). Accumulation of misfolded proteins in mitochondria activates a mitochondrial unfolded protein response involving CHOP- and C/EPBβ-mediated upregulation of nuclear encoded mitochondrial Hsp (Zhao et al., 2002). Further work is required to investigate the relevance of these pathways to motor neuron diseases and how mtHsp are affected by agents that upregulate other stress-responsive genes.

STRESS-SENSING THROUGH MEMBRANE PERTURBATION

Most of our information on mechanisms of activating the heat shock response comes from studies using hyperthermia as the stressor, with denatured proteins believed to be the major activator of the response. Triggers could be more subtle under typically encountered physiological and environmental stresses that upset neuronal homeostasis. Vigh and his group have identified increased fluidity and altered structure of membrane microdomains as an important stress sensor, stimulating production of Hsp even in the absence of general protein denaturation (Vigh et al., 1998, 2007; Balogh et al., 2005). Membrane fluidizing alcohols increased intracellular Ca^{2+}, hyperpolarized mitochondrial membrane potential, and increased Hsp comparable to a 42° heat shock (Balogh et al., 2005). These authors postulate that one mechanism by which stress-induced membrane changes signal is through activation of growth factor receptors and downstream signaling pathways that affect Hsf1 activation. This pathway could have particular relevance to neurons because of the high unsaturated fatty acid content of their membranes (Vigh et al., 1998). Interestingly, the hydroxylamine family of co-inducers of the heat shock response includes arimoclomol, which delayed disease progression in $SOD1^{G93A}$ transgenic mice (Kieran et al., 2004) and is being tested in clinical trial as a treatment for ALS (see chapter authored by Greensmith). In addition to increasing the amount of Hsf1 bound to DNA in response to stress, these agents alter membrane fluidity by intercalating into the membrane (Hargitai et al., 2003; Torok et al., 2003). Given that NSAIDS, other co-inducers that promote Hsf1-HSE binding, are not effective, at least in cultured motor neurons (Batulan et al., 2005), the importance of membrane sensors of neuronal stress needs to be explored.

ROLE OF ASTROCYTES IN PROTECTING MOTOR NEURONS AGAINST STRESS

Motor neurons rely heavily on astrocytes as their protectors. Many neurons have low reserves of glutathione (GSH) (Beiswanger et al., 1995) and astrocytes contribute to neuronal defense against oxidative stress by secreting GSH, which is then taken up by neurons as either the intact molecule or constituent amino acids for resynthesis (Shih et al., 2003). In general, cytoprotective mechanisms including anti-oxidants and Hsp are highly stress-inducible in astrocytes compared to motor neurons (Batulan et al., 2003; Shih et al., 2003). Evidence points to astrocytes supplying Hsp in addition to other neuroprotective factors. Cells can secrete Hsc/Hsp and take them up from the extracellular fluid, and applying Hsp70 exogenously is neuroprotective (Tytell et al., 1986; Houenou et al., 1996; Guzhova et al., 2001; Tidwell et al., 2004; Robinson et al., 2005; Trougakos and Gonos, 2006; Calderwood et al., 2007) (see Section 3). Astrocytic Hsp may be particularly important for protecting synapses (Bechtold et al., 2000; Bechtold and Brown, 2000; Chen and Brown, 2007b) (see chapter authored by Brown). It is possible that any of the above mechanisms could contribute to the neuroprotective effects of Hsp inducers and co-inducers.

ROLE OF SMALL HEAT SHOCK PROTEINS (sHSP) IN NEUROPROTECTION

Although the focus of this discussion has been on the importance of Hsp70/Hsp40 for protein folding, sHsp are particularly effective in protecting cells from oxidative stress, e.g., Hsp25/27 (the murine and human products of the gene designated *HSPB1* according to HGNC nomenclature). Hsp25 is upregulated in motor neurons following axotomy and promotes regeneration in a manner that involves a complex partnership with p38 and Akt (Murashov et al., 2001; Kalmar et al., 2002b); however, Hsp25 is not upregulated in spinal motor neurons of $SOD1^{G93A}$ transgenic mice, a model of ALS (Batulan et al., 2003; Maatkamp et al., 2004). sHsp (Hsp25, αB-crystallin) are upregulated in reactive astrocytes under many pathological conditions, including in spinal cord of mutant SOD1 transgenic mice (Batulan et al., 2003) and in Schwann cells surrounding regenerating peripheral axons (Hirata et al., 2003), which could facilitate maintenance and regeneration of neurons and their processes (*vide supra*). For additional information on neuroprotective effects of Hsp25 see chapter authored by Currie.

In cultured motor neurons, $Hsf1^{act}$ is not particularly effective in inducing Hsp25 expression, implicating other transcription factors in gene regulation, e.g., Sp1 (Gaestel et al., 1993), which mediates upregulation by oxidative stress (Ryu et al., 2003). The coordinate upregulation of phase II anti-oxidant enzymes and sHsp could be a strategy for combating diseases with a large oxidative stress component. However, antioxidants in general have not proven particularly effective when tested in ALS clinical trials (Qureshi et al., 2006).

IMPORTANCE OF CONSTITUTIVELY EXPRESSED HSC/HSP IN PROTECTING MOTOR NEURONS FROM STRESS

Most studies have used stress-inducible Hsp70 as a marker of the heat shock response because its low level of constitutive expression and high thermal inducibility makes it easy to identify and quantify. However, large neurons such as motor neurons express high levels of other Hsc/Hsp, including Hsc70, which could be sufficient to confer stress-resistance under normal physiologically conditions (Manzerra and Brown, 1992; Manzerra et al., 1997; Chen and Brown, 2007a). Tapping into mechanisms governing transcription/translation of these stress protein families is an alternative to the conventional stress-inducible Hsp. Hsc70 and Hsp70 are not always co-regulated. For example, *hsp70* increases in response to hyperthermia and ischemia via Hsf1-mediated mechanisms, *hsc70* is upregulated by ethanol mainly through Sp1-binding elements (Wilke et al., 2000), and cadmium upregulates both, but with different time courses and interaction of transcription factors (Hung et al., 1998).

THE IMPORTANCE OF MULTIPLE CHAPERONES, COFACTORS AND PROTEIN DEGRADING PATHWAYS WORKING TOGETHER AS NETWORKS

The related issue for longer term effectiveness of Hsp-mediated therapy is the availability of co-chaperones and co-factors that enable refolding and degradation in addition to sequestering misfolded proteins. Although Hsp70 alone is protective over the short term in culture models, protection is dramatically enhanced by co-upregulation of the co-chaperone, Hsp40, and other Hsp (Ehrnsperger et al., 1997; Takeuchi et al., 2002; Patel et al., 2005; Batulan et al., 2006). Overexpression of Hsf1 extends lifespan, yet the contribution of individual Hsp is comparatively minor (Hsu et al., 2003; Murphy et al., 2003; Morley and Morimoto, 2004). Crossing mice overexpressing polyglutamine-expanded androgen receptor (a model of SBMA) with Hsp70 transgenics ameliorated motor function; however, no therapeutic effect was achieved by crossbreeding mutant SOD1 and Hsp70 transgenic mice (a model of ALS) (Liu et al., 2005). These examples illustrate that the effectiveness of individual Hsp varies according to pathogenic mechanisms and that Hsp70 alone can be poorly effective in the long term, despite its ability to intervene at multiple points to prevent apoptosis as well as to chaperone unfolded proteins (Arya et al., 2007).

CONSTITUTIVE VERSUS STRESS-INDUCED UPREGULATION OF HSC/HSP

Co-inducers of Hsp (NSAIDS, Hydroxylamines, Geranyl Geranyl Acetone)

Ideally, therapies that upregulate Hsp should do so by a mechanism that matches the level of cellular stress in order to avoid toxicity. Co-inducers of Hsf1-mediated *hsp* expression in other cells are not particularly effective in motor neurons (e.g,

NSAIDS). However, the hydroxylamine, arimoclomol (Kieran et al., 2004) and geranyl geranyl acetone (GGA) (Katsuno et al., 2005) have shown benefit in transgenic mouse models of motor neuron disease and are interesting candidates for further development. GGA is an acyclic isoprenoid compound with low toxicity that has been used widely in Japan as an oral anti-ulcer drug. GGA increased expression of Hsp70, Hsp90 and Hsp105 in SH-S5Y cells and in spinal cord of transgenic mice expressing androgen receptor with a pathogenic (SBMA-causing) number of polyglutamine repeats (AR97Q), but not wild type (AR24Q) (Katsuno et al., 2005). However, Grp78, Hsp25, Hsp40, Hsp60, and thioredoxin were not induced. An Hsf1-mediated mechanism was supported by an increase in amount of nuclear Hsf1 and hyperphosphorylation of Hsf1 (gel shift) in spinal cord extracts, but such experiments cannot distinguish between activation of Hsf1 in motor neurons versus other cells. Several studies have associated activation of PKCδ by GGA with activation of Hsf1 (Yamanaka et al., 2003; Fujiki et al., 2006); however, activation of PKC failed to enable Hsp70 expression in cultured motor neurons subjected to hyperthermia (Taylor et al., 2007a).

Inducers of Hsp that do not Require Additional Stress (Hsp90 Inhibitors, Celastrol)

Some of the most potent Hsp inducers in motor neurons, including the Hsp90 inhibitors discussed above, do not require additional stress, such as heat shock or oxidative stress. However, toxicity limits use of these compounds therapeutically. In spinal cord cultures, geldanamycin is less cytotoxic than 17-AAG, radicicol or novobiocin at concentrations that induced Hsp, but does not permeate the blood-brain-barrier (Batulan et al., 2006). Chemical modifications to improve therapeutic index and bioavailability could produce more viable candidates.

Celastrol, derived from the *Celastraceae* family of plants and used in traditional Chinese medicine to treat infection and inflammation, was identified in culture-based screens for compounds preventing aggregation and neurotoxicity of huntingtin. Celastrol induces DNA-binding and hyperphosphorylation of Hsf1 with upregulation of multiple Hsp including Hsp70, Hsp40 and Hsp25. Celastrol can act alone or synergistically with heat stress, lowering the temperature threshold for activation and augmenting *hsp* promoter activity (Westerheide et al., 2004). Celastrol attenuated neuronal loss and extended lifespan in SOD1^{G93A} transgenic mice and importantly, increased Hsp70 expression in spinal motor neurons (Kiaei et al., 2005).

Several other drugs, constituents of herbal medicines, and hormones have been shown to act as inducers or co-inducers of Hsp70 in other systems including paeoniflorin and glycyrrhizin (Yan et al., 2004), valproate (Pan et al., 2005), lithium (Bijur and Jope, 2000), and 17-β-estradiol (Lu et al., 2002). Lithium was not effective in cultured motor neurons (Taylor et al., 2007a). The mechanisms by which many of these compounds activate Hsf1 and/or *hsp* transcription are not clear and it is difficult to predict whether they would be effective in motor neurons.

IMPLICATIONS FOR SCREENING COMPOUNDS FOR THE ABILITY TO ACTIVATE HSP EXPRESSION IN MOTOR NEURONS

Although purified motor neuron cultures have been used for studies of neuronal development and pathogenesis of disease, these cells do not survive long term or achieve the same state of differentiation as when cultured with support cells including astrocytes. Our laboratory has used dissociated spinal cord cultures, but the presence of astrocytes, which mount a conventional heat shock response, limits methods that can be used to study the mechanisms underlying the high threshold for stress-induced activation of Hsp in motor neurons and screening of compounds to lower this threshold. Further exploration of the mechanisms of heat shock gene regulation in motor neurons requires a more robust, homogeneous culture system that displays motor neuronal properties. Although it is important to understand the mechanisms underlying the high threshold for activation of the heat shock response in motor neurons, the complexity of these regulatory systems makes hypothesis-driven approaches to therapeutic design difficult. High throughput methods for screening of chemical libraries might be more efficient. However, validation of "hits" in a motor neuronal test system should be carried before time consuming protection studies in animal models or clinical trials. The Amyotrophic Lateral Sclerosis Association (ALSA) has formed partnerships to explore stem cell-derived motor neurons as such screening tools (see http://www.alsa.org/files/cms/Research/RAT_Spring07.pdf). Further work also is required to validate culture models for representation of mechanisms of hsp regulation operant *in vivo*, both experimental animals and humans.

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CHAPTER 11

USE OF VIRAL GENE DELIVERY SYSTEMS TO INVESTIGATE THE NEUROPROTECTIVE ROLES OF HSP70 AND HSP40 PROTEINS

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Abstract: We describe the characteristics of neuron specific adenoviral (Ad) and lentiviral (LV) vectors that make them particularly suited to the study of (*hsp70*, *hsp40* and *hsj1a*) gene function in neurons. Ad vectors are particularly well suited to in vitro studies and we demonstrate that the overexpression of Hsp70 and Hsp40 in primary hippocampal neurons increased luciferase activity following a denaturing stress. Furthermore, the Ad mediated expression of Hsp70 and Hsp40 was shown to protect hippocampal neurons from a denaturing excitotoxic stress. An example of how RNA interference (RNAi) technology can be combined with Ad vector delivery to mediate the effective knockdown of chaperone genes is also given. Lentiviral vectors are best suited to in vivo studies and we demonstrate they can be used to generate animal models of neurodegenerative disease. In this example LV vectors expressing EGFP-tagged polyglutamine constructs were injected into the striatum to generate a chronic model of polyglutamine disease. Furthermore, the lentiviral-mediated expression of Hsj1A was shown to decrease the number of neurons containing inclusions. These and other studies suggest that unlike other HSP40 members Hsj1A does not promote chaperone activity but instead ubiquityates proteins and hence targets them for degradation via the ubiquitin proteosome system

Keywords: Adenoviral; lentiviral; polyglutamine; Hsp70; Hsp40 Hsj1A neurodegenerative

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VIRAL VECTOR SYSTEMS SUITED TO THE TRANSDUCTION OF NEURONS

Viruses have naturally evolved to replicate their genetic material whilst evading the immune response of the host and these characteristics have helped in the development of efficient replication-deficient gene transfer systems. The first viral vector systems created for direct genetic manipulation of the nervous system were based on Herpes Simplex Virus (HSV) (Ho and Mocarski, 1988, 1989), and these have since been extensively used to mediate neuronal gene transfer in vivo (Glorioso and Fink, 2004). However, HSV based systems have limited in vitro use, and in the CNS will mediate an inflammatory response, and replication incompetent systems will only express transgenes for relatively short periods of time. Additional viral based vector systems developed to study neuronal gene function include adeno-associated virus (AAV), lentivirus (LV). and adenovirus (Ad) (Hermens and Verhaagen, 1998). Both adenoviral and lentiviral vectors are examples of recombinant viral vectors, produced by homologous recombination and propagated in producer cell lines expressing the viral genes deleted from the vectors that are required for replication. The ability of these viral systems to transduce post-mitotic neuronal cells with great efficiency combined with substantial developments in safety and ease of production have made them the most widely used to study neuronal gene function.

ADENOVIRAL VECTORS

Ads are double stranded DNA viruses that deliver their genome to the nucleus and replicate with high efficiency, features that make them excellent gene transfer vectors. Adenoviruses naturally target the respiratory tract but in 1993 Ad vector systems were demonstrated to be a viable means of gene transfer to the nervous system (Akli et al., 1993; Davidson et al., 1993). The majority of Ad gene transfer studies since have used human Ad serotype, type 5 which contain a double stranded, linear DNA genome of approximately 36 kb in length and contain overlapping transcription units on both strands, known as inverted terminal repeat sequences (ITRs). Although, helper dependent (Palmer and Ng, 2005) Ad vectors can accommodate transgenes up to 36 kb in length, the vast majority of studies used E1/E3 deleted vectors that can only house transgenes up to 8 kb in size. The majority of Ad serotypes gain entry to cells following the binding of the Coxsackie-adenovirus receptor (CAR) that is expressed on almost every cell type (Bergelson et al., 1997). Following a number of cytoplasmic steps the viral particle, consisting of viral DNA and numerous other viral encoded proteins, is then imported through the nuclear pore into the nucleus. Once inside the nucleus, viral encoded proteins form a complex with the cellular nuclear matrix to facilitate initiation of the primary transcription events. The viral DNA does not, under normal circumstances, integrate into the host cell genome, but remains episomal within the nucleus. Interestingly, the E1A region of wild type Ad vectors activates the transcription of heat shock

proteins. However, recombinant vectors are E1 deleted and will not therefore elicit a heat shock response.

Ad vectors are highly effective at transducing primary cultures of cells and will transduce glial cells preferentially in mixed neuronal cultures. The use of a neuron-specific promoter plus powerful transcription enhancer elements however, allows transgene expression to be driven exclusively and very efficiently to neurons (Glover et al., 2003, Lee et al., 2005). This simple modification probably makes Ad vectors the system of choice for in vitro studies of neuronal gene function (Figure 1). In this chapter we have described the use of Ad vectors to investigate Hsp70 and Hsp40 refolding and neuroprotective functions in primary hippocampal neurons.

Ad Vectors have however also been used extensively for in vivo studies of neuronal gene function (Whitfield et al., 2001) and the use of neuron specific systems greatly facilitates such studies. However, AAV and lentiviral vectors have characteristics that make them more suited to in vivo use than Ad vectors. For

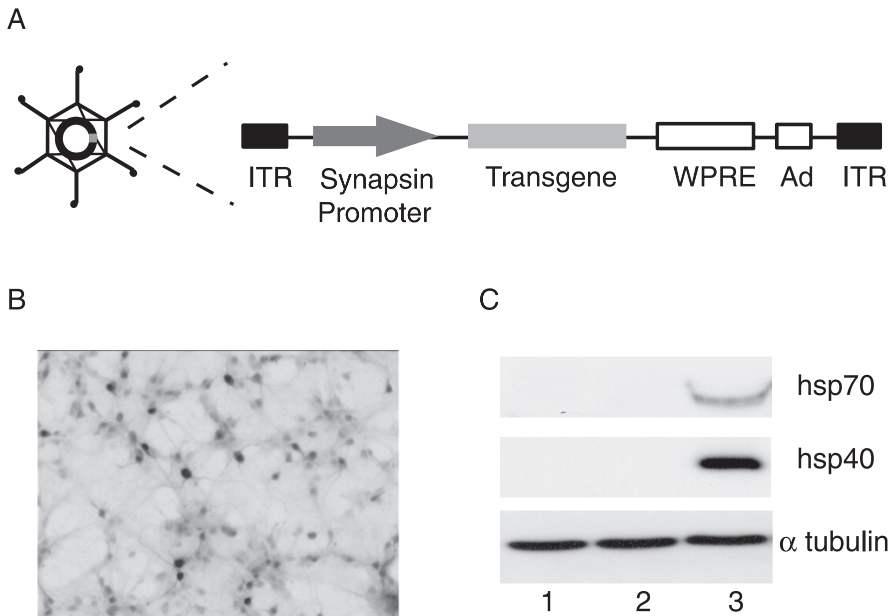


Figure 1. Adenoviral vectors to transduce primary neuronal cultures (**A**) Schematic representation of the Ad vectors used, the synapsin promoter in combination with a powerful enhancer element (WPRE) is used to drive transgene expression. (**B**) Fluorescent Photomicrograph demonstrating that Ad synapsin vectors drive expression entirely to neurons in a mixed hippocampal culture. (**C**) Western blot showing Ad-mediated expression of Hsp70 and Hsp40 in neuronal cells (lane 3). Untransduced cells and those transduced with an empty viral vector control (Ad0), Lanes 1 and 2 respectively, clearly demonstrate low endogenous levels of hsp70 and hsp40

example, they do not generate an immune response and spread more effectively following their stereotactic injection.

Vectors systems incorporating regulatable expression elements have also been developed, such as tetracycline (Harding et al., 1998) and RU486 (Burcin et al., 1999) inducible systems. However, these systems are not ideal and problems with 'leakiness' (unwanted promoter activity) remain. Since Ad vectors remain episomal rather than integrating, leakiness due to integration events can to some extent be avoided as the ratio of the two regulatable elements can be controlled. Such systems have already been used both in vitro and in vivo (Harding et al., 1997, 1998). Furthermore, the tetracycline inducible system has recently been combined with the use of cell-specific promoters to drive neuron-specific, and glial-specific, regulatable transgene expression (Ralph et al., 2000; Glover et al., 2002, 2003; Lee et al., 2004).

LENTIVIRAL VECTORS

Lentiviruses were among the first viruses to be developed for gene therapy and have subsequently become the most commonly used RNA virus vectors (Kay et al., 2001). They infect cells by an interaction between specific host cell surface receptors ('entry receptors') and co-receptors and viral envelope glycoproteins. After binding to its receptor, the viral particle is internalised through virus-cell membrane fusion and the virus core, containing the RNA genome is released into the cytoplasm 'uncoated' and the viral RNA genome is reverse transcribed into linear double-stranded proviral DNA by the viral enzyme reverse transcriptase.

The development of lentivectors (e.g. Human immunodeficiency virus (HIV) and Equine infectious anaemia virus (EIAV)) as gene transfer/therapy agents required the removal of all viral genes, except those required to complete a single round of replication. If possible these genes are preferentially supplied in *trans* from either transient transfection of plasmids or the creation of stable cell lines expressing viral genes. HIV-1 is the most commonly used lentiviral vector. It can accommodate 8 Kb inserts and is often pseudotyped with the vesicular stomatitis virus G (VSV-G) envelope protein. This reduces the potential to form wild type virus (Naldini et al., 1996b; Kafri et al., 1999) and increases cell tropism, enabling VSV-G pseudotyped vectors to transfect both dividing and non-dividing cells in vitro (Reiser et al., 1996; Naldini et al., 1996b) and following direct injection in the brain (Naldini et al., 1996a). The most recently developed lentiviral vectors contain a number of enhancer elements and mediate very strong expression when they are used in the CNS. Furthermore, they do not elicit an immune response and, as they are integrating vectors, transgene expression will last for the lifetime of the animal. Lentiviral vectors are hence suited to in vivo studies of neuronal gene function and such an application is described below. They can also be used to transduce primary neurons but, due to the charged VSVG coat protein, transduction is relatively inefficiently and therefore expression levels tend to be weaker than that obtained with Ad vectors.

In the past few years, extraordinary developments in RNA interference (RNAi) based methodologies have seen small interfering RNAs (siRNA) become the method of choice for researchers wishing to target specific genes for silencing. Viral vectors carrying siRNA expression cassettes have been developed to achieve delivery to a range of cell types (including neurons) and longer-term expression, leading to a more persistent silencing effect. This recent technology allows viral vectors to be employed to study neuronal gene function using overexpression and gene knockdown techniques. In the final part of this report we describe the delivery of a small hairpin shRNA (shRNA) to knockdown the Hsp40 protein, Hsj1a.

INVESTIGATING THE FUNCTION OF HSP70 AND HSP40 PROTEINS IN NEURONS

The HSP70 family constitutes the most conserved of the high molecular weight Hsp's in both non-neuronal and neuronal systems. Human cells contain several Hsp70 family members including the stress-inducible Hsp70, constitutively expressed Hsc70, mitochondrial Hsp75 and GRP78, localized in the endoplasmic reticulum. All members of the HSP70 family are monomeric three domain proteins possessing ATPase activity. They contain a highly conserved 44 kDa N-terminal ATPase domain, a variable C-terminal domain and the peptide binding domain (between the N- and C-termini) which plays a central role in specific functions like protein folding and association, transport of proteins through membranes and regulation of the heat shock response (James et al., 1997). Hsc70 catalyzes the disassembly of clathrin cages which are involved in intracellular protein transport. Overall the function of the HSP70 family can be summarized as to increase the yield of native functional proteins in the cell either by directly binding and refolding proteins or regulating processes to degrade non-native or non-functional proteins (Gabai et al., 1997; Strickland et al., 1997) Considering these functions the neuroprotective effects of Hsp70 were generally attributed to its ability to: (a) stabilize partially denatured proteins, which otherwise may aggregate and/or bind non-specifically to cellular proteins and interfere with their normal cellular function; (b) quickly replace and remove proteins irreversibly damaged following stress. However, a number of studies on non-neuronal cells have now suggested that Hsp70 may inhibit apoptosis by interacting with MAPK signaling pathways (Gabai et al., 1997; Mosser et al., 2000), by preventing cytochrome c release or by binding with apoptotic activating factor-1 (APAF-1) (Beere et al., 2000). While studies in the CNS have shown that Hsp70 (Kelly et al., 2001a) and a carboxy-terminal domain mutant of Hsp70 (a mutant that does not mediate refolding) can protect from ischaemic injury (Sun et al., 2006). Further studies have shown that the protection from ischaemic injury mediated by Hsp70 overexpression is associated with Hsp70 binding apoptosis-inducing factor (AIF) (Matsumori et al., 2005) and apoptosis protease activating factor (Apaf-1) (Matsumori et al., 2005).

Recent work has shown that Hsp70 and other high molecular weight chaperone proteins may be directed to different protein substrates by interacting with

co-chaperone proteins. Studies on prokaryotic systems showed that dnaK (the bacterial homologue of Hsc70) was directed to different protein substrates by interacting with proteins known collectively as DnaJ's (Silver and Way, 1993). Many eukaryotic DnaJ (termed hsp40's) homologues have now been isolated, suggesting that DnaJ proteins participate in the reactions catalyzed by eukaryotic hsp70 family members. In addition to a co-chaperone function, many DnaJ proteins have also been shown to act as chaperones. Cheetham et al. (1992) isolated a human alternatively spliced DnaJ/hsp40 homologue (Hsj1a and Hsj1b) and showed that the isoforms were stress inducible and preferentially expressed in the brain (Cheetham et al., 1992; Silver and Way, 1993). Hsp40 was isolated from Hela cells, and was shown to be highly induced by stress in neurons and to accumulate in the nucleus and nucleolus during stress. Recently, a number of groups have shown that proteins of the HSP40 family increase Hsp70 chaperone activity by enhancing the ATPase activity of Hsp70 (Freeman et al., 1995; Minami et al., 1996). This action is dependent on the interaction of the J-domain of Hsp40 with the ATP binding domain of Hsc70/Hsp70 (Michels et al., 1997, 1999). A number of negative and additional positive regulators of Hsc70 and Hsp70 have been identified. Hsp70 interacting protein (Hip) facilitates refolding by stabilizing the ADP-bound state of Hsp70/Hsc70. Hip directly binds to the ATPase domain of Hsp70 when it is converted to the ADP-bound state by proteins of the HSP40 family (Hohfeld et al., 1995).

Many human degenerative diseases are characterised by the progressive degeneration of neurons (with their eventual death) and accompanying production of abnormal intracellular inclusions containing Hsp's and once normal cellular proteins (e.g. neurofibrillary tangles in Alzheimer's disease). These observations suggest that either stress proteins are associated with the aetiology of these diseases, or that their up-regulation represents an unsuccessful attempt to remove the abnormal protein aggregates. A number of recent studies have also shown that the overexpression of *hsp70* and *hsp40* genes could be neuroprotective for stroke (Kelly et al., 2001b; Klettner, 2004; Giffard et al., 2004) and the polyglutamine (PolyQ) disorders (Wyttenbach et al., 2000; Ho et al., 2001). These observations together with other reports that implicate the UPS system in PD suggest that manipulating Hsp expression could be prophylactic for many human neurodegenerative conditions.

INVESTIGATING HSP70 AND HSP40 PROTEIN FUNCTION IN PRIMARY NEURONS USING ADENOVIRAL VECTORS

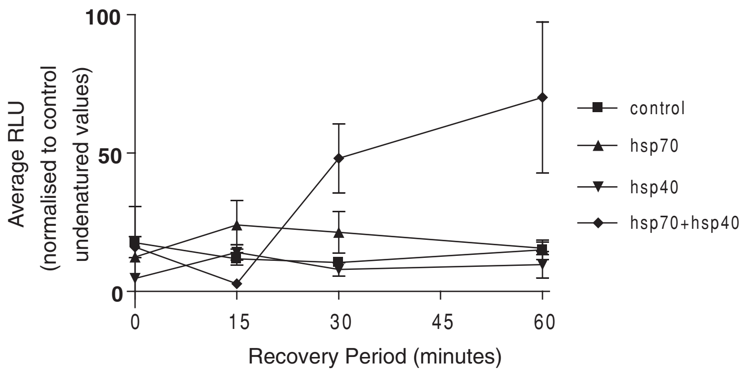
Ad vectors can be used to over-express genes of interest or to express small hairpin RNAs (shRNA) that mediate the silencing of genes by the process of RNA interference. To investigate Hsp70 and Hsp40 function in neurons we have used both approaches.

AD-MEDIATED OVER-EXPRESSION STUDIES

The chaperone function of Hsp70 was investigated using a neuron-specific Ad vector system to transduce primary hippocampal neuron and over-express luciferase, *hsp70*

and *hsp40* transgenes (Figure 1). The results show that Hsp70 alone mediated a small increase in luciferase activity 15 minutes after a denaturing stress. However, when Hsp70 and Hsp40 were expressed together a dramatic increase in luciferase activity was seen. Experiments have also shown that pre-existing chaperone expression levels vary between cell types and influence the effectiveness of exogenous Hsp70 and Hsp40 at refolding denatured luciferase (not shown) and therefore it is important to be cautious when making comparisons.

Having shown that Hsp70 plus Hsp40 can increase protein refolding following a stress in neuronal cells we also investigated whether they mediated a neuroprotective effect in primary hippocampal neurons. Figure 2B shows that the Ad mediated expression of either Hsp40 or Hsp70 protected hippocampal neurons (as assessed by



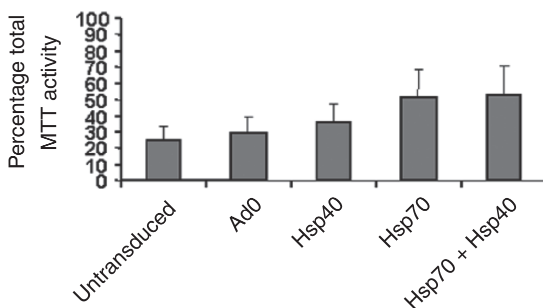


Figure 2. Investigating Hsp70 function in neuronal cells using adenoviral mediated transduction (**A**) Neuronal cells were transduced with Ad vectors expressing luciferase, Hsp70 and Hsp40 and luciferase activity measured following a denaturing heat stress. (**B**) Hippocampal neurons were transduced with Ad vectors expressing Hsp70 and Hsp40 and then exposed to an excitotoxic dose of glutamate

MTT assays) from a denaturing glutamate shock. However, the greatest protective effect was mediated by the expression of Hsp70 alone and Hsp70 plus Hsp40.

Taken together these results suggest that Hsp70 together with Hsp40 is protecting cells from an excitotoxic stress by stabilizing/refolding cellular proteins. However, recent work in non neuronal systems and a viral transfer study we conducted in superior cervical ganglion neurons have shown that Hsp70 mediates powerful anti-apoptotic effects by preventing the activation of the transcription factor c-Jun. The induction of a stress response will therefore result in Hsp70 (together with other hsps such as Hsp27 and the Hsp40s) inhibiting the activation of apoptosis while simultaneously acting as a chaperone to stabilize proteins and prevent denaturation and damaging adventitious associations leading to aggregate formation.

VIRAL-MEDIATED RNA INTERFERENCE TO MEDIATE GENE KNOCKDOWN

RNA interference-based gene silencing, in the form of small interfering RNAs (siRNA), small hairpin RNAs (shRNA) or microRNA, has emerged in recent years as a valuable tool for studying gene expression both in cell culture and in vivo (Bantounas et al., 2004; Zhou et al., 2006); Direct transfection (for example by lipid-based protocols) of these silencing agents has certain disadvantages: Firstly, they are degraded quickly by intracellular nucleases making long term studies (and potential therapeutic applications) virtually impossible. Moreover, certain cell types (e.g. primary neurons) have traditionally been very difficult to transfect with naked nucleic acids. To increase the longevity of the knockdown, siRNA sequences were adapted to include a spacer that mediated the formation of a hairpin structure (shRNA), which allowed the sense and antisense sequences to form base pairs. Thus, vector-based systems for the expression of shRNA were developed, whereby the silencing nucleic acid is expressed under the control of a polIII (e.g. U6, H1) promoter (Figure 3A). More recently microRNA shuttles have been used to deliver shRNAs and an important feature of this method is that polII promoters (e.g. CMV or Synapsin) can be used in the viral vector (Figure 3B). The inclusion of any of these expression cassettes in viral vectors has thereby allowed for efficient delivery and long-term silencing of the gene of interest.

Recently two neuronal enriched members of the HSP40 family termed HSJ1a and HSJ1b, which may act as another link between the molecular chaperone and ubiquitin proteasome systems, were identified (Westhoff et al., 2005). Both proteins were shown to regulate the ATPase activity and substrate binding of Hsp70. Moreover Hsj1a and Hsj1b contain ubiquitin-interacting motifs (UIMs) that bind ubiquityated proteins and target them to the proteasome (Chapple et al., 2004). In a previous study we used adenoviral delivery systems with in vitro models of SBMA and polyglutamine expansion diseases to study the function of Hsj1 proteins. The results showed that in our models Hsj1 proteins did not promote Hsp70 chaperone activity but they were highly effective at reducing aggregation in vitro by increasing protein ubiquitylation and targeting to the UPS (Howarth et al., 2007). However to

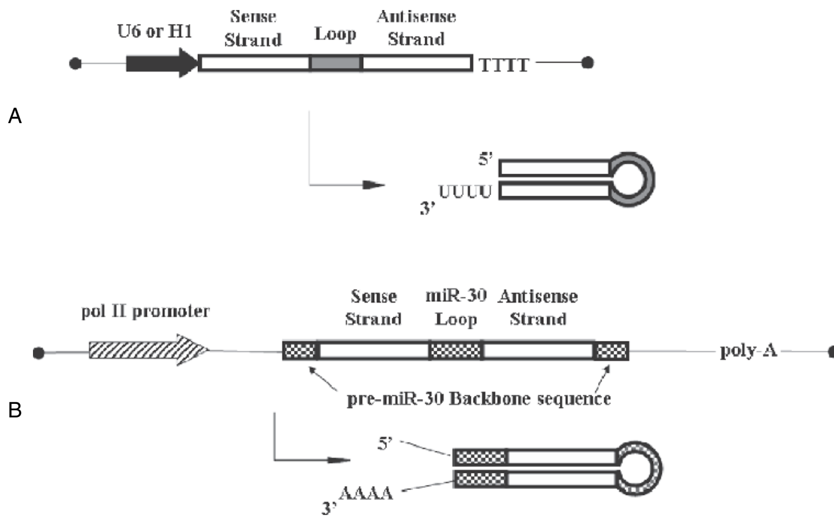


Figure 3. ShRNA/miRNA expression cassettes used in viral constructs to mediate long term gene silencing. (**A**) polIII-driven shRNA. (**B**) polII-driven miR30-based shRNA

investigate the role constitutively expressed Hsj1A plays in preventing aggregate formation in human disease a knockout study in adult animals would be desirable. In an attempt to achieve this we have used a strategy that combines RNAi with viral delivery (illustrated in Figure 4). Briefly, this procedure involved the design of five shRNA sequences targeted against the *hsj1A* gene sequence (Figure 4A), and their transfection into N2A cells followed by western blotting for Hsj1A. The sequence shown to be most effective at preventing Hsj1A expression was then cloned under the control of the U6 promoter into an adenoviral vector (Figure 4B). The Ad-U6-shhsj1 vector was then shown to mediate the very effective knockdown of endogenous Hsj1A at a (low) multiplicity of infection (MOI) of 10. Studies are ongoing using this vector but our preliminary experiments using models of polyglutamine disease indicate that this proteins normal function is to minimise aggregate formation.

USING VIRAL VECTORS TO INVESTIGATE HSP40 PROTEIN FUNCTION AND THERAPEUTIC POTENTIAL USING IN VIVO MODELS OF DISEASE

In recent years major steps have been taken to improve the in vivo utility of adenoviral and lentiviral vectors such that they mediate: (i) efficient transduction. (without altering normal cellular physiology); (ii) targeting to specific cell types; (iii) long-term expression in the CNS. For example, we have recently shown that an adenoviral system incorporating the synapsin promoter and woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) could mediate long-term

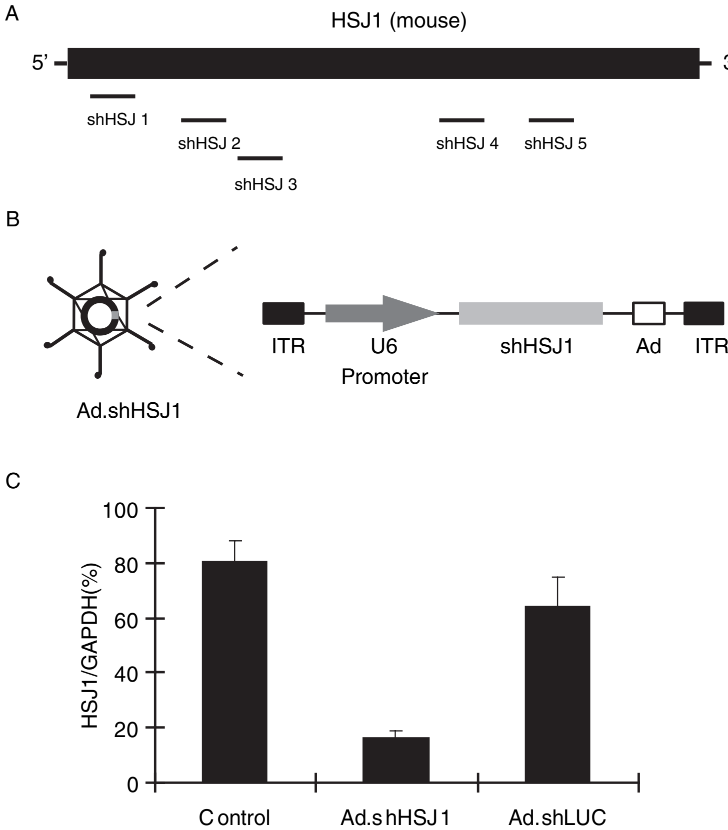


Figure 4. Viral RNAi Knockout strategy for Hsj1A. Five shRNAs directed against the *hsj1A* gene are designed and cloned into expression vectors (**A**). Western blotting and RTPCR are used to determine the shRNA that mediates the greatest knockdown of Hsj1a expression in N2A cells and this sequence is then cloned into an Ad U6 vector plasmid (**B**). N2a cells were transduced with the Ad vector expressing the shRNA against hsj1A and expression assessed by TaqMan analysis (quantitative RTPCR) (**C**)

transgene expression exclusively in neurons (Glover et al., 2002, 2003). In addition we have also developed a VSVG pseudotyped HIV lentiviral system incorporating the synapsin promoter to ensure entirely neuron specific expression (Figure 4). However, since the VSVG-pseudocoat protein confers a neurotropism to the viral particle and LV do not induce any immune response they are the system we chose to carry out in vivo investigations.

To characterise the spread and transduction efficiency of the lentiviral vectors within the striatum we initially injected a vector expressing EGFP under the control of the synapsin promoter (Figure 5A). The results showed that a single injection mediated the very efficient transduction of neurons over a considerable area of the striatum (Figure 5B). Indicating LV vectors could be used to generate animal models

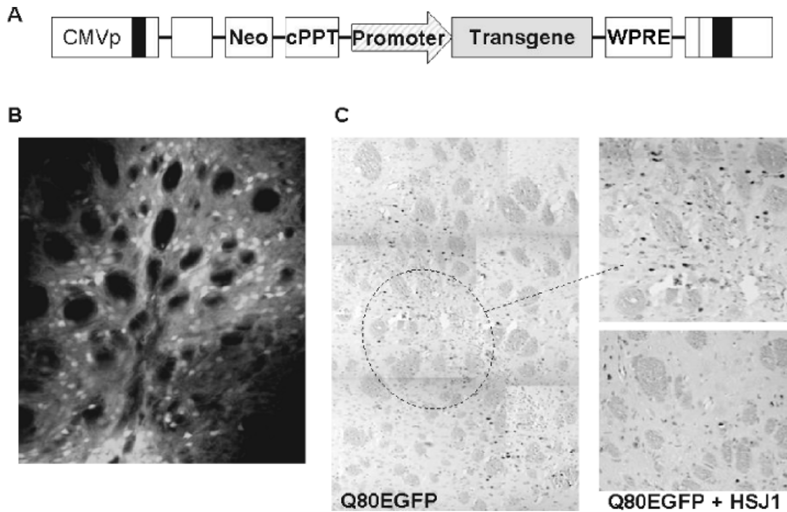


Figure 5. Developing in vivo models of neurodegenerative disease using lentiviral transduction. Schematic representation of the HIV based LV vectors used. The transgene of interest is cloned under into the vector under the control of a neuron specific synapsin promoter or the pleitropic CMV promoter. Expression of the transgene is enhanced by the cPPT and WPRE enhancer elements (**A**). A single injection of a LV vector expressing EGFP into the striatum mediates the long-term (<6 months) and widespread expression of EGFP in striatal neurons (**B**). Immunocytochemical analysis (with an anti-EGFP Ab) of haematoxylin and eosin stained brain sections showed that the intrastriatal injection of the HIV-1-Syn1-Q80EGFP-WPRE led to the production of nuclear inclusions, while the injection of HSJ1a dramatically reduced the formation of inclusions (**C**)

of neurodegenerative disease and to achieve this we generated LV vectors expressing polyglutamine repeats linked to EGFP (Q19EGFP and Q80EGFP fusion proteins) and expressing Hsj1A. The injection of the control vector (HIV-1-Syn1-Q19EGFP-WPRE) did not result in the formation of EGFP-positive inclusions, but produced very faint, diffuse EGFP staining throughout the whole cell. In contrast, injection of the HIV-1-Syn1-Q80EGFP-WPRE vector led to the widespread formation of nuclear inclusions in neurons of the striatum (Figure 5C). The injection of a LV expressing Hsj1A together with the vector expressing Q80-EGFP dramatically reduced the number of neurons containing EGFP-positive inclusions (Figure 5C). A full analysis of the kinetics of lentiviral mediated inclusion formation and of Hsj1A mediated neuroprotection have recently been described.

Together these viral studies of Hsp70 and HSP40 family mediated neuroprotective effects show that when expressed alone Hsp70, Hsp40, and Hsj1a can suppress aggregate formation. Hsp70 and Hsp40 mediate this effect by increasing (Hsp70) chaperone mediated refolding and their expression in primary hippocampal neurons was sufficient to protect neurons from an excitotoxic stress. However, in a separate study we have shown that Hsp70 overexpression blocks c-Jun mediated transcription and is thereby mediates powerful anti-apoptotic effects. The induction of a stress

response will therefore result in Hsp70 (together with other Hsps such as Hsp27 and the HSP40s) inhibiting the activation of apoptosis while simultaneously acting as a chaperone to stabilize/protect target proteins and hence prevent their denaturation.

Hsj1a has been shown to act in a different manner to other members of the HSP40 family. We and others found that it suppresses aggregate formation by increasing polyglutamine protein ubiquitylation and targeting to the UPS (Westhoff et al., 2005; Howarth et al., 2007). Importantly, the in vivo viral studies also showed that the lentiviral-mediated expression of Hsj1a protected neurons from polyglutamine mediated inclusion formation in the striatum.

The UPS plays an important role in targeting proteins for degradation and molecular chaperones can target substrate proteins to the UPS for degradation under some circumstance (Esser et al., 2004). For example carboxy-terminal of Hsc70 interacting protein (CHIP) uses Hsp70 and Hsp90 family members to recognise misfolded protein substrates, which it then directs for degradation via the UPS. The function of CHIP is known to be enhanced by Bag-1 and on formation of the Bag-1/Hsc70/CHIP complex, CHIP mediates the attachment of ubiquitin moieties to the ubiquitin-like domain (UBL domain) found on all Bag-1 isoforms (Alberti et al., 2002, 2003). This domain enables Bag-1 to function as a physical link (or 'coupling factor') between the Hsc70/Hsp70 chaperone system and the proteasome. Since HSJ1 proteins contain DnaJ and UIM motifs they could act to mediate chaperone function or fulfil a similar role to Bag-1 proteins (acting as a switch between chaperone refolding activities of Hsc70 and it's targeting to the UPS).

Study of the nervous system has been particularly challenging compared to other organs, since neurons are post-mitotic and exist as a myriad of discrete cell types that have an extremely heterogeneous structure and function. The historic difficultly of efficiently transfecting neurones has been overcome by the use of attenuated viral vectors (e.g. Ad, LV, AAV and Herpes simplex). These viral vectors all have the ability to enter and express in quiescent cells at relatively high efficiency. In this study we have given examples of how powerful neuron specific Ad and LV vectors can currently be used to study neuronal gene function and to model disease and potential gene therapy strategies.

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CHAPTER 12

HEAT SHOCK PROTEINS AT THE SYNAPSE: IMPLICATIONS FOR FUNCTIONAL PROTECTION OF THE NERVOUS SYSTEM

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Abstract: The protective effects of prior heat shock against cell death have been well established. Comparatively little attention has been given to the determination of whether this type of 'preconditioning' treatment protects critical neural processes such as synaptic function from subsequent stress. Synapses are key sites of information transfer in the nervous system and their functionality must be preserved under stressful conditions to prevent communication breakdown. Synaptic connections are vulnerable regions of neurons involved in the physiological process of 'neurotransmission' that link neurons into functional networks. The combined application of molecular biology and neurophysiology techniques has demonstrated that prior heat shock protects neurotransmission and synapses are able to function under conditions that would normally be disruptive. Selective overexpression of Hsp70 enhances the level of synaptic protection. Biochemical isolation of synaptic fractions and immunocytochemistry has localized a set of constitutive and stress-inducible heat shock proteins to components of the synapse. Constitutively expressed Hsc70 protein is enriched in neural tissue compared to non-neural tissues. Following hyperthermia, an enhancement of Hsc70 is apparent in synapse-rich areas of the brain in concert with the appearance of stress-inducible Hsp70, Hsp32 and Hsp27 at synapses. Induction of the heat shock response protects the nervous system at the functional level and permits neurotransmission events to proceed at synapses during stressful conditions. Synaptic function is disrupted during the progression of neurodegenerative diseases and upregulation of heat shock proteins could mitigate that dysfunction

Keywords: Heat shock response; heat shock proteins; synapses; neurotransmission; neuroprotection; neurodegenerative diseases

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INTRODUCTION

In response to adverse stimuli, cells activate a highly conserved 'heat shock response' in which a set of stress proteins, termed 'heat shock proteins' (Hsps), are induced. These proteins play important roles in cellular repair and protective mechanisms. Induction of the heat shock response protects cells from subsequent stress that would normally be lethal. This phenomenon, termed 'induced thermotolerance', was initially investigated in tissue culture systems. However, the ability of the heat shock response to protect against cell death induced by a range of stressful stimuli has also been demonstrated in the intact nervous system (reviewed by Brown, 1994, 2007; Brown and Sharp, 1999; Franklin et al., 2005). Comparatively little progress has been made regarding the mechanisms by which the heat shock response protects cells at a functional level. Are there important physiological processes unique to the nervous system that must be protected during stressful conditions? Synapses are critical points of information transfer between neurons. Their functionality must be preserved during stressful conditions to prevent communication breakdown in the nervous system. Synaptic connections are vulnerable regions of neurons involved in the physiological process of 'neurotransmission' that functionally link neurons into communication networks. This review explores whether induction of the heat shock response protects neurons at the functional level of the synapse and whether this strategy could impact synaptic dysfunction that is a feature of neurodegenerative diseases.

THE HEAT SHOCK RESPONSE IN THE NERVOUS SYSTEM

The classic heat shock response was initially studied in non-neural tissue culture systems. This provided the impetus for subsequent investigations that demonstrated that the response is physiologically relevant as it could be induced in the mammalian nervous system following a fever-like increase in body temperature or tissue injury (Brown, 1994, 2007; Brown and Sharp, 1999; Franklin et al., 2005). A robust induction of Hsp70, Hsp32 and Hsp27 is triggered in brain cells following hyperthermia (Manzerra et al., 1993, 1997; Foster and Brown, 1997; Bechtold and Brown, 2000; Bechtold et al., 2000; Franklin et al., 2005).

Stressful stimuli can elicit two reactive responses: the heat shock response and activation of the cell death pathway. Most studies on the in vivo effects of hyperthermia on mammalian tissues have focused on the induction of Hsps. However, a fever-like increase in body temperature has been shown to induce cell death in dividing cell populations of the testis and thymus of the adult rat but not in mature, postmitotic cells of the brain (Khan and Brown, 2002). Hyperthermia did induce cell death in brain cells at early stages in development when neural cells undergoing cell division are present. These results suggest that actively dividing cell populations in vivo are more prone to cell death induced by hyperthermia than fully differentiated, postmitotic neural cells (Khan and Brown, 2002). Correlation of the patterns of cell death and Hsp70 expression revealed that cells inducing Hsp70 after whole

body hyperthermia were not triggered into cell death (Belay and Brown, 2003). Interestingly, populations of neurons in the adult brain rat, such as hippocampal neurons and Purkinje neurons, did not induce Hsp70 after hyperthermia and were not triggered into cell death (Belay and Brown, 2006). High expression of constitutively expressed Hsc70 was noted in these neuronal cell populations, suggesting that neuronal expression of Hsc70 may play roles in 'preprotecting' neurons from stressful stimuli (Belay and Brown, 2006).

PRIOR HEAT SHOCK PROTECTS THE NERVOUS SYSTEM AT THE FUNCTIONAL LEVEL OF THE SYNAPSE

Prior exposure to sublethal temperatures induces heat shock proteins and protects cells from death that would normally result from exposure to lethal temperatures and other forms of stress (Morimoto et al., 1997). The nervous system follows this general rule as induction of the heat shock response by 'preconditioning' protects the nervous system against subsequent insults (Brown, 1994; Yenari, 2002). Protective effects of prior heat shock treatment have been reported in both neural cells grown in tissue culture and in the intact nervous system. For example, prior heat shock at 42°C protects rat embryos from developmental neural defects caused by heat shock at 43°C (Walsh et al., 1987, 1989). Prior heat shock also protects retinal photoreceptors from cell death induced by bright light (Barbe et al., 1988). The time course of Hsp70 induction parallels the time course of the protective effect and intraocular injection of exogenous Hsp70 reduces the susceptibility of the retinal cells to light damage (Tytell et al., 1993; Yu et al., 2001).

The protective effects of prior heat shock treatment against cell death have been well established, but comparatively little is known with respect to whether this type of 'preconditioning' protects critical neural processes such as synaptic function from subsequent stress. Synapses are critical sites of information transfer in the nervous system and their functionality must be maintained under stressful conditions to prevent communication breakdown. Synapses are vulnerable regions of neurons involved in the physiological process of 'neurotransmission' that link neurons into functional networks. Exploration of the neural heat shock response at the level of the synapse requires the combined application of molecular and cellular techniques to measure induction of Hsps and neurophysiological techniques to measure neurotransmission events at synapses. Drosophila is an organism that is highly appropriate for studies that combine molecular and cellular biology with neurophysiology.

Interestingly the heat shock response was first described in Drosophila (Ritossa, 1962) and much of the early work on Hsps was carried out on this organism. In Drosophila, synthesis of most cellular proteins is down-regulated during thermal stress while the predominant Hsp70 is rapidly induced and plays a major role in protective mechanisms (Tissieres et al., 1974; Parsell and Lindquist, 1993; Feder et al., 1996). The Drosophila system lends itself to genetic

and molecular manipulation, but it is also ideal for studies on synaptic neurophysiology as the large size and accessibility of the larval neuromuscular junction makes it the premier experimental preparation for investigation of synaptic transmission (Keshishian et al., 1996). A macropatch electrode can be positioned over individual, visualized synaptic ‘boutons’ to facilitate neurophysiological recordings of pre- and postsynaptic events that modulate synaptic transmission (Cooper et al., 1995; Stewart et al., 1996). Drosophila larvae were subject to heat shock that strongly induced expression of Hsps, particularly Hsp70. At time points thereafter, larvae were harvested and a macropatch electrode was utilized to record synaptic activity at individual, visualized boutons as the temperature of the preparation was raised in a stepwise fashion (Karunanithi et al., 1999). Increasing the temperature of the preparation resulted in failure of synaptic transmission, however, prior heat shock of the larvae sustained synaptic performance at high test temperatures through both pre- and postsynaptic alterations. Beneficial presynaptic modifications resulting from the larval ‘preconditioning’ were apparent since nerve impulses released more quantal units at high temperature and exhibited fewer failures of neurotransmitter release. In addition, beneficial postsynaptic modifications were reflected by the constant amplitude of quantal currents. The time course of these protective modifications of synaptic physiology paralleled the time course of Hsp70 induction. The protective effects of the prior heat shock on synaptic physiology was maximal at peak Hsp70 levels and declined as stress-induced Hsp70 decayed. These observations demonstrate that stress-induced neuroprotective mechanisms are operative that maintain the functionality of synapses by modifying their physiological properties.

Prior heat shock conferred protection to Drosophila synapses during subsequent thermal stress by stabilizing quantal size and reducing the decline of quantal emission at individual synaptic boutons. Expression of Hsp70 was not detectable in nonheat-shocked larvae, however, following heat shock ‘preconditioning’, it became the most prominent induced protein. To investigate whether Hsp70 is an important component of the synaptic protective response, transgenic Drosophila were engineered to contain 12 extra copies of the Hsp70 gene. Elevation of temperature induced a greater amount of Hsp70 in the transgenic larvae compared to an excision line that shared the same chromosomal sites of transgene insertion and flanking sequences but lacked the extra copies of the Hsp70 gene. This selective overexpression of Hsp70 enhanced the level of synaptic performance as assayed by measuring quantal content and percentage of success of synaptic transmission (Karunanithi et al., 2002). Use of a Drosophila mutant that fails to accumulate inducible Hsp70 revealed the compensatory upregulation of constitutively expressed Hsps and the preservation of synaptic thermoprotection (Neal et al., 2006). Heat shock ‘preconditioning’ has been shown to protect synaptic transmission in slice preparations from the mammalian brain (Kelty et al., 2002). This study also demonstrated that addition of exogenous Hsp70 to the medium of the brain slices protected synaptic transmission from thermal stress. These studies demonstrate that the protection conferred to the nervous system by heat shock ‘preconditioning’ has been extended to the level of synaptic function. Targeting Hsp70 to motor neurons

induced structural plasticity of axonal terminals that resulted in increased transmitter release at neuromuscular junctions at high temperature (Xiao et al., 2007). This protected larval locomotor activity from hyperthermia in Drosophila.

ASSOCIATION OF HEAT SHOCK PROTEINS WITH THE SYNAPSE

Hsps are composed of constitutively expressed members that are present in unstressed cells and inducible members that are expressed in response to stressful stimuli. Biochemical fractionation of tissue from the mammalian brain indicates that constitutively expressed Hsp90, Hsp60 and Hsc70 are associated with synaptic elements in the unstressed animal, suggesting that they play roles in normal synaptic function (Bechtold et al., 2000). Constitutive members of the Hsp70 multigene family are involved in the regulated release of neurotransmitters at synapses that depends on repeated cycles of exocytosis and endocytosis of synaptic vesicles within the presynaptic element (Bronk et al., 2001; Zinsmaier and Bronk, 2001). Together with cysteine-string protein, Hsc70 is a component of synaptic vesicle fusing complexes that form a synaptic chaperone machine (Chamberlain and Burgoyne, 2000; Bronk et al., 2001, 2005; Tobaben et al., 2001; Zinsmaier and Bronk, 2001; Dawson-Scully et al., 2007). Hsc70 is also associated with postsynaptic elements such as the postsynaptic density (Bechtold et al., 2000). DNAJ-like proteins, cysteine-string protein and Hsp40 have been identified at the synapse (Kohan et al., 1995; Suzuki et al., 1999; Ohtsuka and Suzuki, 2000). Following a physiological relevant increase in body temperature, Hsp70 is induced and is found associated with biochemically isolated synaptic elements including the postsynaptic density (Bechtold et al., 2000). Immunoelectron microscopy has provided visual confirmation of the localization of stress-inducible Hsp70, and also constitutively expressed Hsc70, to synaptic elements (Bechtold et al., 2000). Subcellular fractionation and immunoelectron microscopy have also demonstrated that hyperthermia-induced Hsp27 and Hsp32 are present in synaptic elements (Bechtold and Brown, 2000). Early induction of Hsp70 by chronic hypoxia stress has been shown to be critical for maintaining expression levels of presynaptic proteins (Fei et al., 2007). This study reports a direct interaction between Hsp70 and the presynaptic protein syntaxin. The positioning of Hsps at the synapse could facilitate the repair of stress-induced damage to synaptic proteins and contribute to neuroprotective mechanisms at the synapse.

LIPID RAFTS AND HEAT SHOCK PROTEINS

Lipid rafts are specialized plasma membrane microdomains that are enriched in cholesterol and sphingolipids that serve as major assembly and sorting platforms for signal transduction complexes (Brown and London, 1998; Simons and Toomre, 2000). The brain is enriched in lipid rafts. More than 1% of total brain protein is recovered in a lipid raft fraction, whereas less than 0.1% of total protein is associated with lipid rafts isolated from non-neural tissue (Maekawa et al., 2003). It

has been proposed that lipid rafts are important components of synapses critical for the maintenance of synaptic stability (Suzuki, 2002; Hering et al., 2003). A wide range of neurotransmitter receptors and constitutively expressed Hsp90, Hsc70, Hsp60 and Hsp40 are present in lipid rafts isolated from regions of the rat brain (Chen et al., 2005). Within 1 hr of hyperthermia, stress-induced Hsp70 was detected in lipid rafts isolated from the cerebellum (Chen et al., 2005). These observations indicate that constitutively expressed Hsps participate in the normal functioning of lipid rafts during neurotransmission events at synapses. After hyperthermia, the presence of stress-inducible Hsp70 in lipid rafts suggests a role in preserving the functional stability of lipid rafts and their associated signal transduction complexes at synapses.

STRESS-INDUCED ENHANCEMENT OF CONSTITUTIVELY EXPRESSED HSC70 IN SYNAPSE-RICH AREAS OF THE BRAIN

Hsp70 is a multi-gene family composed of stress-inducible Hsp70 and other members that are constitutively expressed (Hsc70). It is noteworthy that Hsp70 and Hsc70 exhibit similar molecular structures and biochemical features (Hightower et al., 1994). Heat shock 'preconditioning' triggers the synthesis of stress-inducible Hsp70, hence protective mechanisms have tended to focus on this stress-inducible Hsp. However, Hsc70 has also been hypothesized to play a role in thermotolerance and stress resistance (diIorio et al., 1996). In vitro studies on purified mammalian Hsc70 have reported that the protein undergoes a conformational change that activates its peptide/unfolded-protein binding activity as temperature is increased (Leung et al., 1996). This heat-induced Hsc70 conformational change may be associated with acquired thermotolerance (Leung et al., 1996).

Our studies have noted that constitutively expressed Hsc70 protein is enriched in the mammalian nervous system compared to non-neural tissue and is present at high levels in neuronal cell bodies (Manzerra et al., 1993, 1997; Manzerra and Brown, 1996). After thermal stress, overall neural levels of Hsc70, as determined by Western blotting, do not change. However, confocal immunocytochemistry detected an enhancement of Hsc70 in synapse-rich areas of the cerebral cortex that were identified by the synaptic marker synaptophysin (Chen and Brown, 2007a). The functioning of Hsc70 protein requires the assistance of Hsp40 that delivers protein candidates to Hsc70 and stimulates the ATPase actitivity of Hsc70 to mediate correct protein folding (Fan et al., 2003). The association of Hsc70 with Hsp40 was investigated by co-immunoprecipitation. This showed that Hsc70 and Hsp40 were associated in the unstressed brain, as expected, in Hsc70/Hsp40 chaperoning machinery engaged in the folding of newly synthesized proteins (Chen and Brown, 2007a). Thermal stress can induce protein unfolding (Lepock, 2005). Interestingly co-immunoprecipitation demonstrated that the association of Hsc70 and Hsp40 increased in the brain after heat shock (Chen and Brown, 2007a). Confocal immunocytochemistry revealed an increased co-localization of Hsc70 and Hsp40 in synapse-rich areas after heat shock. This could reflect the association of Hsc70

and Hsp40 in a synaptic chaperone machine that refolds synaptic proteins that have been perturbed by stress (Chen and Brown, 2007a).

These results could be interpreted to suggest that the heat shock response in the nervous system involves not only the induction of stress-inducible Hsps but also the translocation of constitutively expressed Hsc70 to synapse-rich areas where it participates with Hsp40 in neuroprotective mechanisms that preserve synaptic function during times of stress. An alternative possibility to explain the stress-induced enhancement of Hsc70 in synapse-rich areas is 'local translation' of Hsc70 mRNA at the synapse (see subsequent section entitled 'mRNA transport into post-synaptic dendrites of neurons'). A protective role for Hsc70 may be particularly relevant to differentiated neurons that characteristically exhibit high levels of Hsc70 and do not synthesize stress-inducible Hsp70 after thermal stress (Manzerra et al., 1993; Foster et al., 1995; Marcuccilli et al., 1996; Batulan et al., 2003). In contrast to neurons, glial cells demonstrate a strong induction of Hsps in response to hyperthermia in both tissue culture systems and in the intact nervous system (Brown, 1994; Foster et al. 1995; Foster and Brown, 1997; Brown and Sharp, 1999; Franklin et al., 2005). More severe stress, such as ischemia, results in induction of Hsp70 in neurons (Franklin et al., 2005).

TRANSFER OF HEAT SHOCK PROTEINS BETWEEN CELL TYPES IN THE NERVOUS SYSTEM

Transfer of Hsps between cell types in the nervous system was suggested by early work for the Tytell laboratory (Tytell et al., 1986). Heat shock induces the synthesis of Hsp70 in glial cells located in the sheath surrounding the squid giant axon and the rapid transport of this glial stress protein to the adjacent axonal process. This 'glial to neuron' transfer may provide a mechanism for fast delivery of neuroprotective Hsps to cellular processes distant from the neuronal cell body. Subsequent work has indicated that application of exogenous Hsps at neural injury sites is an effective strategy to maintain neuronal viability (Tytell et al., 1993; Houenou et al., 1996; Guzhova et al., 2001; Yu et al., 2001; Tidwell et al., 2004; Robinson et al., 2005; Tytell, 2005). Interestingly, hyperthermia results in the appearance of stress-inducible Hsp27 and Hsp32 in perisynaptic glial processes that surround and nurture synapses (Bechtold and Brown, 2000). These Hsps are subsequently transported to synaptic compartments. For additional discussion on extracellular release of Hsps in the nervous system and their effects on other neural cells, see Chapter 13 by Tytell et al.

mRNA TRANSPORT INTO POSTSYNAPTIC DENDRITES OF NEURONS

Using the electron microscope, Steward and Levy (1982) detected polyribosomes in the distal dendritic processes of neurons in the dendate gyrus. This raised the possibility that mRNAs could be transported to synapses and locally translated in

response to synaptic stimulation. Subsequent studies demonstrated that synapse-rich biochemical fractions could corporate labeled amino acids into protein (Rao and Steward, 1991; Weiler and Greenough, 1991; Torre and Steward, 1992). A functional role for dendritic protein synthesis was suggested by the observation that local protein synthesis in dendrites was required for the rapid enhancement of synaptic transmission by exposure to growth factor (Kang and Schuman, 1996). Current evidence suggests that dendritic protein synthesis contributes to various aspects of memory processing and synaptic remodeling and plasticity (Sutton and Schuman, 2006). It is now accepted that mRNAs localize to postsynaptic dendrites and that translation of these mRNAs is regulated in response to neuronal activity (Martin and Zukin, 2006; Schuman et al., 2006). Central questions that are being investigated include (1) what mRNAs are present in dendrites (2) how are they transported from the neuronal nucleus to postsynaptic dendrites (3) how is translation of these mRNAs regulated by synaptic activity and (4) what is the function of local translation at the synapse of these mRNAs (Martin and Zukin, 2006; Hirokawa, 2006; Schuman et al., 2006; Pfeiffer and Huber, 2006). Dendritic mRNAs have been found to encode a variety of proteins that extend beyond those involved in modulating synaptic plasticity. For example, synapse-associated mRNAs have been found to encode receptors, channels, signaling molecules, cytoskeleton proteins, adhesion molecules and factors involved in vesicle trafficking, protein synthesis and protein degradation (Moccia et al., 2003; Sung et al., 2004; Zhong et al., 2006).

Are mRNAs encoding heat shock proteins transported into the dendritic processes of neurons? Examination of the intracellular localization of mRNA encoding constitutively expressed Hsc70 protein demonstrated that it is localized to the cytoplasm of neuronal cells bodies in unstressed animals (Foster and Brown, 1996). Following a physiological relevant increase in body temperature, transport of hsc70 mRNA into dendritic processes was apparent in a range of neuronal cell types. These neuronal cell types did not induce hsp70 mRNA after hyperthermia, however hsp70 mRNA was strongly induced in glial cells and transported into cellular processes of these glial cells (Foster and Brown, 1996). The localization of hsc70 mRNA and hsp70 mRNA in the cellular processes of neural cells could provide a mechanism for local control of the synthesis of Hsps in cellular compartments, including the synapse, that are remote from the cell body.

HEAT SHOCK PROTEINS AND PROTEIN MISFOLDING

Neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) have been termed 'protein misfolding disorders' (Selkoe, 2004; Muchowski and Wacker, 2005; Brown, 2007; Haass and Selkoe, 2007). They are characterized by accumulation of intracellular and extracellular protein aggregates, disruption of synaptic function and selective neuronal loss in the nervous system. These neurodegenerative diseases differ widely in frequency and impact different classes of neurons, however, increasing evidence supports the view that they exhibit common molecular mechanisms associated with

protein misfolding and aggregation (Forman et al., 2004; Ross and Poirier, 2004; Selkoe, 2004; Muchowski and Wacker, 2005). Recent evidence suggests that soluble oligomers of misfolded proteins interfere with synaptic function in Alzheimer's disease and other neurodegenerative disorders (Haass and Selkoe, 2007).

Manipulation of the cellular stress response involving induction of Hsps offers a therapeutic strategy to counter conformational changes in neural proteins that trigger cascades resulting in neurodegenerative diseases (Sherman and Goldberg, 2001; Meriin and Sherman, 2005; Muchowski and Wacker, 2005; Westerheide and Morimoto, 2005; Brown, 2007). Hsps are protein repair agents that provide a line of defense against misfolded aggregation-prone proteins (Muchowski and Wacker, 2005). Animal models of neurodegenerative diseases have demonstrated the beneficial effects of upregulation of Hsps on disease progression (Kieran et al., 2004; Kalmar et al., 2005; Muchowski and Wacker, 2005). Hsp70 overexpression has been reported to functionally protect synapses at the level of neurotransmission (Karunanithi et al., 2002) and synaptic function is disrupted in neurons during disease progression (Li et al., 2000, 2003; Lee et al., 2003; Smith et al., 2005a, b; Cummings et al., 2007; Haass and Selkoe, 2007). This has led to the quest for pharmacological agents that can induce the heat shock response in the nervous system as a therapeutic approach to counter neurodegenerative diseases. Upregulation of a combination of Hsps, rather than a single Hsp, will likely yield added benefits (Jana et al., 2000; Patel et al., 2005). It has been suggested that the finely balanced, concerted action of a set of Hsps could best be achieved by the development of agents that target activation of heat shock transcription factor 1 (HSF1), the master regulator of stress-inducible genes (Westerheide and Morimoto, 2005; Zourlidou et al., 2007).

A collaborative drug screen aimed at identifying candidates from a panel of existing drugs has identified celastrol as a potential neuroprotective agent (Abbott, 2002; Heemskerk et al., 2002). The screen utilized tissue culture assays to score drugs on their ability to suppress aspects associated with neurodegenerative diseases such as protein aggregation. Celastrol has been used in traditional Chinese medicine to treat various ailments such as inflammation (Pinna et al., 2004) and rheumatoid arthritis (Tao et al., 2002). This compound exhibits neuroprotective properties in animal models of neurodegenerative diseases such as Parkinson's disease (Cleren et al., 2005), ALS, (Kiaei et al., 2005), and Huntington's disease (Cleren et al., 2005; Wang et al., 2005). Celastrol has been shown to activate heat shock transcriptional factor HSF1 in undifferentiated neuroblastoma cells (Westerheide et al., 2004).

Recent studies have demonstrated that celastrol induces a set of Hsps in differentiated neurons grown in tissue culture (Chow and Brown, 2007). Our current work indicates that these celastrol-induced Hsps are transported down neuronal processes towards synaptic termini. The differentiation status of neurons is of particular importance because differentiated neurons in both in vivo and in vitro systems have been reported to be refractory to Hsp induction following conventional heat shock (Manzerra et al., 1993; Foster et al., 1995; Dwyer et al., 1996; Marcuccilli

et al., 1996; Hatayama et al., 1997; Batulan et al., 2003). The celastrol experiments were carried out on human and rodent differentiated neurons in order to explore species-specific differences in Hsp induction patterns that might influence the translation of observations on animal-based models of neurodegenerative diseases to the actual human condition. This led to the finding that celastrol induced a wider set of potentially neuroprotective Hsps, including Hsp70B', in differentiated human neurons compared to rodent neurons (Chow and Brown, 2007).

The human genome includes members of the Hsp70 multigene family, such as Hsp70B', that are not present in the genomes of rodents (Parsian et al., 2000; Noonan et al., 2007). The Hsp70B' gene arose after the divergence of rodents and humans and hence is not present in animal models of neurodegenerative diseases as a potential beneficial factor to combat misfolded aggregation prone-proteins. The Hsp70B' protein and stress-inducible Hsp70 share 84% sequence identify, however, differences in the substrate binding pocket and activation profiles may confer Hsp70B' with a distinct cellular role (Noonan et al., 2007). Hsp70B' has not been studied in the field of human neurodegenerative diseases. We are presently investigating the binding partners and potential neuroprotective properties of Hsp70B', in addition to determining whether it localizes to synaptic termini and protects synapses from stressful stimuli. Celastrol is a promising candidate as a therapeutic agent to counter neurodegenerative diseases with the attractive feature of upregulating a wider set of Hsps, including Hsp70B', in differentiated human neurons compared to rodent neurons (Chow and Brown, 2007).

NEURONAL EXPRESSION OF CONSTITUTIVE HEAT SHOCK PROTEINS AND FREQUENCY OF NEURODEGENERATIVE DISEASES

Constitutively expressed Hsc70 is enriched in the mammalian nervous system relative to non-neural tissue and is present at high levels in neuronal cell bodies (Manzerra et al., 1993, 1997; Manzerra and Brown, 1996). Following thermal stress, Hsc70 is enhanced in synapse-rich areas of the brain where it could play roles in synaptic protective mechanisms (Chen and Brown, 2007a). Levels of Hsc70 have been compared in different classes of neurons that are affected in different neurodegenerative diseases (Chen and Brown, 2007b). Motor neurons in the spinal cord impacted in a low frequency disease such as ALS, demonstrated very high levels of Hsc70, whereas neurons in the hippocampus and entorhinal cortex affected in a high frequency disease such as Alzheimer's, showed comparatively low levels of Hsc70. Intermediate levels of Hsc70 were apparent in neurons of the substantia nigra that are impacted in an intermediate frequency disease such as Parkinson's disease. The differing levels of constitutively expressed Hsc70 in different neuronal populations may confer a variable buffering capacity against protein misfolding disorders that correlates with the relative frequency of these diseases in the human population (Chen and Brown, 2007b). Variable levels of Hsc70 could be present at synaptic termini in the different neuronal cell types, resulting in variability in the degree of

potential synaptic protection. Neurons may rely on their constitutive levels of Hsc70 as a 'pre-protection' mechanism for defense against aggregation-prone misfolded proteins that accumulate following stressful stimuli or during of the progression of neurodegenerative diseases.

CONCLUDING REMARKS

Synapses are critical sites of information transfer in the nervous system and their functionality must be preserved under stressful conditions to prevent communication breakdown. Heat shock proteins localize to components of the synapse and play roles in neurotransmission events including the functional protection of synapses against stressful stimuli. Synaptic dysfunction is a feature of neurodegenerative disorders and manipulation of the heat shock response is a potential strategy to mitigate disruption of synaptic function that occurs during disease progression.

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PART III

EXTRACELLULAR HEAT SHOCK PROTEINS AND THE NERVOUS SYSTEM

CHAPTER 13

RELEASE OF HEAT SHOCK PROTEINS AND THEIR EFFECTS WHEN IN THE EXTRACELLULAR SPACE IN THE NERVOUS SYSTEM

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Abstract: The ability of heat shock proteins (Hsps) to make cells more resistant to most types of metabolic stress has great implications for all post-mitotic cells, especially those of the nervous system. Preventing the loss of neurons is a more parsimonious approach to treatment of injury and disease than is replacement because of the difficulty in reconstructing the complex architecture of the nervous system, the basis for its function storage of information. The discoveries that the 70 kD Hsps are released and that neurons can take them up from the extracellular fluid provides a rationale to investigate how to use them to rescue injured neurons teetering between life and death. We present some of the history behind those discoveries and review the current understanding of the release and uptake of the 70 kD Hsps, discussing the distinct significance these observations have for neurons and some hypotheses about how extracellular Hsps protect neurons from potentially lethal injuries

Keywords: Exogenous; extracellular; Hsp70; Hsc70; neuronal injury; apoptosis

Abbreviations: bov, bovine; exo-Hsps, exogenously administered and/or extracellular Hsps; Hsc70, constitutive form of 70 kD Hsp; Hsp, all heat shock proteins; Hsp70, inducible form of 70 kD Hsp; Hsp/c70, both inducible and constitutive forms of 70 kD Hsps; hum, human; PC12 cells, rat phaeochromocytoma neuron-like cell line; recom, recombinant protein

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BACKGROUND AND HISTORY OF EXTRACELLULAR HSPS

For more than two decades after the initial discovery of the heat shock protein response by Ritossa (1962, 1963, 1996), the 70 kD heat shock protein and other members of this group discovered subsequently, all were considered to be intracellular proteins. This assumption arose from other discoveries in cell biology during that period showing that proteins destined to be packaged into secretory vesicles all were synthesized via the rough endoplasm reticulum and had a similar peptide sequence at their amino termini, the signal sequence, that targeted them to secretory vesicles (Stroud and Walter, 1999). However, beginning in the mid-1980s, reports began to be published suggesting that Hsp70 could be released, that it was present in extracellular fluid, and that it could be taken up from that compartment (reviewed in Tytell, 2005). Then, with the discovery that Hsp70 was present in normal human blood (Pockley et al., 1998), a large number of publications have appeared documenting the presence of Hsp70 in blood and other extracellular fluids like the cerebrospinal fluid (Steensberg et al., 2006). However, because this protein is found in all cells and tissues, which ones are the primary sources of extracellular Hsp70 remains to be determined. Nonetheless, there is no longer any doubt that extracellular Hsp70 has functional significance for both the immune system and tolerance to metabolic stress.

HOW ARE HSPS RELEASED FROM CELLS?

This issue is addressed in detail in Chapter 1 of the first of the volumes in this series (Asea, 2007), so only a few pertinent points will be discussed here. Asea (2007) makes the point that all of the Hsps are known to be intracellular proteins and lack the signal peptide that would target them to secretory vesicles for conventional release via secretory vesicles. His Figure 1 summarizes the two other potential release processes: (1) unregulated leakage from cells whose membranes are damaged because of physical disruption subsequent to trauma or necrosis or (2) regulated release of membrane-bounded vesicles called exosomes. The blebbing of small membrane-bounded vesicles from the surfaces of cells has been recognized for a long time in a few types of cells known to release material by the process called apocrine secretion. These are the secretory epithelial cells of the mammary gland, apocrine glands in the skin, ciliary glands of the eyelid, and the wax-producing cells of the external ear canal. More recently, exosome production has been observed in a wide variety of cells (de Gassart et al., 2003; Fevrier and Raposo, 2004). The significance of this process is that it provides a means for a cell to release small volumes of its cytoplasmic constituents. Since Hsps are relatively abundant cytoplasmic constituents, with Hsp70 comprising 0.29% of total brain protein (Gutierrez and Guerriero, 1995) and Hsc70 being 2%–3% of total spinal cord protein (Aquino et al., 1993), exosomes released by neurons and glial will contain Hsps. In fact, they also can contain functional mRNAs (Valadi et al., 2007), implying that the cell receiving the exosomes potentially could manufacture more of the protein than

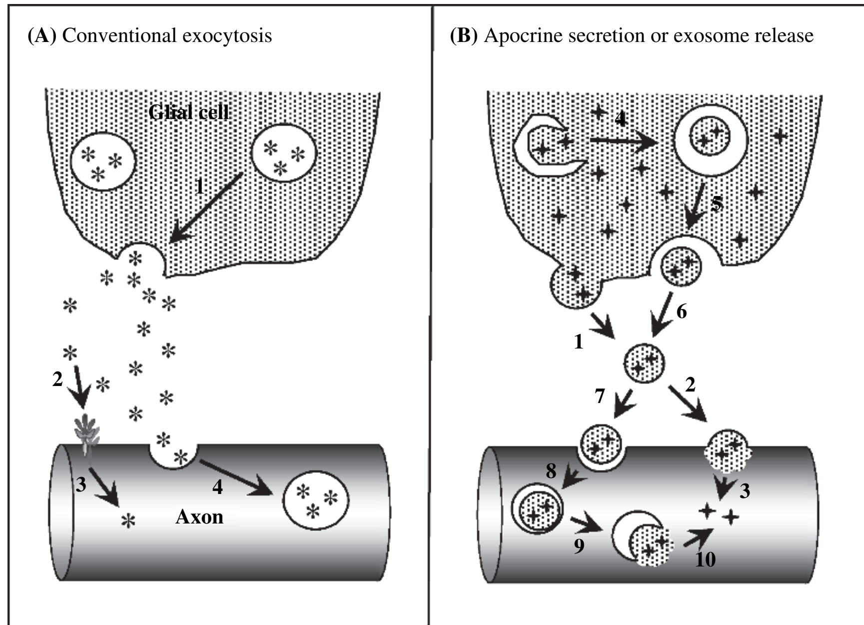

Figure 1. Potential mechanisms for the release and uptake of Hsps. For the purposes of these examples, release occurs from a glial cell and uptake by an axon, but these hypothetical transfers could occur between any groups of cells. **Panel A** presumes that an unknown mechanism allows Hsp70 or Hsc70, represented by asterisks, to become concentrated in secretory vesicles. (Hereafter, the proteins will be jointly designated as Hsp/c70.). Arrow 1 indicates conventional exocytosis of the Hsp/c70, so that it is present in the extracellular fluid. Arrow 2 indicates interaction of the exo-Hsp/c70 with the axonal membrane, reflected by a change in its shape. Arrow 3 indicates diffusion of Hsp/c70 through the plasma membrane by an unknown mechanism so that it is in the cytoplasm of the axon and is free to interact with other cytoplasmic components as endogenously synthesized Hsp/c70 would. Arrow 4 indicates uptake of Hsp/c70 by conventional pinocytosis, after which it is present in endocytotic vesicles. From there it may diffuse through the endosomal membrane to enter the cytoplasm or it may remain inside as the endosome cycles through the endolysosomal pathway of the neuron. How that process might affect stress tolerance is not known. **Panel B** depicts two ways that exosomes containing glial cytoplasmic Hsp/c70 (4-pointed stars) could wind up in the cytoplasm of the axon. On the left side, arrow 1 indicates conventional apocrine secretion, in which small vesicles containing a mixture of cytoplasmic constituents bud off the cell surface . This process is the way that milk is secreted by the mammary gland and mucus by goblet cells lining the intestinal tract. Alternatively, some of the cytoplasm of the donor may be enclosed within a vesicle by the well-known process of autophagy (arrow 4). Autophagy's role in the production of exosomes is discussed in the text. The multivesicular body (small vesicles within a larger vesicle) resulting from autophagy can then be released by the glial cell via exocytosis (arrows 5 and 6). Thus, both apocrine secretion and exosome release yield the same result, a membrane enclosed vesicle containing cytoplasmic constituents in the extracellular space. The released vesicle then may interact with the axon in either of two hypothetical ways. It may fuse with the plasmalemma of the recipient cell, releasing its contents, including Hsp/c70, into the neuron's cytoplasm (indicated by arrows 2 and 3). Alternatively, the released vesicle may be phagocytosed by the neuron, forming another multivesicular body. Then the inner vesicle membrane may fuse with the outer vesicle membrane in a form of intracellular exocytosis, releasing the Hsp/c70 and other cytoplasmic constituents from the glial cell into the cytoplasm of the neuron (indicated by arrows 8–10). (This figure modified from Tytell (2005)

what is contained in the exosomes. It seems that this alternative release process may be a common means by which cells release Hsps, as exosomes released from maturing red blood cells and from normal and hyperthermically stressed blood-borne mononuclear cells contain Hsc- or Hsp70 (Mathew et al., 1995; Geminard et al., 2001; Lancaster and Febbraio, 2005).This possibility is especially important for axons, as explained in the next section.

Though exosomes may account for Hsp release by cells in many situations, recent work shows that secretion via conventional secretory vesicles is also an option. Evdonin et al. (2006) found that two types of transformed human keratinocyte cells released Hsp70 from granules that included the secretory vesicle marker, chromogranin A and that the secretion was blocked by an inhibitor of conventional exocytosis, brefeldin A. However, this paper does not address the mechanism by which Hsp70 becomes concentrated in secretory vesicles, despite its lack of a signal sequence. Since this phenomenon has not been reported in other cells, it remains to be determined if it is specific to these keratinocytes.

If exosome-based Hsp70 release is its primary route for leaving a cell, one still needs to explain how it becomes a soluble protein in the blood or other extracellular fluids. The possibility that it can diffuse through lipid bilayers, though unexpected for a 70 kD protein, is supported by a number of observations suggesting that Hsp70 is an amphipathic protein. The first hint that this might be the case was in the observation by Guidon and Hightower that both Hsc70 and Hsp70 purified from rat brain included nonesterified fatty acids (Guidon and Hightower, 1986a, b). A few years later, Alder et al. reported the surprising observation that Hsp70 added to a solution on one side of an artificial lipid bilayer formed an ion conducting channel in that bilayer (Alder et al., 1990). Despite these intriguing early observations, six years passed before this property of Hsp70 was again examined. Negulyaev et al. (1996) found that potassium channel activity was increased in a cultured monocyte cell line treated with a mixture of soluble, extracellular Hsc- and Hsp70. Then Arispe and co-workers, using artificial lipid bilayers and liposomes, found that soluble Hsc- and Hsp70 formed potassium-conducting channels and promoted the spontaneous fusion of liposomes (Arispe and De Maio, 2000; Arispe et al., 2002). Both these activities of the two proteins were modulated by ATP and ADP, though in different ways. ATP increased the frequency of channel opening, whereas ADP inhibited it. Conversely, ADP in low concentration promoted liposome fusion induced by Hsc70, but ATP inhibited it. For Hsp70, both nucleotides inhibited its liposome-fusing activity. These results are the only ones that have shown clear distinctions in functional activities of the two forms of the protein. What they imply for the in vivo functions of Hsc- and Hsp70 remains to be examined.

The above observations leave us with several intriguing possibilities concerning the release, uptake and effects of extracellular70 kD Hsps. First, there is no doubt that the 70 kD Hsps can be released from cells, but three routes are suggested, exosomes, conventional secretory vesicles, and diffusion through the plasmalemma (see Figure 1). Once in the extracellular space, the proteins can interact with the membranes of cells, possibly altering ion conductance, cell-to-cell interactions,

and/or diffusing through the membrane into the cytoplasm. Each of these events will have distinct functional outcomes for the cells with which the 70 kD Hsps are interacting and it is likely that the specific events triggered by the Hsps will be both context and cell-type dependent.

THE SPECIAL SIGNIFICANCE OF HSP RELEASE AND UPTAKE IN THE NERVOUS SYSTEM

In the nervous system, neurons, especially large ones with long axons (i.e., tens or hundreds of times greater in length than the cell body diameter), have a refractory stress response, failing to turn on the stress protein synthesis system after stresses such as hyperthermia or ischemia (Mathur et al., 1994; Voisin et al., 1996; Batulan et al., 2003; Tidwell et al., 2004; Robinson et al., 2005). Thus, the greater vulnerability of neurons to metabolic stress may be partly a consequence of their poor Hsp response. Another reason for the stress vulnerability of neurons with long axons arises from the fact that axons lack the full complement of protein synthetic machinery found in the cell body. For that reason, they are largely dependent on the latter, as well as the system of axonal transport, for protein renewal (Gallant, 2000; Brown, 2003). This fact leads to one more handicap for the neuronal stress protein response that is specific to the axon and is typically overlooked. Hsp90, and -70 are transported from the neuronal cell body towards the axon terminal at a slow rate, about 2 mm/day (Clark and Brown, 1985; Waegh and Brady, 1989; Black et al., 1991; Bernstein et al., 2001). Hsp25 axonal transport rate has been estimated to be faster, about 20 mm/day (Murashov et al., 1998), but is still about 10-fold slower than the 200 mm/day rate of fast axonal transport (Cyr and Brady, 1992). These slow transport rates mean that, even if a neuron could respond robustly to an injury to part of its axon several centimeters away, it would take at least 24 hours for the newly synthesized stress-induced Hsps to reach the site of injury, clearly too long to be of use. However, these neurons and their axons and dendrites are surrounded by glial cells that do respond in the typical way to metabolic stress, showing prominent increases in Hsp content (Manzerra et al., 1993; Koroshetz and Bonventre, 1994; Voisin et al., 1996; Krueger et al., 1999). Thus, if glial cells can release exosomes containing either or both Hsps and their mRNAs, then they can serve as a local source of additional protein or make it possible for axons (and dendrites as well) to synthesize the protein via translation of the message at any points along their lengths, compensating for the problem of slow transport over long distances.

In fact, glia to axon transfer of Hsp70 in the giant axon of the squid was the first observation suggesting that intact, normally functioning cells could release Hsps and that they could be taken up by neighboring cells (Tytell et al., 1986). That observation was replicated in another invertebrate with large axons, the crayfish (Sheller et al., 1998), but for vertebrate models, the evidence is more limited. Hsp70 was observed to be synthesized in the glial sheath of the severed frog sciatic nerve, taken up by the sciatic nerve axons and retrogradely transported (Edbladh

et al., 1994). In the rat cerebellum, hyperthermia strongly stimulated the production of Hsp27 and -32 in Bergman glial cells, after which those Hsps were also localized in Purkinje cell synaptic terminals (Bechtold and Brown, 2000); the relatively short interval between these events suggested that the glia were the source of the Hsps, not the neuron cell bodies. This possibility is supported by the observations that mammalian glial cells in culture release Hsp70 (Guzhova et al., 2001; Taylor et al., 2007). Furthermore, the Hsp70 released from astrocytes is in the form of exosomes (Taylor et al., 2007). As yet, no one has shown directly in the mammalian nervous system that glia-derived Hsps are taken up by neurons and render them more resistant to metabolic stress. However, it is likely that this uptake occurs, since our observations and those of others show clearly that soluble extracellular Hsc- and Hsp70 can be taken up by neurons (Houenou et al., 1996; Guzhova et al., 2001; Yu et al., 2001; Tidwell et al., 2004; Tytell, 2005; Novoselova et al., 2005; Robinson et al., 2005; Robinson et al., 2007).

NEUROPROTECTIVE AND NEURODEGENERATIVE EFFECTS OF EXTRACELLULAR, EXOGENOUS HSPS

In the 1980s, conventional wisdom held that a protein the size of Hsp70 could not pass through the cell membrane without the involvement of a pore or membrane transporter. Despite this bias, we and a few others were intrigued by the observed release and cell-to-cell transfer of the protein (Tytell et al., 1986; Hightower and Guidon, 1989). Additionally, the strong association between Hsp70 content and neuronal survival shown by the many stress-preconditioning papers published from the late 1970s on, prompted a few researchers to try administering the protein itself to the injured neural tissue or cells. This unconventional approach was motivated by the goal of developing therapeutic uses of the potent survival-promoting activity of Hsps, since the preconditioning model was not applicable for the treatment of unpredictable, acute injury of the nervous system. The results obtained to date confirm that extracellular Hsc- and Hsp70 can promote survival and function of neurons subjected to a wide variety of stressful conditions, including hyperthermia, lack of neurotrophic factors, ischemia, free radical damage, and physical trauma; these are summarized in Table 1. Additionally, one report made the novel observation that Hsp70, -90, and -32 all stimulated the activity of microglia, promoting phagocytosis of β-amyloid, whereas Hsp27 did not (Kakimura et al., 2002). Regarding Hsp27, the only other report in which this member of the group was tested also showed it did not have a protective effect in concussive brain injury, though it did alter the K^+ channel function, but in a negative way (Armstead and Hecker, 2005). One other observation suggests that exo-Hsps also may be useful in treatment of chronic neurodegenerative conditions. Human neuroblastoma cells transfected with the Huntington's disease polyglutamine-repeat gene were protected by addition of a mixture of Hsc- and Hsp70 to the culture medium, the treated cells showing fewer and smaller polyglutamine protein aggregates (Novoselova et al., 2005). This effect is consistent with the well-known protein folding functions of the Hsps and indicates that the exo-Hsps must have entered the cytoplasm

Table 1. Summary of effects of exo-Hsps on neural and glial cells and tissues in vitro and in vivo

Model used	Hsps used	Effects	Ref.
In vitro			
Neuroblastoma cells	bov skeletal muscle Hsc/Hsp70	Increased survival after heat stress and resistance to drug-induced apoptosis	Guzhova et al. (2001)
Rat microglia	hum-Hsp90, recom hum-Hsp70, recom rat-Hsp32, recom hum-Hsp27	All except Hsp27 stimulated cytokine production and phagocytosis of β-amyloid	Kakimura et al. (2002)
Rat brain slice	recom hum-Hsp70	Synaptic transmission preserved during hyperthermia	Kelty et al. (2002)
Rat cortical neurons	recom bov-Hsc70	Inhibition of ischemia-induced Hsp70 increase	McLaughlin et al. (2003)
PC12 cells	recom bov-Hsc70, recom hum-Hsp70	Increased apoptosis	Arispe et al. (2004)
Rat olfactory cortex anoxia	bov skeletal muscle Hsc/Hsp70	Preservation of glutaminergic neurotransmission	Mokrushin et al. (2004)
Rat cortical neurons	bov skeletal muscle Hsc/Hsp70	Preservation of glutaminergic neurotransmission	Mokrushin et al. (2005)
Human neuroblastoma cells transfected with Huntington gene	bov skeletal muscle Hsc/Hsp70	Promoted survival by reducing poly-Q protein inclusions	Novoselova et al. (2005)
Chick embryonic motor neurons	recom bov-Hsc70 or hum-Hsp70	Promoted survival in absence of trophic factors	Robinson et al. (2005)
Chick embryonic motor neurons	Full length & substrate-binding portion of hum-Hsc70	Promoted survival after free radical damage	Robinson et al. (2007)
In vivo			
Rat retina	bov skeletal muscle Hsc/Hsp70	Reduction of light-induced photoreceptor degeneration	Yu et al. (2001)
Mouse sciatic nerve	bov brain Hsc70, bov skeletal muscle Hsc/Hsp70, recom bov- Hsc70, recom hum-Hsp70	Reduction of axotomy-induced apoptosis of dorsal root ganglion neurons and spinal motor neurons	Houenou et al. (1996) and Tidwell et al. (2004)
Pig brain, neonatal	recom hum Hsp70 and Hsp27	Preservation of post-injury K^+ channel-related cerebrovasodilation by Hsp70, but not Hsp27, after concussive injury	Armstead and Hecker (2005)
Chicken embryo	hum recom Hsc70	Prevention of developmental programmed motor neuron death	Robinson et al. (2005)

(In chronological order, then alphabetically by author)

of the neurons. All these observations suggest that Hsc- and Hsp70 present in the extracellular fluid surrounding neurons are as broadly neuroprotective as the endogenously synthesized proteins have been show to be in preconditioning and transfection-induced overexpression experiments. It is also apparent that Hsp90 and -32, like Hsp/c70, may have neuroprotective activities when present in the extracellular space, but, except for one observation of the release of Hsp90α by cultured vascular smooth muscle cells (Liao et al., 2000), little is known about whether these two Hsps occur naturally outside the cell and how they interact with cells from the extracellular space.

In only one case has extracellular Hsp/c70 been found to cause neuronal death. Arispe et al. (2004) showed that as little as 0.3 μg/ml of Hsc70 or Hsp70 added to the culture medium of PC12 cells caused a significant increase in cell death. This unexpected toxicity was a result of the presence of phosphatidylserine on the extracellular side of the plasma membrane of PC12 cells, a normal feature for them, but, for other neurons, it occurs only at the beginning of apoptosis. They found also that the toxicity was increased when either ATP or ADP was added with Hsc70, whereas only ATP increased the toxicity of Hsp70, a functional distinction between the two proteins that they had noted earlier in their interactions with liposomes (Arispe et al., 2002). These results, they suggested, may be caused by the formation of ion channels in the plasma membrane and may explain other observations that overexpression of Hsp/c70 can be toxic (Feder et al., 1992), a possibility that must be taken into account when considering neuroprotective strategies using the Hsps.

ENDOGENOUS SOURCES OF EXTRACELLULAR HSPS

As mentioned at the beginning of this chapter, the case for the physiological relevance of extracellular Hsps received a major boost when Pockley and coworkers showed that soluble Hsp70 was present in normal human blood (Pockley et al., 1998). That observation prompted many more studies that have found changes in blood levels of Hsp70 with various types of injuries, diseases, and even after vigorous exercise (Giraldo et al., 1999; Walsh et al., 2001; Pockley et al., 2002; Febbraio et al., 2002; Pittet et al., 2002; Zhu et al., 2003; Campisi and Fleshner, 2003; Njemini et al., 2003; Dybdahl et al., 2004; Kimura et al., 2004; Lancaster et al., 2004; Fleshner and Johnson, 2005; da Rocha et al., 2005; Johnson et al., 2005; Marshall et al., 2006). These numerous observations have not, unfortunately, provided a coherent explanation of the functional significance of circulating Hsp70 and what the alterations mean. There is some evidence that the protein may enter the bloodstream simply as a result of cell damage or death after injury, analogous to the appearance in the blood of cardiac cell cytoplasmic components, like creatine kinase and troponin, after a heart attack. For example, Hsp70 increased in the blood as a function of time on a heart bypass pump during open-heart surgery (Dybdahl et al., 2004) or the extent of surgical trauma during liver resection (Kimura et al., 2004). From this perspective, the finding of a negative correlation between blood Hsp70 concentration and recovery after traumatic brain injury is not surprising (da Rocha et al., 2005). However, the opposite also has been reported (Pittet

et al., 2002). Conflicting results exist as well for the functional impact of circulating Hsp70. Asea and colleagues found extracellular Hsp70 to have pro-inflammatory effects on monocytes (Asea et al., 2000) and a recent review by Fleshner proposed that an increase in circulating Hsp70 serves as a "danger signal" and stimulates the innate immune response (Fleshner and Johnson, 2005). Conversely, others found evidence for anti-inflammatory effects of extracellular Hsp70 (Yoo et al., 2000; van Eden et al., 2005), even in relation to microglial cell activation in the brain (Yenari et al., 2005). The best explanation for these opposing reports is that the biological impact of extracellular Hsp70 must be highly context-and concentration-dependent, meaning that it is contingent on when, where, and how much of it is present. Details, recent findings, and current hypotheses on the immunomodulatory functions of extracellular Hsp70 can be found in reviews by Asea and Calderwood in volume 1 of this series (Asea, 2007; Calderwood et al., 2007).

Another event that causes temporary increases in blood Hsp70 concentrations is exercise, a context that seems very different than the acute injury and infection-related events described above, but may have some physiological effects in common. For example, exercise can cause microscopic damage to skeletal muscle (Miyake and McNeil, 2003), which may account, in part, for the exercise-induced increase in skeletal muscle Hsps (Thompson et al., 2003), and it is well known to alter the responsiveness of the immune system (Nieman, 2007). Thus, it is not surprising that Febbraio and colleagues have shown that vigorous exercise in healthy individuals acutely elevates blood Hsp70 concentration (Walsh et al., 2001). Some of this Hsp70 may be derived from specific organs, such as the liver (Febbraio et al., 2002) and the brain (Lancaster et al., 2004). From subsequent in vitro studies, they provide evidence that the Hsp70 is released via exosomes (Lancaster and Febbraio, 2005), but this creates a puzzle, especially with respect to release from the brain. How would Hsp70-containing exosomes released from the brain parenchyma enter the bloodstream, given the blood-brain barrier? Other work shows that a primary trigger for Hsp70 release into the blood is the activation of α1-adrenergic receptors (Johnson et al., 2005; Johnson and Fleshner, 2006) and the authors suggest that the exosome is the vehicle for that release. However, as described previously for the brain, it is not known how exosomes released from cells would get into the bloodstream unless they were produced by cells in direct contact with the blood. Thus, the simplest explanation for the phenomenon is that the blood vessels themselves are the source of the blood-borne Hsp70. If that is the case, then the apparent release of Hsp70 into the blood by the brain and liver may be accounted for by the high vascularity of those organs. Much work needs to be done to sort out these questions.

HOW DO EXTRACELLULAR 70 KD HSPS PROMOTE NEURONAL SURVIVAL?

In all of the injury models in which exo-Hsc70 or Hsp70 have been shown to promote neuron survival, apoptosis is the primary mode of neuronal death. Thus, our studies of the details of the neuroprotective effects of the protein have used

a well-established model of neuron apoptosis, the primary embryonic chick spinal motoneuron (Milligan et al., 1994). When these neurons are prepared for culturing, they require trophic factors in order to survive and extend neurites. The optimal mixture of trophic factors is provided by an extract of chick skeletal muscle (MEx) added to the culture medium. Without MEx, the intracellular signals leading to apoptosis are initiated by 16 hours, committing the neurons to die (Li et al., 2001). Hsc70, Hsp70, or a mixture of both, can be substituted for MEx to promote neuron survival, but must be added to the culture medium within the first 12 hours; the Hsps are ineffective after that time (Robinson et al., 2005). This interval precedes the first detectable step in motoneuron apoptosis, the translocation of Bax from the cytoplasm to organelles (Li et al., 2001). Therefore, the proteins, jointly termed exo-Hsp/c70, must be inhibiting signals at or before the decrease in mitochondrial membrane potential and release of cytochrome C.

The possibility that exo-Hsp/c70 inhibits early kinase-mediated events in the commitment of a cell to undergo apoptosis implies that this function may be separate from its well-known protein folding role. This speculation was raised in a review by Gabai and Sherman (2002) and, in support of it, they referred to earlier, intriguing reports that mutant Hsp72 lacking ATPase activity still prevented heat-induced death of fibroblasts transfected to express such mutant proteins; it also inhibited c-Jun NH_2-terminal kinase (JNK) (Volloch et al., 1999; Yaglom et al., 1999; Park et al., 2001). We examined the same question in oxidatively stressed cultured motor neurons by pretreating them with the substrate-binding domain of Hsc70 (Robinson et al., 2007). Not only was it neuroprotective, it was effective at $0.1\,\mu M$, which was one-tenth the concentration of intact Hsc70 needed to achieve a similar effect. The ATPase domain, in contrast, had no protective effects at the same concentrations. Changes in kinase activity were not monitored in these experiments, but we did assess mitochondrial membrane potential and found that it was preserved by pretreatment with both the intact Hsc70 and the substrate-binding domain, but not by the ATPase domain. These results support the possibility that the extracellular proteins enter the neurons and can interact with mitochondria in protecting them from oxidative stress. We also found that the substrate-binding domain could rescue neurons when added immediately after, rather then before, exposure to peroxide-induced oxidative stress (unpublished observations), but this required a higher concentration, $1\,\mu M$, then when it was added 4 hours before the peroxide. Why the post-injury treatment required a 10-fold higher concentration of the substrate-binding domain to produce significant protection is not known. Although it is clear that extracellular Hsp/c70 can enter cells from the extracellular space (Houenou et al., 1996; Guzhova et al., 1998; Fujihara and Nadler, 1999; Guzhova et al., 2001; Yu et al., 2001; Tidwell et al., 2004; Novoselova et al., 2005; Robinson et al., 2005) and affect elements of several signal transduction pathways that improve neuronal survival, there is no evidence as yet for the existence of any membrane receptor type of interaction, as there is for cells of the immune system (Asea et al., 2000, 2002; Asea, 2005) (see also volume I of this series (Asea and De Maio, 2007)). However, other types of interactions

with neuronal membranes must be considered in light of the observations presented earlier in this review on the membrane interactions of Hsp/c70. Vigh and co-workers have for some time proposed that plasma membrane fluidity changes may be a trigger for induction of the Hsp response (Vigh et al., 1998; Balogh et al., 2005). Furthermore, Hsp70 has been found to be associated with lipid rafts in neuronal tissue (Chen et al., 2005) and to stabilize lysosomal membranes in stressed tumor cells and transformed fibroblasts (Nylandsted et al., 2004). Thus, further study is needed on the possibility that stabilization of the plasma membrane and the membranes of intracellular organelles is one of the actions of exo-Hsp/c70.

In addition to direct effects on injured neurons, one needs to consider that in vivo, Hsp/c70 and other Hsps may promote neuronal survival by eliciting beneficial responses of the surrounding glial and other non-neuronal cells. This possibility is suggested by the previously mentioned stimulation of microglial phagocytosis of amyloid by Hsp90, -70, and -32 (Kakimura et al., 2002). It serves as a reminder that the potential positive and negative effects of exogenous Hsps will require an understanding of how they may alter the functions and interactions of all cells in the nervous system, not just neurons.

CONCLUSIONS

There is extensive evidence that the 70 kD Hsps are normal constituents of the extracellular fluid and that they vary with physiological stress. That fact, together with the growing number of reports that administration of the proteins enhances neuronal survival under a wide variety of stressful conditions, makes the potential for the development of therapeutic uses of the proteins very likely. The focus of much of the research on traumatic injury and degenerative diseases of the nervous system has been and remains on regeneration and replacement. However, we suggest that the potential for Hsps to rescue injured neurons offers an approach that is more expedient and achievable sooner. One of the key issues that needs to be addressed to begin translating this research into practical clinical treatments is to determine the interplay between the immunomodulatory and cytoprotective effects of the Hsps because inflammatory responses occurring after acute nervous system injury and in many chronic neurodegenerative diseases cause much greater loss of neurons than those directly affected by the injury or disease process.

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The therapeutic potential of Hsp70 received further support recently in the report by Gifondorwa et al. that regular, repeated intraperitoneal injections of human recombinant Hsp70 in a transgenic mouse model of amyotropic lateral sclerosis inhibited the onset of paralysis and extended lifespan (Gifondorwa, D. J., M. B. Robinson, C. D. Hayes, A. R. Taylor, D. M. Prevette, R. W. Oppenheim, J. Caress, and C. E. Milligan. Exogenous delivery of heat shock protein 70 increases lifespan in a mouse model of amyotrophic lateral sclerosis (2007) J Neurosci 27 13173–13180).

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CHAPTER 14

SILENCING OF METASTASIS-ASSOCIATED GENE 1 (MTA1) STIMULATES HSP70 CELLULAR RELEASE AND NEURITE EXTENSION IN NEUROBLASTOMA CELLS

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Abstract: The MTA family has recently been demonstrated to play an important role in influencing the status of chromatin remodeling due to its presence within the nucleosome remodeling histone deacetylase complex (NuRD complex). In addition, MTA is associated with metastatic cancer tissues such as breast, gastric and esophageal carcinoma. In several human metastatic breast cell lines the expression level of MTA is highly upregulated; however, little is known about the role of MTA in neuroblastoma and related disorders. We recently demonstrated that silencing the mta gene in neuroblastoma cells using siRNA specifically directed against the mta1 gene, induces the release of Hsp72 and stimulates neurite extension. This chapter will briefly address the biological significance of silencing the mta1 gene in neuroblastoma cells

Keywords: Chaperokine; heat shock proteins; HSP release; metastasis; MTA1; neuroblastoma; neurodegeneration; neurite extension

Abbreviations: APC, antigen presenting cells; DC, dendritic cells; DRG, dorsal root ganglion; ERK, extracellular signal regulated kinase; *hsp*, heat shock protein gene; HSF, heat shock factor; HSP, heat shock protein; Hsp70, inducible form of the seventy-kilo Dalton heat shock protein; HDAC, histone deacetylage complex; ISS-N1, intronic splicing silencer N1; mta, metastasis-associated gene; MTA, metastasis-associated protein; NGF, nerve growth factor; NK, natural killer; NuRD complex, nucleosome remodeling complex; RA, retinoic acid; RNAi, RNA interference; SMA, spinal muscular atrophy; TSA, trichostatin A; VA, valproic acid

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INTRODUCTION

MTA1 (metastasis-associated protein 1) is a member of a rapidly growing gene family. To date there are three known forms of mta genes: mta1, mta2 and mta3. Recent data suggests that MTA proteins regulate/modulate transcription of certain genes by influencing the status of chromatin remodeling (Kumar et al., 2003). MTA1 was originally discovered as an essential component of the cyclin-dependent kinase (CDK7)-activating kinase (CAK). This complex consists of a catalytic subunit, CDK7, a regulatory subunit cyclin H, and MTA1 (Tassan et al., 1995). The MTA1 protein consists of three major motifs: the N-terminal ZINC finger region, the central coiled-coil region, and the C-terminal cyclin-like region. Initially, two studies demonstrated MTA1 to be a component of nucleosome remodeling histone deacetylase complex (NuRD complex) which is involved in chromatin remodeling (Xue et al., 1998; Zhang et al., 1998); however, the nature of its target or targets remains unidentified (Mazumdar et al., 2001). MTA1 has been demonstrated to be a critical regulator of the metastatic process in both human and rodent mammary tumors (Toh et al., 1994; Debies and Welch, 2001), and more recently in other tumor types as well. Genes known to regulate breast cancer metastasis are currently divided into two categories, these include (1) metastasis activators, including mta1, MEK1, ras, MEK1, proteinases, adhesion molecules, chemoattractants/receptors, autotaxin, PKC, S100A4, RhoC, osteopontin and, (2) metastasis suppressors, including Nm23, E-cadherin, TIMPs, KiSS1, Kai1, Maspin, MKK4, BRMS1. A current model for the mechanism by which MTA1 regulates metastasis is in part by its role in the multiprotein Mi-2-nucleosome remodeling and deacetylating (NURD) complex by controlling the epithelial-tomesenchymal transition (EMT) (Nigg, 1996).

Heat shock proteins (HSP) were originally described for their role as chaperones induced by temperature shock as well as various other kinds of stress including environmental (U.V. radiation, heat shock, heavy metals and amino acids), pathological (bacterial, parasitic infections or fever, inflammation, malignancy or autoimmunity) or physiological stresses (growth factors, cell differentiation, hormonal stimulation or tissue development), that induced a marked increase in intracellular HSP synthesis known as stress response (Lindquist and Craig, 1988). This is achieved by activating the trimerization and nuclear translocation of cytoplasmic heat shock factor-1 (HSF-1) to the heat shock element (HSE) within the nucleus and consequent transcription of HSP. By binding unfolded, misfolded or mutated peptides or proteins and transporting them to the endoplasmic reticulum (ER), HSP prevent potential aggregation of proteins and/or cell death. Within the ER the peptides are released in an ATP-dependent fashion and refolded (Fink, 1999).

Recently, an additional role has been ascribed to HSP as both a cytokine and a chaperone, a chaperokine (for review, see (Asea, 2003, 2004; Asea and DeMaio, 2007)). When found in the extracellular milieu, Hsp70 induces a plethora of immune responses. For example, as early as 2–4 hours post exposure of APC to extracellular Hsp70, there is a significant release of cytokines including TNF-a, IL-1β, IL-6 and IL-12 (Asea et al., 2000b, 2002a), GM-CSF (Srivastava, 2002); nitric oxide, a potent apotogenic mediator (Panjwani et al., 2002); chemokines

including MIP-1, MCP-1 and RANTES (Lehner et al., 2000; Panjwani et al., 2002). This part of the immune response does not require peptide, since both peptide-bearing and non peptide-bearing extracellular Hsp70 is capable of inducing pro-inflammatory cytokine production by APCs (Asea et al., 2000a). However, peptide is required for specific $CD8^+$ CTL responses (Srivastava et al., 1994; Srivastava 2000, 2005). Extracellular Hsp72 induces the DC maturation by augmenting the surface expression of CD40, CD83, CD86 and MHC class II molecules on DC (Basu et al., 2000; Singh-Jasuja et al., 2000; Asea et al., 2002a; Noessner et al., 2002) and migration of DC (Binder et al., 2000) and NK cells (Gastpar et al., 2005).

Taken together, these studies indicate that there is a clear dichotomy now exists that the effects of HSP based on its relative location, intracellular versus extra-cellular, and the target cell it binds to and activates. The upregulation of intra-cellular Hsp70 is generally cytoprotective and induces the cell's anti-apoptotic mechanisms (Jaattela et al., 1998), represses gene expression (Tang et al., 2001), modulates cell cycle progression (Hut et al., 2005) and is anti-inflammatory (Housby et al., 1999). On the other hand, the upregulation of extracellular Hsp70 is generally immuno-stimulatory and stimulates pro-inflammatory cytokine synthesis (Asea et al., 2000c; Asea, 2005), augments chemokine synthesis (Lehner et al., 2000; Panjwani et al., 2002), upregulates co-stimulatory molecules (Asea et al., 2002b; Bausero et al., 2005) and enhances anti-tumor surveillance (Srivastava et al., 1994; Srivastava, 2000, 2005).

Using RNAi technology, we recently demonstrated that silencing the mta1 gene resulted in an enhanced release of Hsp72 and stimulated neurite extension of neurob-lastoma cells. In this chapter, we will briefly discuss the biological significance of silencing the mta1 gene in neuroblastoma cells.

METASTASIS ASSOCIATED GENES (MTA)

MTA1 has been demonstrated to be highly associated with metastasis of cancer (Yaguchi et al., 2005) in metastatic cancer tissues such as breast, gastric and esophageal carcinomas. In several human metastatic breast cell lines the expression levels of MTA1 are highly upregulated (Toh et al., 1994, 1997, 1999). Interestingly, the mta1 gene is also found to be involved in migration and invasion properties of cells. Deeper invasion and higher rate of penetration into lymph nodes have been observed in patients suffering from colorectal and gastric carcinomas overexpressing MTA1 (Toh et al., 1999).

Studies by Nawa and colleagues previously demonstrated that silencing of mta1 gene expression by antisense oligoneucleotides results in the inhibition of growth of human breast cancer cells (Nawa et al., 2000). Using RNAi technology, we recently demonstrated that siRNA directed against the mta1 gene in human SH-SY5Y neuroblastoma cells dramatically stimulates neurite extension (Figure 1). Neurite extension could be detected as early as 48 hours post transfection and lasted up to 144 hours without significant toxicity (*manuscript in preparation*). The exact mechanism by which MTA1 negatively regulates neurite growth in

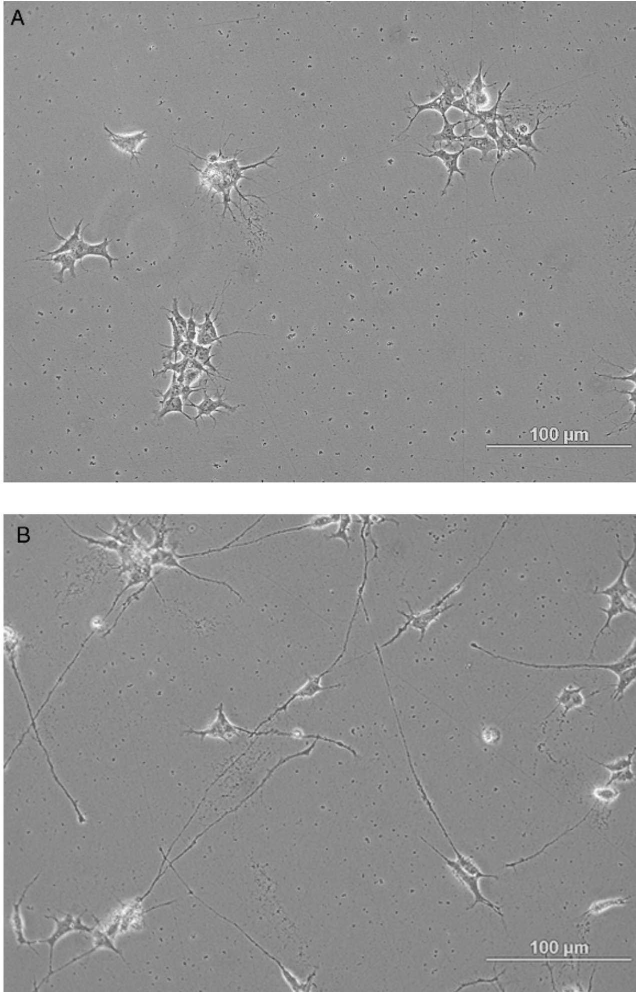

Figure 1. Silencing mta1 gene stimulates neurite growth in SH-SY5Y neuroblastoma cells. SH-SY5Y (10^4) cells were plated on Falcon CultreSlides (BD Labware, Franklin Lakes, NJ) overnight and transfected for 24 hours with (**A**) scrambled siRNA or (**B**) siRNA against MTA1. After which 500 µL ice-cold absolute methanol was added and kept on ice for 20 min. Cells were washed and incubated in 1X Perm/wash buffer (BD Biosciences) for 15 min. The wash step was repeated with TBS before the addition of Vectashield mounting medium (Vector Labs). Results show microscope pictograms of a phase contrast images of the cells. Results are a representative of seven independently performed experiments with similar results

neuroblastoma cells is currently unknown. Our preliminary results indicate that heat shock fator-1 (HSF-1), which is the transcriptional regulator of heat shock protein genes, and MTA1, are found in close proximity within the nucleosome remodeling histone deacetylase complex (NuRD complex) in the nucleus, thereby acting as an

endogenous repressor of HSF-1. Silencing the mta1 gene releases this repression and results in the upregulation of intracellular heat shock proteins and its subsequent release into the surrounding milieu (*manuscript in preparation*).

The biological significance of the released Hsp70 is currently under investigation. However, it is intriguing to speculate that this released Hsp70 might be taken up by neighboring cells to afford protection in a fashion similar to that described by Guzhova and colleagues (Guzhova et al., 2001). In this study, the authors demonstrated that glia cells released Hsp70 spontaneously and under thermal stress conditions. Interestingly, the authors demonstrated that the released Hsp70 was taken up by surrounding neuronal cells and this resulted in an increase in tolerance to thermal stress and to staurosporine-induced apoptosis (Guzhova et al., 2001).

NEUROBLASTOMA AND HEAT SHOCK PROTEINS (HSP)

Neuroblastoma is a childhood cancer of the sympathetic nervous system. Kaarniranta and colleagues examined the regulation of *hsp70* gene expression in various transformed and primary neurons and showed that neuronal cells manifest different *hsp70* gene expression patterns which range from undetected to detectable response to transcriptional and post transcriptional regulation during thermal stress (Kaarniranta et al., 2002). The results suggest that primary rat hippocampal neurons show a lack of HSF1 or induction of the *hsp70* gene. In Neuro-2a neuroblastoma cells, the *hsp70* gene response is regulated at the transcriptional level. IMR-32 neuroblastoma cells respond to thermal stress by the classical HSF-1 driven transcriptional regulatory mechanisms (Kaarniranta et al., 2002).

Apoptosis, or programmed cell death, is an important feature for the proper differentiation and development of the nervous system. Hsp70 participates in inhibition of apoptosis and necrosis and protection from oxidative stress that are related to neurodegenerative diseases (Fan et al., 2005). In this study, the authors have shown that HDJ-1 (homologue of Hsp40), when overexpressed in SK-N-SH cells, showed significant increase in protection against MPP toxicity. Also, apoptotic and necrotic rates in HDJ-1 transfected cells were significantly lower than those in the heat shock treated cells. On the basis of the above results the authors have hypothesized that the effects of HSP, such as reducing the incidence of apoptosis and necrosis, preserving mitochondrial functions and minimizing oxidative stress may bring about a novel approach for Parkinson disease therapy (Fan et al., 2005). Similarly, Hsp27 was found to be very effective in protecting PC12 cells from apoptosis. Gorman and co-worker overexpressed Hsp27 in PC12 cells and found its overexpression to protect cells from apoptosis caused by exposure of a neurotoxin 6-Hydroxydopamine (6-OHDA) (Gorman et al., 2005).

Previous studies have clearly shown that Hsp27 and Hsp70 are important for survival of neurons in both CNS and PNS (Brar et al., 1999; Wagstaff et al., 1999; Patel et al., 2005; Tsai et al., 2005; Dodge et al., 2006). Hsp27 is a member of α-crystallin-related small heat shock protein family (Kappe et al., 2003) and is present in various mammalian tissues. Hsp27 is constitutively expressed in many

motor and sensory neurons of the brainstem and spinal cord (Plumier et al., 1997). Overexpression of Hsp27 was found to prevent cellular polyglutamine toxicity (Schmidt et al., 2002). Similarly overexpression of Hsp70 reduces aggregate formation in cultured neuronal cells expressing truncated androgen receptor protein with an extended polyglutamine tract (Kobayashi et al., 2000).

Recently, a study by Dodge and colleagues revealed a positive role of Hsp27 in dorsal root ganglion (DRG) neuronal survival showing that *hsp27* has neuroprotecive effects on neurotrophin – independent survival of adult DRG neurons (Dodge et al., 2006). The findings of this study show that the level of Hsp27 expression between neonatal and DRG neurons is different both *in vivo* and *in vitro* and that this expression in adult neurons may be a contributing factor to their NGF-independent survival. It is likely that the association with Akt and other important signaling intermediates contributes its mode of action (Dodge et al., 2006).

CURRENT THERAPEUTICS FOR NEURODEGENERATIVE DISORDERS

To date there is no cure for neuroblastoma. However, there are certain drugs specifically histone deacetylase inhibitors which have shown therapeutic potential for the cure of neurodegenerative disorders. For example, valproic acid (VA) has been found to activate the extracellular signal regulated kinase (ERK) pathway and promote neurite extension in SH-SY5Y cells. VA also increased the expression of genes regulated by ERK pathways including growth cone-associated protein 43 and Bcl-2 (Yuan et al., 2001). Furthermore, VA has been found to be very effective in mouse models of Amyotrophic Lateral Sclerosis (ALS) (Sugai et al., 2004). In this work authors have shown neuroprotective effect of VA employing both *in vitro* and *in vivo* models of ALS. Another drug, sodium butyrate, has been shown to minimize hypoacetylation of histone, thus ameliorrating neurodegenerative phenotypes in mouse models for dentatorubral-pallidoluysian atrophy (Ying et al., 2006). In this study, biochemical analysis of Atro-118Q mouse (DRPLA mouse) revealed hypoacetylation of histones in the brain tissue suggesting that global gene repression is a possible mechanism for neurodegeneration in mouse models for dentatorubral-pallidoluysian atrophy. Although the exact mode of action of the above drugs is not clear, it is interesting to hypothesize that VA and sodium butyrate might exert neuroprotective effect through novel mechanism based on nucleosome rearrangement of the chromatin, eventually leading to modulation of the disease phenotypic traits. Several studies have also shown neuroprotective effect of retinoic acid (RA). Recently using cell line SH-SY5Y, it has been shown that RA induces activation of P13K/Rac1 signaling pathways and represents a potential mechanism of regulation of neuronal differentiation in neuroblastoma (Pan et al., 2005). In this study, using SH-SY5Y cells authors have demonstrated that RA promotes activation of Rac1, which leads to neuronal differentiation via p13k-mediated mechanism. Further, they have shown that activation of Rac1 by RA was independent of tGase (Pan et al., 2005). In addition to the above-mentioned drugs, in recent years much emphasis

has been given for the use of HSP as a therapeutic agent for the prevention of neurodegenerative disease. Recently, Novaselva and colleagues have shown the neuroprotective effect of the mixture of Hsp70/Hsc70 in a Huntington disease model. In their study they have demonstrated that Hsp70/Hsc70 in Huntingtin exon 1 transfected neuroblastoma cells and SK-N-SH cells significantly reduced the apoptotic effect of aggregated protein by 40–50% (Novoselova et al., 2005). Finally, in recent year's RNA-mediated therapeutics have shown tremendous potential for the cure of neurodegerative disorders and cancer. For example recent discovery of unique and novel drug target ISS-N1, for spinal muscular atrophy (SMA), a neuromuscular disease of infants and children for the correction of defective SMN2 gene. Using antisense oligonucleotide technology to target ISS-N1 increased the levels of SMN protein in SMA patient cells (Singh et al., 2006).

Proper organization and differentiation of neuronal cells are prerequisites for the development of a functional nervous system (Feng and Walsh, 2001; Dent and Gertler, 2003). In pathological situations such as Alzheimer's disease, the success of neuronal regeneration after central or peripheral nerve injury requires guided cell orientation and neurite extension (Yang et al., 2005); thus, reestablishing an ordered arrangement of neuronal cells in vitro is a crucial first step towards normal reconstruction and/or formation (Tuszynski, 2002; Yang et al., 2005). We believe HSP have potential to protect neuronal cells from variety of stresses through various mechanisms. Therefore, HSP-based technology should be explored as a potential therapy for neurodegenerative disorders.

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CHAPTER 15

EXTRACELLULAR CHAPERONES AND AMYLOIDS

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Abstract: The pathology of more than 40 human degenerative diseases is associated with fibrillar proteinaceous deposits called amyloid. Collectively referred to as protein deposition diseases, many of these affect the brain and the central nervous system. In many cases the amyloid deposits are extracellular and are found associated with newly identified abundant extracellular chaperones (ECs). Evidence is discussed which suggests an important regulatory role for ECs in amyloid formation and disposal in vivo. This is emerging as an exciting field. A model is presented in which it is proposed that, under normal conditions, ECs stabilize extracellular misfolded proteins by binding to them, and then guide them to specific receptors for uptake and subsequent degradation. In this scenario, EC receptors are a critical part of a quality control system which protects the brain against dangerously hydrophobic proteins/peptides. However, it also appears possible that in the presence of a high molar excess of misfolded protein, such as might occur during disease, the limited amounts of ECs available may actually exacerabate pathology. Further advances in understanding of the mechanisms that control extracellular protein folding are likely to identify new strategies for effective disease therapies

Keywords: Extracellular chaperones; receptor-mediated endocytosis; amyloid; protein quality control; aggregate toxicity; fibril formation

INTRODUCTION

The term "molecular chaperone" was first developed in the late 1970's when referring to the ability of nucleoplasmin to inhibit inappropriate interactions between histones and DNA (Laskey et al. 1978). The meaning of this term is continuing to evolve but two key properties of molecular chaperones are (i) selective binding to non-native protein conformations to form stable complexes, and (ii) inhibition of the irreversible aggregation of non-native protein conformations (Fink 1999). Many molecular chaperones maintain other proteins in "folding-competent" conformations, which are returned to the native conformation by the involvement of other

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refolding chaperones. Previous studies have overwhelmingly focussed on intracellular molecular chaperones and their roles in protein folding within the cell. The discoveries that a range of serious human diseases are related to protein aggregation phenomena, and that molecular chaperones affect these processes, has led to a recent explosion of research in this area. The pathology of more than 40 human degenerative diseases is associated with the deposition of fibrillar proteinaceous aggregates called amyloid. Collectively referred to as *protein deposition diseases*, these include various sporadic (e.g. Alzheimer's (AD) and Parkinson's (PD) diseases), familial, and transmissible degenerative disorders (e.g. spongiform encephalopathies such as Creutzfeldt-Jakob disease (CJD)), many of which affect, amongst other tissues and organs, the brain and the central nervous system (CNS). In many cases, molecular chaperones have been found physically associated with amyloid deposits, although the reason for this association remains to be established.

It is notable that many neurodegenerative disorders are associated with *extracellular* amyloid deposits. It has only recently become apparent that extracellular counterparts to the intracellular molecular chaperones exist. These proteins share functional characteristics with the small heat shock proteins in that they are able to efficiently stabilize misfolded proteins to prevent them aggregating but are not capable of independently refolding such proteins. Strikingly, in all cases tested, at least one or more of these "extracellular chaperones" (ECs) are associated with amyloid deposits found outside cells (Table 1). The role of these abundant ECs in the formation of amyloid deposits in vivo is emerging as an exciting field. In this chapter, we first briefly discuss what is understood about the molecular basis of amyloid formation before examining the available evidence that ECs influence this process and discussing the potential roles of ECs in amyloid formation and disposal in vivo.

AMYLOIDS AND THE BRAIN

Amyloid Formation

The hallmark of a wide range of debilitating and incurable human pathologies is the abnormal presence of extracellular deposits in a variety of organs and tissues, including the brain and CNS (e.g. AD, CJD), peripheral organs such as the heart, liver, and spleen (e.g. systemic amyloidoses), and skeletal tissues and joints (e.g. haemodialysis-related amyloidosis). The extent of in vivo deposition can vary significantly depending on the disease state as well as the location of the deposits. For instance, deposits associated with neurodegenerative diseases of the brain typically weigh as little as a few grams whilst systemic deposits can accumulate to quantities of as much as several kilograms (Pepys 1995).

Amyloid fibrils arise when a specific protein or protein fragment converts from an otherwise soluble form into insoluble filamentous aggregates. Key fibril-forming proteins associated with amyloid deposits and diseases have included full-length globular proteins, bioactive peptides (e.g. calcitonin), and fragments of whole

Table 1. Summary of protein deposition disorders in the CNS and the extracellular chaperones known to be associated with them

Disease	Aggregating protein/peptide	Location	EC associated with deposit
Alzheimer's disease	Aβ peptide	Extracellular	Clusterin (Calero et al., 2000), α_2M (Fabrizi et al., 2001), Hp (Powers et al., 1981), SAP (Perlmutter et al., 1995)
Spongiform encephalopathies	Prion Protein	Extracellular	Clusterin (Chiesa et al., 1996), (Freixes et al., 2004), α_2M (Adler and Kryukov, 2007), SAP (Ishii et al., 1984)
Parkinson's disease	α-synuclein	Intracellular	(Clusterin Sasaki et al., 2002b), SAP (Kalaria and Grahovac, 1990), α_2M (Nicoletti et al., 2002)
Dementia with lewy bodies	α-synuclein	Intracellular	(Clusterin Sasaki et al., 2002b), SAP (Kalaria and Grahovac, 1990)
Frontotemporal dementia with Parkinsonsism	Tau	Intracellular	Unknown
Amytrophic lateral sclerosis	Superoxide dismutase 1	Intracellular	Unknown
Huntington's disease	Huntingtin with poly Q expansion	Intracellular	Unknown
	Ataxins with poly Q expansion	Intracellular	Unknown
Spinocerebellar ataxia's	TATA box-binding protein with poly Q expansion	Intracellular	Unknown
Spinal and bulbar muscular atrophy	Androgen receptor with poly Q expansion	Intracellular	Unknown
Hereditary dentatorubral-pallidoluysian atrophy	Atrophin-1 with poly Q expansion	Intracellular	Unknown
Familial British dementia	ABri	Extracellular	Clusterin (Ghiso et al., 1995), SAP (Rostagno et al., 2007)
Familial Danish Dementia	ADan	Extracellular	Clusterin (Lashley et al., 2006), SAP (Rostagno et al., 2007)
Familial amyloid polyneuropathy	TTR	Extracellular	Unknown
Age related macular degeneration	Drusen components	Extracellular	(Clusterin Sakaguchi et al., 2002), SAP (Ambati et al., 2003)
Gelatinous drop-like corneal dystrophy	Keratoepithelin	Extracellular	Clusterin (Nishida et al., 1999), SAP (Stix et al., 2005)
Lattice type I corneal dystrophy	M1S1	Extracellular	Clusterin (Nishida et al., 1999), SAP (Stix et al., 2005)
Pseudoexfoliation (PEX) syndrome	PEX components	Extracellular	Clusterin (Zenkel et al., 2006), SAP (Schlotzer-Schrehardt et al., 1992)
Down's syndrome	Aβ	Extracellular	Clusterin (Kida et al., 1995), SAP (Kalaria and Grahovac, 1990)

proteins produced by specific cellular processing (e.g. the processing of amyloid precursor protein (APP) to yield Aβ) or by natural degradation. In recent years, it has become apparent that the self-assembly of proteins into amyloid fibrils is not restricted to the relatively small number of proteins associated with the protein deposition diseases. An increasing body of evidence points to this phenomenon as being a generic property of polypeptide chains, suggesting that most, if not all, proteins have the potential to form amyloid fibrils if exposed to appropriate conditions (Dobson 1999). What triggers the in vivo conversion of a protein from its native state into amyloid fibrils is not known, however, several important determinants of the fibrillation of proteins have been identified, including hydrophobicity and net charge (Chiti and Dobson 2006). At present, a detailed understanding of the mechanism for the transition from soluble precursors to mature amyloid fibrils is lacking. Despite this, it seems that the aggregation behaviours of various amyloid-forming peptides and proteins are strikingly similar. Often described as a hierarchical process involving multiple stages of assembly, the kinetics of amyloid formation (as measured by ThT fluorescence or light scattering) is generally characterized by an initial "lag" or nucleation phase, followed by a rapid exponential "growth" or polymerization phase (Jarrett and Lansbury 1993), and lastly, by a plateau phase in which no further polymerization occurs (Figure 1).

The lag (or nucleation) phase is defined as the time required for the formation of the soluble (prefibrillar) oligomers or nuclei. This phase begins with the destabilization and partial unfolding of the native protein, leading to the formation of an ensemble of intermediately folded species. In vitro studies show that structural perturbation leading to protein unfolding can be achieved by exposing the native protein to one or more chaotropic conditions, including elevated temperature, low pH, oxidative stress, molecular crowding, and protease-mediated degradation (Kelly 1998). In vivo, the fate of the proteins within the ensemble of partially unfolded intermediates depends on the environmental conditions present at the time of their existence. Some proteins may be re-folded, whilst others may be degraded. A subset of proteins within this ensemble will undergo non-ordered aggregation to form nuclei. When viewed by transmission electron (TEM) or atomic-force (AFM) microscopy, these species may appear as small beads that are linked together to produce what are often described as amorphous aggregates or micelles (Yong et al. 2002). The nucleation phase, which constitutes the rate-limiting step in amyloid formation, is relatively slow, due largely to unfavourable protein association equilibria rather than to intrinsically slow association rates (Jarrett and Lansbury 1993). After nucleation and immediately preceding the polymerization phase, the nuclei transform into an ensemble of various assemblies called protofibrils that exhibit increased levels of complexity and order. During the polymerization phase, these protofibrils rapidly grow via the addition of either monomers or oligomers that are formed after the initiation of the aggregation process, leading to the formation of well-ordered protofilaments. The lateral association of such protofilaments, accompanied by minor structural reorganization, is thought to give rise to the formation of mature amyloid fibrils (van Gestel and de Leeuw 2007).

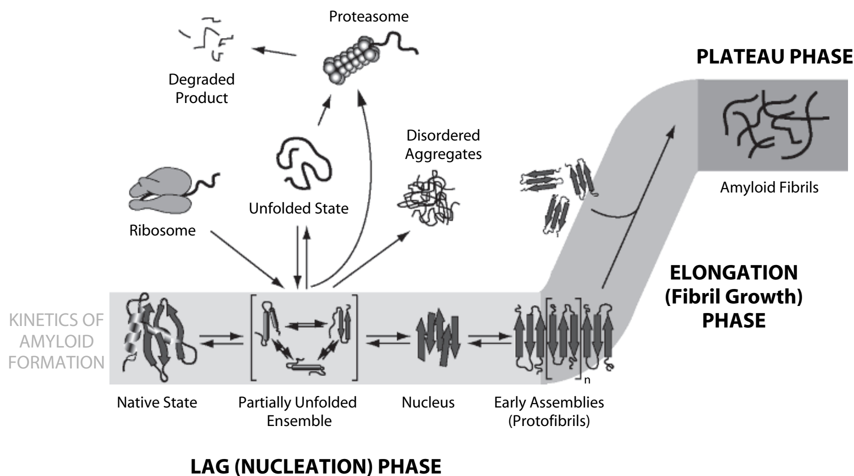

Figure 1. A schematic representation of some of the fates that await polypeptide chains in vivo. Newly synthesized polypeptides fold into their biologically active, native conformation, often via the transient formation of one or more partially unfolded intermediates. Failure to do so may result in the polypeptide experiencing other fates, including degradation or irreversible aggregation to form either disordered (amorphous) aggregates or highly organized amyloid fibrils. The formation of amyloid fibrils occurs via a nucleation dependent mechanism in which soluble prefibrillar oligomers or nuclei are formed during the lag phase. These oligomeric species then act as templates to sequester other aggregation-prone intermediates, leading to rapid fibril growth (represented by the exponential elongation phase) and subsequently, to the formation of insoluble mature amyloid fibrils. The plateau phase represents the steady state when maximum fibril growth has been reached. Adapted from (Chiti and Dobson 2006)

Regardless of their individual protein composition, the molecular architecture of all amyloid fibrils shares some striking similarities. Despite a lack of information detailing the structure of amyloid fibrils at the atomic level, techniques such as X-ray fibre diffraction, solid-state NMR, and cryoelectron microscopy have given insights into how proteins pack together to form such highly ordered structures. Typically, amyloid fibrils exist as long, unbranched, but often twisted structures that are 6–12 nanometres in diameter, bearing a characteristic “cross-β” X-ray fibre diffraction pattern (Sunde and Blake 1997). Each fibril is usually comprised, within its core structure, of two to six “protofilaments” that wind around each other to form supercoiled rope-like structures. Protofilaments are believed to be principally stabilized by intra- and intermolecular interactions (e.g. hydrogen bonds) and are composed of β-sheets whose strands lie perpendicular to the long axis of the fibril (Serpell 2000; Serpell et al. 2000).

Amyloid Pathology in the Brain

Protein deposition diseases are typically characterized by protein aggregates in specific regions of the brain, neuronal death and brain function decline. In the case of

AD, pathological symptoms include both large anatomical and microscopic changes, and are generally characterized by gross diffuse atrophy, primarily in the frontal, temporal and parietal regions of the cortex, and extensive neuronal loss. Microscopic features, commonly detected by histological staining procedures, include extracellular amyloid plaques and intracellular neurofibrillary tangles (Gorevic et al. 1986). Pathological features of PD, on the other hand, include the accumulation of Lewy bodies within the cytoplasm of pigmented neurons of the cerebral cortex. Yet, for CJD, no obvious gross pathology, besides neuronal loss and gliosis, is ever observed due to the short course of the lethal disease. Despite advances in understanding of the pathological basis of the various amyloid-related neuropathies, the actual cause/s of disease remains undetermined and are the subject of intense investigation. Numerous theories have been advanced to account for the pathology of amyloid diseases. Many of these theories particularly focus on the toxic nature of amyloids and how this relates to neuronal loss in the brain (reviewed in Stefani and Dobson (2003)).

One theory proposes that disease pathology might simply be attributed to the shear bulk of deposited material present in the affected organs and tissues, such as the brain. It suggests that the extensive accumulation of amyloid deposits causes physical disruption to the cellular architecture, subsequently leading to organ dysfunction. Another theory suggests that the formation of pores or channels in neuronal cells by oligomeric species formed from the self-association of partially unfolded proteins may be a critical determinant of amyloid-related neuropathy. Consistent with this idea, atomic force microscopy (AFM) has shown that Aβ, the primary constituent of senile plaques, can form pore-like structures in vitro (Chromy et al. 2003; Lashuel et al. 2003). In addition, ion flux data indicate that synthetic Aβ can form cation-selective channels across model membranes, leading to increased membrane permeability, disruption of calcium homeostasis and associated cellular degeneration (Arispe et al. 1993). Similar pore forming activity has also been reported for amylin, the major component of type-II diabetes-associated islet β-amyloid (Mirzabekov et al. 1996) and Cu/Zn superoxide dismutase (SOD1), which is associated with amyotrophic lateral sclerosis (ALS) (Chung et al. 2003).

A more recent and widely accepted theory proposes that soluble, inherently toxic species are the primary cause of pathogenesis in disorders such as AD. Until recently, neuronal cell death was attributed to the presence of mature fibrils, the most common form of proteinaceous aggregates present in pathological deposits. Yet, an increasing quantity of data suggests that the highly toxic species are in fact the pre-fibrillar structures (e.g. oligomers and protofibrils), rather than the mature fibrils into which they develop. Exemplifying this, small, soluble and diffusible Aβ oligomers (often refered to as amyloid β-derived diffusible ligands or ADDLs) have been shown to be highly toxic to cultured neuronal cells, even at nanomolar concentrations (Dahlgren et al. 2002). The demonstration that these species are, in addition to being highly cytotoxic, readily diffusible throughout the brain may explain why neuronal loss is commonly observed at sites distant from those of the amyloid deposits. By proposing that the primary toxic species are the early aggregates, this

theory also provides a plausible explanation for the lack of correlation between the extent of deposition of mature fibrils in the form of amyloid plaques in the diseased brain and the severity of clinical symptoms (Katzman et al. 1988).

Protein aggregation is often accompanied by the production of reactive oxygen species (ROS), although the reason/s for this is not entirely understood. There is evidence to indicate that oxidative stress, arising from the ageing process or from the production of ROS, contributes to disease pathology. The observation that Aβ deposition in the brain is positively correlated with regions of oxidative stress and neurodegeneration supports this notion (Hensley et al. 1995). Lending further support is the demonstration that antioxidants such as lipoic acid and resveratrol can protect cells against aggregate-mediated toxicity (Savaskan et al. 2003; Jesudason et al. 2005). Disease pathology is believed to arise because oxidative stress reduces the expression levels and activity of the multicatalytic proteosome (MCP), which is normally responsible for ubiquitin ATP-dependent degradation of aberrant protein structures. The reduction of available MCP leads to the accumulation of oxidized and damaged proteins at levels that are sufficient to induce neuronal cell death (Keller et al. 2000).

Potential Control Mechanisms?

Inside cells the collective functions of chaperones, the ubiquitin-proteasome system and lysosome-mediated autophagy is usually sufficient to prevent accumulation of misfolded proteins. However, under certain conditions the capacity of this quality control system is exceeded and protein aggregates accumulate (Muchowski and Wacker 2005). Intracellular levels of molecular chaperones in the brain are thought to be lowered with increasing age (Hay et al. 2004), which may contribute to the often late onset of protein deposition diseases. In the nematode *Caenorhabditis elegans*, overexpression of intracellular heat shock proteins (HSPs) promotes longevity. Furthermore, in the same organism, lifespan is shortened by a reduction in the level of heat shock factors (transcriptional activators), which presumably results in lower levels of HSPs (Westerheide and Morimoto 2005). These results suggest that intracellular chaperones are vital in the continuous fight against misfolded protein aggregation and amyloid formation. As a corollary, it appears likely that in diseases involving extracellular protein deposition, the extracellular processes that normally operate to inhibit protein aggregation and to clear protein deposits may be impaired – they are certainly overwhelmed. This is supported by the observation that, apart from people carrying mutations that result in increased production of Aβ (e.g. presenilins or APP genes), the rate of formation of Aβ does not increase with age (Deane et al. 2004b) but its normally efficient removal from the cerebrospinal fluid (CSF) is slowed in aged humans (Zlokovic et al. 2000).

Understanding of the mechanisms controlling protein folding in extracellular spaces, especially in the CNS, is in its infancy. However, there is evidence to suggest that there are such mechanisms. For example, although CSF is regularly released into the venous system through the arachnoid villi, the half lives of proteins in

the CSF differ from protein to protein, and even differ between various proteolytic fragments of the same original protein (Savage et al. 1998), suggesting a selective mechanism of removal. Recently it was proposed that there is an extracellular protein quality control system consisting of abundant secreted ECs that recognize and facilitate the disposal of non-native or dangerously hydrophobic proteins via receptor-mediated cell uptake (Yerbury et al. 2005b). These proteins may patrol the CNS for misfolded proteins and under normal conditions prevent their aggregation and mediate their clearance.

EXTRACELLULAR CHAPERONES

In the extracellular environment, protein concentrations may be high, as in human plasma (about 70 mg/ml), or lower such as in human CSF (120–640 μg/ml) (Williams and Marshall 2001). Exposure to stresses capable of inducing protein unfolding is common (e.g. sheer stress in extracellular fluids, hyperthermia, oxidative stress, transient pH fluctuations), and ATP is 1000 times less abundant than inside cells (Farias et al. 2005). In the event of a large-scale presentation of extracellular non-native protein(s), such as might occur during amyloid disease, only ATP-independent, physically abundant extracellular chaperone(s) could reasonably be expected to provide an effective line of defense. Intracellular chaperones (e.g. Hsp70, Hsp90) are present in human plasma and CSF and are associated with cell surfaces (in particular cancer cells); they are thought to be released from dead or dying cells. Many potentially important extracellular roles have been proposed for these chaperones, such as cancer cell invasiveness (Eustace et al. 2004), immune presentation (Becker et al. 2002) and signaling (Whittall et al. 2006). However, these "normally intracellular" chaperones are present extracellularly at very low (ng/ml) levels and require ATP to carry out protein refolding. Thus, the capacity of the low levels of Hsp70 and similar chaperones present extracellularly would be quickly exceeded. Therefore, it is far more likely that bulk processing of non-native proteins is dealt with by much more abundant proteins with chaperone properties, herafter referred to as extracellular chaperones (ECs), which have only recently been identified.

Four secreted glycoproteins, clusterin (Humphreys et al. 1999b), haptoglobin (Yerbury et al. 2005a), α2-macroglobulin (French et al. 2008) and serum amyloid P component (SAP) (Coker et al. 2000), have been shown to exhibit chaperone properties in vitro. These four proteins share some notable similarities. All four are secreted glycoproteins widely distributed in most physiological fluids, including plasma and CSF (Barrett 1981; Baltz et al. 1982; Bowman and Kurosky 1982; Humphreys et al. 1999b). Moreover, all bind to a broad range of ligands (Barrett 1981; Capiau et al. 1986; Katnik et al. 1987, 1993; Aruga et al. 1993; Zahedi 1996; Ashton et al. 1997; Langlois et al. 1997; Zahedi 1997; Kurdowska et al. 2000; Wilson and Easterbrook-Smith 2000; Kimura et al. 2001; Sen and Heegaard 2002) and have been found associated with clinical amyloid deposits in vivo (Table 1) (Powers et al. 1981; Baltz et al. 1982; Van Gool et al. 1993;

McHattie and Edington 1999; Calero et al. 2000). In addition all have been shown to mediate receptor-mediated endocytosis of ligands (see below). In terms of the chaperone action, clusterin is currently the best characterized.

Clusterin

Clusterin is a protein secreted from many different cell types that is found in human plasma, CSF and seminal fluid at concentrations of approximately 100, 2, and 1000 μg/ml (Fritz et al. 1983; Murphy et al. 1988; Choi-Miura et al. 1992), respectively. It can also transit from the cell secretory system to the cytosol during certain stress conditions, although the reasons why this occurs remain to be established (Nizard et al. 2007). Clusterin mRNA is nearly ubiquitous in animal tissues, being found in locales as diverse as the rat prostate gland and quail neuroretinal cells. The expression of clusterin is increased in a wide variety of models of stress and disease, including withdrawal of growth factors and exposure to noxious agents (Jenne and Tschopp 1992). The clusterin promoter contains a highly conserved 14 bp element which is recognized by the transcriptional regulator heat shock factor 1 (HSF1) (Michel et al. 1997). HSF1 activates expression of heat shock proteins (which protect cells from stresses) and clusterin (Michel et al. 1997). Across different mammalian species, the amino acid sequence of the protein is maintained at the level of 70%–80% (Jenne and Tschopp 1992). Clusterin is encoded by a single gene and the translated product is internally cleaved to produce its α and β subunits prior to secretion from the cell. Matrix-assisted laser desorption ionization mass spectrometry has identified two primary forms of human plasma clusterin at about 58 and 63.5 kDa, which probably represent different glycoforms. The exclusively N-linked glycosylation is variable in nature and extent, ranging from 17% to 27% (by weight) (Kapron et al. 1997). The function of the glycosylation is unknown, although it may be involved in recognition by cell surface receptors (Stewart et al. 2007). Probably because of the substantive and variable glycosylation, when analyzed by SDS PAGE, clusterin migrates as a broad band corresponding in position to a mass of 70–80 kDa. In aqueous solution at physiological pH, clusterin exists in a range of oligomeric forms; mildly acidic pH favours partial dissociation of oligomers into individual α-β heterodimers (Hochgrebe et al. 2000). Current insights into clusterin structure are largely reliant upon predictions based on sequence analyses, which suggest that the protein has significant contiguous regions of disordered (possibly molten globule) conformation that separate other regions of well-defined secondary structure, such as amphipathic α-helical regions and coiled-coil α-helices (Dunker et al. 2001, 2002). On this basis, clusterin has been categorized as an intrinsically disordered protein (Dunker et al. 2001, 2002). The high level of disorder, variable glycosylation and tendency to form oligomers have so far limited attempts to structurally characterize clusterin by approaches such as NMR, X-ray crystallography and mass spectrometry.

Recent studies have demonstrated that clusterin has chaperone activity with a potent ability to influence the amorphous and fibrillar aggregation of many

different proteins. Clusterin potently inhibits stress-induced protein aggregation by ATP-independent binding to exposed regions of hydrophobicity on non-native proteins to form soluble, high molecular weight complexes (Humphreys et al. 1999a; Poon et al. 2000; Yerbury et al. 2007). Immunoaffinity depletion of clusterin from human plasma renders proteins in this fluid more susceptible to aggregation and precipitation (Poon et al. 2002). Clusterin lacks the ability to independently refold heat-stressed, non-native enzymes but, like the small heat shock proteins, is able to preserve heat-inactivated enzymes in a state competent for subsequent ATP-dependent refolding by Hsc70 (Poon et al. 2000). However, because there is no currently known abundant refolding-competent EC, the physiological significance of this remains uncertain. During amorphous aggregation of proteins, clusterin appears to interact with slowly aggregating species on the off-folding pathway.

By complexing with misfolded extracellular proteins, ECs like clusterin may mediate their cellular uptake and degradation (Yerbury et al. 2005b). Clusterin has long been known to interact with the cell surface receptor megalin (LRP2) and to complex with Aβ to mediate its uptake by megalin and subsequent degradation (Hammad et al. 1997). It also interacts with other members of the low density lipoprotein (LDL) receptor family – it binds to chicken LR8 and an LDLR-related protein (Mahon et al. 1999), and uptake of clusterin-leptin complexes by apoER2 and VLDLR has been proposed to facilitate leptin clearance (Bajari et al. 2003). Furthermore, clusterin and LRP1/megalin have been implicated in the clearance of cellular debris by non-professional phagocytes (Bartl et al. 2001).

Haptoglobin

Haptoglobin (Hp) is a secreted acidic glycoprotein (20% of its total mass is N-linked carbohydrate (Bowman and Kurosky 1982)) produced mainly in the liver and found in most body fluids of humans and other mammals. Normally, it is present in human plasma at 300–2000 μg/ml (Bowman and Kurosky 1982) and CSF at 0.5–2 μg/ml (Sobek and Adam 2003). However, the levels of Hp in human plasma are increased up to 8-fold during inflammation, various infections, trauma, tissue damage and in association with neoplasia, leading to it being designated as an "acute phase protein" (Bowman and Kurosky 1982; Dobryszycka 1997). Hp is encoded by a single gene; uniquely, in humans, there are two principal alleles (Hp1 and Hp2), which results in individual humans expressing one of three major Hp phenotypes (Hp 1-1, Hp 2-1, Hp 2-2). In all cases, Hp can be represented as a multimer of an αβ subunit. In its simplest form (Hp 1-1), Hp consists of a disulfide-linked $(\alpha 1)_2\beta_2$ structure (~100 kDa). However, in Hp 2-1 and Hp 2-2, an additional cysteine residue in the α2 chain allows the formation of a complex series of various sized disulfide-linked αβ polymers (~100–~500 kDa).

Hp binds with extremely high affinity to hemoglobin (Hb) ($K_D \sim 10^{-15}$ M; (Bowman and Kurosky 1982)). Formation of the Hp–Hb complex inhibits

Hb-mediated generation of lipid peroxides and hydroxyl radical, which is thought to occur in areas of inflammation (Dobryszycka 1997). Although it was previously thought that Hp cleared the body of vascular Hb released from damaged red blood cells, recent work has shown that the ablation of Hp expression in mice had no significant effect on Hb clearance following experimentally induced severe hemolysis. Nevertheless, the Hp knock-out mice suffered substantially greater mortality under these conditions, which was attributed to greater oxidative stress (Lim et al. 1998). Therefore, it appears likely that Hp exerts an important anti-inflammatory action in vivo by inhibiting oxidative damage mediated by free Hb (Lim 2001). A variety of other putative functions have also been ascribed to Hp. It has been implicated in immune regulation (Louagie et al. 1993), shown to inhibit cathepsin B activity (Snellman and Sylven 1967) and to have pro-angiogenic effects (Cid et al. 1993). Binding of Hp to human neutrophils has been reported to inhibit respiratory burst activity (Oh et al. 1990). In addition, neutrophils have been shown to take up exogenous Hp and store it within cytoplasmic granules – they subsequently secrete it into the local extracellular environment in response to a variety of pro-inflammatory stimuli (e.g. yeast, TNFα, or the chemotactic peptide fMLP (Wagner et al. 1996; Berkova et al. 1999)). Thus, the available evidence indicates that Hp is likely to play an important role in suppressing inflammatory responses.

Human Hp specifically inhibits the precipitation of a wide variety of proteins induced by a range of stresses (Pavlicek and Ettrich 1999; Yerbury et al. 2005a). All three human Hp phenotypes exert this chaperone action, although at equivalent mass concentrations, at least for one substrate protein tested, Hp 1-1 was the most efficient. Like clusterin, Hp forms stable, soluble high molecular weight complexes with misfolded proteins. Also like clusterin, Hp lacks ATPase activity and has no independent ability to refold misfolded proteins. The possibility that Hp holds misfolded proteins in a state competent for refolding by other chaperones is currently untested. Immunoaffinity depletion of Hp from human serum significantly increased the amount of protein that precipitated in response to stresses (Yerbury et al. 2005a). Thus, Hp has the ability to protect many different proteins from stress-induced amorphous precipitation and its effects in whole human serum suggest that this activity is likely to be relevant in vivo. Currently, there are no published studies of the effects of Hp on amyloid formation, although Hp is found associated with amyloid deposits in vivo (Table 1).

When complexed to Hb, Hp is known to bind to the CD163 cell surface receptor (Graversen et al. 2002). Other receptors to which Hp alone binds are the CD11b/CD18 integrin (Mac-1/CR3), which also binds denatured proteins and the iC3b fragment of complement (Ross 2000), and the CD22 B lymphocyte receptor. Unidentified specific Hp binding sites have also been reported to occur on neutrophils (Oh et al. 1990) and mast cells (El-Ghmati et al. 2002). Thus, it appears feasible that Hp might interact with one or more of these receptors to mediate the clearance and degradation of misfolded extracellular proteins.

α_2-MACROGLOBULIN (α_2M)

α_2-Macroglobulin (α_2M) is a major human blood glycoprotein, comprised of ~10% carbohydrate by mass. It is assembled from four identical 180 kDa subunits into a 720 kDa tetramer; the 180 kDa subunits are disulphide bonded to form dimers, which non-covalently interact to yield the final tetrameric quaternary structure (Jensen and Sottrup-Jensen 1986). α_2M is present in human plasma and CSF at 1500–2000 (Sottrup-Jensen 1989) and 1–3.6 μg/ml (Biringer et al. 2006), respectively. It has a well known ability to inhibit a broad spectrum of proteases, which it accomplishes using a unique trapping method. When exposed to a protease, α_2M undergoes limited proteolysis at its bait region leading to a large conformational change, physically trapping the protease within a steric "cage" (Sottrup-Jensen 1989). The trapped protease forms a covalent linkage with α_2M by reacting with an intramolecular thiol ester bond to yield a conformationally altered form known as "activated" or "fast" α_2M (α_2M*), which exposes a receptor recognition site for low density lipoprotein receptor related protein (LRP) (Sottrup-Jensen 1989). By directly interacting with the thiol ester bond, small nucleophiles such as methylamine can also activate α_2M (Imber and Pizzo 1981).

Aside from its interactions with proteases, α_2M binds to Aβ peptide and β_2-microglobulin, which are associated with Alzheimer's disease (Narita et al. 1997) and dialysis related amyloidosis (Motomiya et al. 2003), respectively, to cytokines and growth factors (Mettenburg et al. 2002), and to a range of hydrophobic molecules including endotoxin, phenyl-Sepharose, and liposomes (Barrett 1981). The binding to hydrophobic molecules does not inhibit the trapping of proteases and is not known to be associated with any conformational changes (Barrett 1981). Previous work has indicated that α_2M-polypeptide complexes are immunogenic (Chu and Pizzo 1993; Binder et al. 2001). α_2M bound peptides are internalised by LRP and fragments of the peptide are subsequently re-presented on the cell surface. This response is identical to the one elicited by peptides chaperoned by intracellular heat shock proteins (Srivastava 2002). A further hint that α_2M might have chaperone properties came from the observation that it inhibits the aggregation of Aβ and protects cells from Aβ toxicity (Du et al. 1997). It was recently shown that α_2M has a promiscuous ATP-independent chaperone action similar to that of both clusterin and haptoglobin. It forms stable complexes with misfolded proteins to inhibit their stress-induced aggregation and precipitation but is unable to independently effect their refolding (French et al. 2008). α_2M is the first known mammalian protein with both protease inhibitor and chaperone-like activities.

Selective removal of α_2M from whole human serum renders proteins in this fluid more susceptible to precipitation, even at 37°C (French et al. 2008), suggesting that the in vitro chaperone properties of α_2M are likely to be relevant in vivo. The effects of clusterin, haptoglobin and α_2M on plasma protein precipitation are additive (French et al. (2008) and unpublished data), suggesting that even though they are promiscuous in their interactions with different substrate proteins, the ECs may provide complementarity with respect to the endogenous extracellular proteins they protect. Interaction with a misfolded substrate protein does not activate α_2M and

the resulting complex is not bound by LRP. However, if the complex subsequently interacts with and traps a protease, then the activated conformation is adopted and the α_2M/protease/misfolded protein complex is bound by LRP (French et al. 2008). Thus, for example, interaction with abundant proteases at sites of inflammation may be one in vivo switch to trigger LRP-mediated uptake of α_2M/misfolded protein complexes. Although LRP is the only known receptor for α_2M, it remains possible that non-activated α_2M/misfolded protein complexes are taken up via other currently unknown cell surface receptors. Like the other ECs, it has been proposed that α_2M patrols extracellular spaces for misfolded proteins and facilitates their disposal via receptor mediated cellular uptake. This activity would contribute to important anti-inflammatory actions of α_2M in vivo.

Serum Amyloid P Component

SAP is a member of the pentraxin family of proteins which are characterised by five identical subunits noncovalently associated to form a disc-like structure. The SAP pentamer consists of five 25 kDa subunits each containing 204 amino acids and a single intra-chain disulfide bond, and constructed from multiple anti-parallel β-strands arranged in two sheets (Emsley et al. 1994). Each human SAP subunit bears an invariant N-linked biantennary oligosaccharide which constitutes more than 8% of the mass of the molecule (Pepys et al. 1994). It has been proposed that SAP circulates as a decamer with two pentameric discs noncovalently bound face to face (Wood et al. 1988; Emsley et al. 1994). Other reports claim that SAP exists as a single pentamer in the body and that the decameric form is obtained only upon purification (Sorensen et al. 1995; Aquilina and Robinson 2003). SAP is synthesised and catabolized in the liver, and is present in human plasma and CSF at concentrations of ~40 μg/mL (Hutchinson et al. 1994) and 8.5 μg/ml (Hawkins et al. 1994), respectively. Whereas SAP is an acute-phase protein in mice (Pepys et al. 1979a), in humans it's plasma concentration is not significantly elevated during acute inflammation (Pepys et al. 1978).

Each subunit monomer has two Ca^{2+}-binding sites and shows Ca^{2+}-dependent binding to many different ligands, including certain oligosaccharides, glycosaminoglycans (Hamazaki 1987), fibronectin (de Beer et al. 1981), C-reactive protein (Swanson et al. 1992), aggregated IgG (Brown and Anderson 1993), C1q (Sorensen et al. 1996), complement C4-binding protein (Sorensen et al. 1996), DNA (Pepys and Butler 1987), chromatin (Breathnach et al. 1989), histones (Hicks et al. 1992), and phosphoethanolamine-containing compounds such as phosphatidylethanolamine (Emsley et al. 1994). Interaction with these ligands localises SAP to elastic microfibrils (Breathnach et al. 1981b), glomerular and alveolar basement membrane, arterioles, bronchioles, sarcolemma of cardiac and smooth muscle (Dyck et al. 1980), and all forms of amyloid (Pepys et al. 1979b).

Previous studies of the chaperone properties of SAP are insufficient in number and depth to allow unequivocal classification of SAP, together with clusterin, haptoglobin and α_2M as a genuine EC. However, one study showed that when

added to a refolding buffer containing denatured lactate dehydrogenase (LDH), SAP markedly enhanced the yield of active LDH. The reaction was supra-stoichometric, as a ratio of SAP pentamer to LDH substrate of 10:1 was needed to recover 25% of enzyme reactivity (Coker et al. 2000). This suggests that SAP is an inefficient refolding-competent chaperone. It is important to note that this in vitro activity was demonstrated in the absence of ATP and any “helper” chaperones. Clearly, further studies of the chaperone properties of SAP and how they might relate to known ECs will be valuable. Of particular interest in the current context, SAP has been found present in all amyloid deposits examined (Breathnach et al. 1981a; Coria et al. 1988; Kalaria et al. 1991; Yang et al. 1992). The interaction between SAP and amyloid fibrils is highly specific, and the abundance of SAP in amyloid fibrils relative to its trace concentration in plasma is extraordinary (Botto et al. 1997). SAP has a protease-resistant β-pleated sheet structure that in the presence of Ca^{2+} is resistant to proteolysis (Kinoshita et al. 1992). Furthermore, it has been shown that SAP inhibits the degradation of several types of amyloid fibrils by proteases. Tennent et al. (1995) suggested that SAP protects amyloid from proteolytic degradation in vivo by binding to fibrils and masking fibrillar conformation.

THE EFFECTS OF EXTRACELLULAR CHAPERONES ON AMYLOID FORMATION AND TOXICITY

Effects of ECs on Amyloid Formation In Vitro

In vivo amyloid formation and deposition is, in terms of its nature, onset, and progression, an extraordinarily complex process that can occur over much of the lifetime of an individual, thus making it difficult to monitor and study over a reasonable timescale. The co-deposition of other components, including proteins (e.g. molecular chaperones), metals, and glycosaminoglycans, adds further complexity to the situation. Such complexity prevents the amyloid-forming process from being fully reproduced in a test tube. Nevertheless, by examining indvidual steps in the process in vitro, it is still possible to gain insight into many facets of in vivo amyloid formation and deposition. In vitro, conditions can be manipulated such that fibril formation can be assessed in a manageable timeframe. Perturbation of the environment, with the specific aim of increasing the population of aggregation-prone intermediates, is a common strategy that usually involves, but is not limited to, the use of polypeptide concentrations that exceed physiological levels, alterations to pH or ionic strength, the use of denaturants, or incubation at high temperatures. Much of the current knowledge regarding the effects of ECs on amyloid formation has been gleaned from in vitro studies that utilise such strategies.

The in vitro chaperone actions of clusterin, haptoglobin, α_2M, and SAP, have been previously reported (see section on Extracellular chaperones). Whilst many studies have highlighted the ability of these EC to suppress amorphous aggregation, their effects on amyloid formation are, apart from clusterin, less well-documented. A report published in 1994 provided the first indication that clusterin is capable

of preventing amyloid fibril formation (Oda et al. 1994). In this report, it was shown that the formation of Aβ fibrils could be inhibited by sub-stoichiometric levels of clusterin, a finding which has since been confirmed by other independent studies (Matsubara et al. 1996; Hughes et al. 1998). Since then, clusterin has been shown to potently inhibit, in a dose-dependent manner, the formation of fibrils derived from a broad range of other unrelated "substrates", including prion protein $Pr_{106-126}$ (McHattie and Edington 1999), Apo C-II (Hatters et al. 2002), chicken egg white lysozyme, calcitonin, κ-casein, α-synuclein, β_2-microglobulin, PI3-SH3, Aβ, a model peptide $CC\beta_w$ (Yerbury et al. 2007), and the naturally occurring amyloidogenic variant of lysozyme, I56T (Kumita et al. 2007). Similar inhibitory capabilities have also been documented for α_2M. Like clusterin, sub-stoichiometric levels of α_2M (at a 1:8 molar ratio of α2M:substrate) were enough to completely inhibit the formation of fibrillar Aβ aggregates (Hughes et al. 1998). Finally, it was shown that in vitro a 1:5 molar ratio of SAP to target protein completely attenuated the fibrillation of $A\beta_{1-42}$ and α_1-antitrypsin-derived C-terminal peptides (Janciauskiene et al. 1995). There are no published data to show whether haptoglobin has similar anti-amyloidogenic properties in vitro.

The ability of sub-stoichiometric levels of clusterin, α_2M, or SAP to inhibit fibrillogenesis suggests that the substrate species involved is not highly populated but is crucial for fibril formation. Studies probing the mechanisms of clusterin's chaperone action have provided some indications as to the identity of such species. Recent studies exploring the interactions between clusterin and amyloid forming proteins revealed that clusterin did not bind to the native form of the substrates tested, nor did it interact with mature fibrils (Kumita et al. 2007; Yerbury et al. 2007). Interestingly, the greatest amount of anti-amyloidogenic effect afforded by clusterin was detected when it was present during the early stages of fibrillogenesis. Yet, based on results obtained by mass spectrometry, the presence of clusterin did not eliminate the appearance of a partially unfolded monomeric intermediate identified as the initial step in the pathway of lysozyme amyloidosis (Kumita et al. 2007). The addition of clusterin during the elongation phase did not greatly alter the kinetics of aggregation of I56T lysozyme (Kumita et al. 2007). Similarly, clusterin was more effective at suppressing fibril formation in solutions of Aβ and SH3 which had been seeded with samples taken from corresponding aggregation mixtures during the lag phase compared with those taken from the late (plateau) phase (Yerbury et al. 2007). This is consistent with data showing that at the same molar clusterin:substrate ratio, as the concentration of amyloid forming protein is increased, clusterin is less able to inhibit the formation of amyloid by Apo C-II, PI3-SH3 and Aβ. The increased concentration of amyloid forming protein increases the abundance of destabilized monomer, favouring self-association into oligomers able to nucleate the aggregation process and thereby shortening the lag phase (Hatters et al. 2002; Yerbury et al. 2007).

Taken together, the above results suggest that clusterin primarily exerts its effects on amyloid formation at or near the nucleation stage. It is likely to be a destabilized pre-fibrillar oligomeric species that are targets for binding by clusterin.

During amyloid formation, conformationally rearranged proteins and oligomeric species are usually present at concentrations low enough to account for the potent sub-stoichiometric effects of the ECs; when in equilibrium with the native fold, destabilised and non-native structures can initiate aggregation when present at an abundance as low as 1% (Canet et al. 2002; Marcon et al. 2005). The binding of ECs such as clusterin and α_2M probably reduces the availability of these species to participate in the nucleation events that normally precedes fibril formation (Figure 2).

The broad-range specificity of clusterin for amyloid forming substrates is not only impressive but suggests that the effects of clusterin on amyloid formation may be generic. This suggests that there may be a structural feature common amongst amyloid-forming peptides and proteins that are specifically recognized by the chaperone. Whether this is also true for other ECs is not known. Nevertheless, under experimental conditions that favour the formation of amorphous aggregates, both clusterin and α_2M have been shown to form high molecular weight complexes with denatured proteins, at least in part, through hydrophobic interactions (see section on Clusterin and French et al. (2008)).

Given that hydrophobicity is considered to be an important universal determinant in the formation of fibrillar protein aggregates (Chiti et al. 2002), it is therefore feasible that the inhibitory effects of clusterin, α_2M, and SAP on in vitro amyloid formation can be attributed to similar hydrophobic interactions between the chaperones and their amyloidogenic substrates. In support of this notion, clusterin and α_2M (activated and non activated) seem to preferentially bind intermediate protein species formed during amyloid-related aggregation but do not bind to native structures of the same proteins (Narita et al. 1997; Lauer et al. 2001; Hatters et al. 2002; Mettenburg et al. 2002; Kumita et al. 2007; Yerbury et al. 2007). Five times more clusterin-Aβ complex was formed when clusterin was incubated for 18 h at 37°C with Aβ monomer than was the case when it was incubated under the same conditions with pre-aggregated Aβ (Matsubara et al. 1995). Similarly, α_2M did not initially form complexes with Aβ but under conditions that promote amyloid formation stable α_2M-Aβ complexes were formed after 2 h (Narita et al. 1997). This pattern of binding may be explained by the different levels of hydrophobicity exposed on the various protein conformations produced at different stages of the amyloid-forming pathway. For example, the level of exposed hydrophobicity on a native protein is generally low with hydrophobic residues buried inside the molecule. However, after the protein has been destabilized some of the originally buried hydrophobic residues will become accessible to the solvent and perhaps chaperones. Subsequent protein aggregation will again bury most of the hydrophobic residues inside the aggregating structure.

The relationship between α_2M and Aβ is complex due to its two functional conformations. There is no doubt that α_2M binds to Aβ with high affinity. However, there are conflicting reports of the particular α_2M conformations that bind to Aβ. There are several reports claiming that Aβ binds to active α_2M but *not* to native α_2M (Narita et al. 1997; Lauer et al. 2001; Mettenburg et al. 2002). In contrast, others

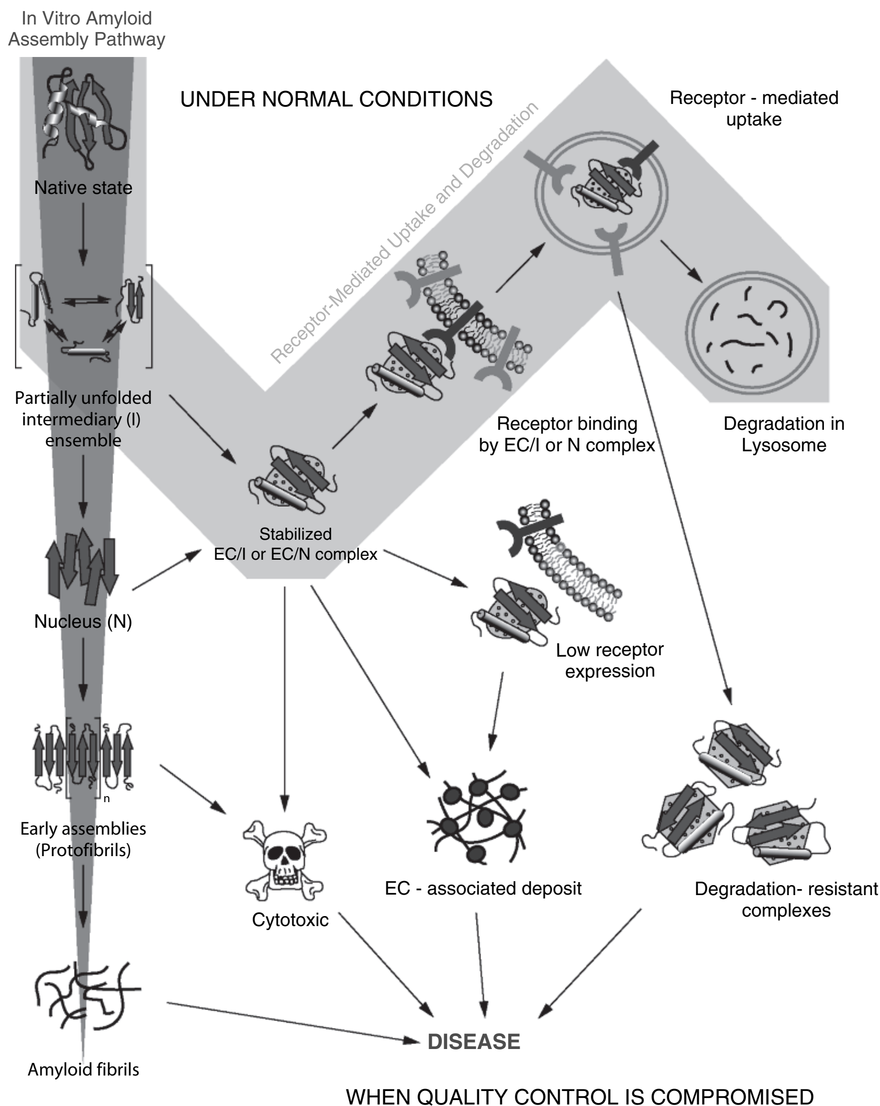

Figure 2. Possible consequences for proteins when extracellular quality control mechanisms fail. An extracellular quality control process has recently been proposed whereby extracellular chaperones (EC) facilitate the clearance of non-native protein structures via receptor-mediated endocytosis and intracellular, lysosomal degradation. The existence of such a quality control mechanism ensures that proteins do not persist beyond their intended lifespan in the extracellular space. Failure of this system, at any of its stages, may potentially result in the onset of disease caused by the accumulation and/or deposition of (i) cytotoxic species generated through the amyloid assembly pathway, (ii) aberrantly folded structures and aggregates such as insoluble complexes of ECs and fibrillar material, or (iii) degradation-resistant complexes (as exemplified by Aβ_{1-42} amyloid fibrils)

claim that native α_2M binds to Aβ (Du et al. 1997; Hughes et al. 1998). Moreover, native α_2M can inhibit fibril formation (Du et al. 1997; Hughes et al. 1998), providing additional evidence that α_2M in its native state can bind Aβ. The binding of Aβ occurs in a site that is distinct from the protease and growth factor binding sites (Mettenburg et al. 2002). It is most probable that the binding occurs to a linear sequence of amino acids, as both activated and native α_2M bind Aβ after they have been denatured (Mettenburg et al. 2002). In a curious combination of functions, activated α_2M seems to be able to feed Aβ inside its "cage" to facilitate its proteolysis by a trapped protease (Qiu et al. 1996; Lauer et al. 2001).

Despite many reports of the ability of ECs to inhibit fibril formation in vitro, there are indications to suggest that they may, at least under certain conditions, actually promote the formation or maintain the persistence of amyloid fibrils. α_2M binds to and protects prion protein from degradation by proteinase K; this protection was not exerted by α_2M binding to and inhibiting proteinase K (Adler and Kryukov 2007). In addition, another study showed that α_2M prevents the trypsin-mediated degradation of fibrils composed of either immunoglobulin lambda (λ) light chains or β2M (Gouin-Charnet et al. 1997). Similarly, the calcium-dependent binding of SAP to amyloid fibrils formed from Aβ, serum amyloid A protein or immunoglobulin light chain, protected the fibrils from subsequent protease-mediated degradation (Tennent et al. 1995). This in turn led to suggestions that SAP may promote the persistence of amyloid deposits in vivo.

For some protein substrates, at low clusterin:substrate ratios (1:50–1:500), clusterin promotes the formation of aggregates with an increased level of thioflavin T fluorescence, suggesting that (at these ratios) it promoted the formation of fibrils from calcitonin, α-synuclein and Aβ. At these low levels, clusterin was found incorporated into insoluble Aβ and SH3 fibrils (Yerbury et al. 2007). Similarly, at low SAP:Aβ ratios, SAP promoted the formation of Aβ aggregates (Hamazaki 1995); at SAP:Aβ of 1:1000, short fibrillar-like structures lacking typical amyloid features were formed which often contained associated SAP molecules (Janciauskiene et al. 1995). In addition, α_2M has been shown to stabilize a conformation of the prion protein resistant to proteinase K (PrPRes) that is thought to initiate aggregation and is associated with prion disease pathology (Adler and Kryukov 2007). Taken together, these results suggest that when ECs are present at low levels relative to the substrate they may act to stabilize an otherwise unstable conformation and facilitate amyloid fibril formation (Figure 3).

The In Vitro Effects of ECs on Amyloid Toxicity

Recent studies have indicated that the location of amyloid deposits in vivo do not correlate well with sites of neurotoxicity (e.g. in AD (Kirkitadze et al. 2002), ALS (Lee et al. 2002), Parkinson's disease (Volles and Lansbury 2003) and familial amyloidotic polyneuropathy (Sousa et al. 2001)). Neuronal losses associated with these disorders may be brought about by toxicity exerted by smaller soluble aggregates (sometimes referred to as oligomers or protofibrils; see section on Amyloid

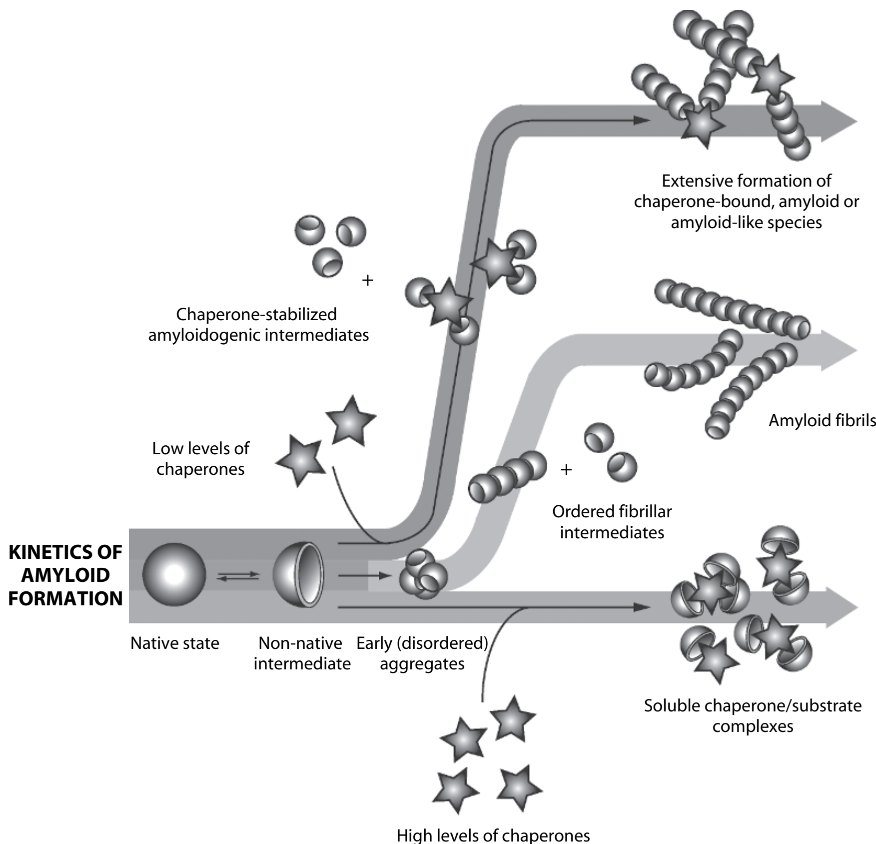

Figure 3. The biphasic effects of chaperones on amyloid fibril formation. Under certain conditions, proteins readily self-associate into highly structured amyloid fibrils. The kinetics of the amyloid assembly process is often depicted as a sigmoidal curve to reflect the three distinct phases (i.e. lag, elongation and plateau; see also Figure 1) that characterizes the fibril formation pathway. This kinetic profile can be detected in vitro using real-time spectroscopic techniques, such as those that monitor fibril formation by measuring the relative binding of amyloid-specific dyes such as Congo-red and Thioflavin T. Such techniques have, in the past, provided clues as to what effects chaperones have on fibril formation. Using various model substrate proteins, it was shown that complete inhibition of fibril formation can be achieved when clusterin and α_2M are present at high (but usually still sub-stoichiometric) levels relative to the substrate protein. Yet, at low EC:substrate ratios, extensive amyloid or amyloid-like fibril formation is observed for some substrate proteins, suggesting that in these cases, ECs may be stabilizing an otherwise unstable conformation and promoting amyloid fibril formation

pathology in the brain) (Chiti and Dobson 2006). These protofibrils have been shown to be more toxic than both the protein/peptide from which they are made and mature fibrils constructed from them (Bucciantini et al. 2002). If this scenario is correct, then it follows that at least under some conditions EC-mediated inhibition of protein aggregation in the brain could actually promote neurotoxicity. When

tested against neuronal cell lines, under certain specific conditions, clusterin and α_2M were shown to promote the neurotoxicity of Aβ (clusterin – PC12 cells (Oda et al. 1995); α_2M – LAN5 cells (Fabrizi et al. 2001)). In stark contrast, using primary rat mixed neuronal cultures, others have demonstrated that clusterin and α_2M can protect cells from Aβ toxicity (Boggs et al. 1996; Du et al. 1997). Even within individual studies, depending on the conditions used, clusterin was shown both to protect cells and to promote cytotoxicity. Complexes of clusterin and Aβ formed at high ratios of clusterin:Aβ (e.g. 1:10) were less toxic to SH-SY5Y cells than Aβ alone; in contrast, complexes formed at low ratios of clusterin:Aβ (e.g. 1:500) were more toxic than Aβ alone (Yerbury et al. 2007). Thus, the effects of ECs, and in particular clusterin, on the toxicity of aggregates are complex and have been shown to depend upon the clusterin:substrate ratio, the stage of amyloid formation at which the aggregates are formed, and the cell type.

It has been postulated that the level of hydrophobicity exposed by aggregates may determine their toxicity (Chiti and Dobson 2006). How this directly relates to the mechanism of their toxicity is uncertain and is currently a hotly researched topic, but it is probably due to interactions of hydrophobic residues with membranes or other molecules essential for normal cellular function (Chiti and Dobson 2006). This, in part, may explain the differential effects of clusterin on toxicity. When the substrate protein is present at a high molar excess, clusterin may be unable to mask all regions of exposed hydrophobicity but instead may stabilize aggregates which retain sufficient exposed hydrophobicity to exert toxicity (Figure 3). In contrast, at higher but still sub-stoichiometric ratios of clusterin:Aβ, toxicity is reduced (Yerbury et al. 2007).

Another potentially important factor to consider when trying to understand the in vivo effects of ECs on amyloid toxicity, is that in addition to their effects on the nature and size of protein aggregates, ECs may also be involved in the physical clearance of protein aggregates. This is illustrated by the demonstration that in the presence of α_2M (but not otherwise), SH-SY5Y cells expressing the α_2M receptor (LRP) are more resistant to Aβ toxicity than cells that do not (Fabrizi et al. 2001). The protective effect of α_2M could be inhibited by RAP (a pan-specific inhibitor of LRP ligand binding). Furthermore, α_2M promoted Aβ toxicity against LRP-negative LAN5 cells but had the opposite effect with LRP-expressing LAN5 transfectants (Fabrizi et al. 2001). Therefore, although ECs may provide cells with some protection by binding to exposed hydrophobic regions on protein aggregates, a further protective mechanism may only come into play when appropriate cell surface receptors are available to mediate uptake and degradation of EC-substrate protein complexes.

ECs and Protein Disposal in the Brain

It has been proposed that ECs are scavengers for hydrophobic proteins in the extracellular space, inhibiting protein aggregation and deposition and guiding misfolded proteins to specific receptors for their internalization and subsequent lysosomal

degradation ((Yerbury et al. 2005b) and see Figure 3). This system of EC directed receptor-mediated endocytosis may play a major role in the maintenance of protein solubility in the extracellular fluids in the brain (interstitial fluid and CSF). Evidence supporting this model includes:

(1) In the CNS, ECs are produced locally in astrocytes and some neuron populations (Lauro et al. 1992; Zwain et al. 1994; Klimaschewski et al. 2001) and can be transported from plasma across the blood brain barrier (BBB) (Zlokovic 1996). The receptors LRP and megalin are expressed on the surface of cells in contact with CSF (i.e. chloroid plexus and ependymal cells (Kounnas et al. 1994) and at the BBB (Zlokovic 1996; Donahue et al. 2006) and LRP is also found on the surface of astrocytes (Arelin et al. 2002) and microglial cells (Laporte et al. 2004).
(2) Clusterin and α_2M are both found complexed with soluble amyloid-forming proteins in human CSF (Ghiso et al. 1993; Adler and Kryukov 2007).
(3) It has previously been shown that clusterin-Aβ complexes bind to megalin on the surface of mouse teratocarcinoma F9 cells, and are subsequently internalised, transported to lysosomes and degraded (Hammad et al. 1997). Similarly, α_2M-Aβ complexes are internalized via LRP expressed on U87 cells and are subsequently degraded (Narita et al. 1997).
(4) The normally rapid removal of radiolabelled Aβ from mouse brain is significantly inhibited by the LDL family inhibitor RAP and antibodies against LRP-1 and α_2M (Shibata et al. 2000). Furthermore, when complexed with clusterin, the rate of clearance of $A\beta_{1-42}$ from the mouse brain across the BBB into plasma is increased by more than 80% and this transport is significantly inhibited by anti-megalin antibodies (Bell et al. 2007).

When Quality Control is Compromised?

If a system of extracellular protein quality control (QC) operates and is efficient, why do protein deposits sometimes develop? Protein aggregation in the brain is generally age-related, and some proteins such as Aβ accumulate as a part of normal aging (Fukumoto et al. 1996). In young, healthy humans the disposal of proteins such as transthyretin or peptides like Aβ is efficient (that is there are no apparent protein deposits). Thus, it is likely that protein deposition may represent an age-dependent overloading of the QC system that ultimately results in toxicity and neurodegeneration. If the QC system is overloaded in amyloid diseases of the brain, it is critical to pinpoint the factors and pathways involved to facilitate identification of new potential therapeutic strategies.

One poorly understood pathway relates to the observation that under some conditions ECs can actually promote the formation of amyloid fibrils; clusterin, α_2M and SAP have been shown to promote fibril formation in vitro (Hamazaki 1995; Adler and Kryukov 2007; Yerbury et al. 2007) (see section on Effects of ECs on amyloid formation in vitro). In addition, in a transgenic mouse model expressing

APP (PDAPP mice), a comparison of clusterin −/− and clusterin +/+ mice showed no difference in the amount of Aβ deposited in the brain, however the amount of thioflavin S positive material was larger in clusterin +/+ mice (DeMattos et al. 2002). Although there was no electron microscopy data to confirm that this was in fact amyloid fibrils, the authors suggest that this demonstrates clusterin promoted fibril formation in this model. In contrast, PDAPP *apoE−/−*, *clusterin−/−* mice had significantly increased Aβ levels in CSF and intersititial fluid and thioflavin-S deposits in the brain (DeMattos et al. 2004). The results of this latter study were interpreted as suggesting that clusterin works cooperatively with ApoE to inhibit the formation of Aβ amyloid in the brain. The interpretations presented from these two studies are seemingly contradictory, and a clear understanding of the in vivo role of clusterin in amyloidogenesis has yet to be achieved. An important factor influencing the nature of the effect of clusterin on amyloid formation, at least in vitro, is the clusterin:substrate protein ratio. At very low ratios of clusterin:substrate, clusterin has been shown to enhance amyloid associated thioflavin T fluorescence suggesting that it increases amyloid formation, while at higher but still sub-stoichiometric ratios have the opposite effect (see section on Effects of ECs on amyloid formation in vitro). A similar biphasic effect on amyloid formation has also been shown for the intracellular yeast chaperone hsp104 (Shorter and Lindquist 2004).

It is possible that at times in local microenvironments of the CNS, the concentrations of ECs are low and that under these conditions they actually promote the formation of amyloid fibrils. Interestingly, under conditions of high substrate protein excess, clusterin becomes incorporated into insoluble aggregates (Yerbury et al. 2007). Sequestration of clusterin into deposits would effectively lower it's availability, and potentially further promote fibril formation. If the other ECs behave similarly, then it is no surprise that all are found associated with plaques (see Table 1, and Figure 3). In humans with AD (Duguid et al. 1989; May et al. 1989) and Creutzfeldt Jacob disease (Duguid et al. 1989; Sasaki et al. 2002a) the levels of clusterin mRNA are increased, however, there is no associated increase in clusterin protein levels in AD CSF (Lidstrom et al. 2001) and the level of clusterin protein in CSF is decreased by 2.5 fold in Creutzfeldt Jacob disease (Piubelli et al. 2006). In addition, decreased levels of clusterin in the eye are thought to be responsible for deposition of Pseudo exfoliation (PEX) material in PEX syndrome (Zenkel et al. 2006) (again clusterin is found associated with PEX deposits).

Another factor that could favour the development of amyloid pathology is a dysfunction in the expression or function of relevant cell surface receptors (see Figure 3). Under some conditions, ECs may stabilize toxic conformations of substrate proteins with exposed hydrophobicity (see section on The in vitro effects of ECs on amyloid toxicity). If receptor-mediated clearance is inadequate, the persistence of such toxic complexes could manifest as neuronal damage. In vitro assays demonstrated that when neuronal cells were in mixed cultures with astrocytes and microglia (which express receptors capable of specifically binding to ECs, e.g.

LDLR family members), by complexing with Aβ both clusterin and α_2M were neuroprotective. However, when neuronal cells were cultured alone, complexes of clusterin or α_2M with Aβ were neurotoxic, suggesting that complexes of EC and aggregating protein may be toxic in the absence of receptor mediated endocytosis pathways. Moreover, there are increased plasma concentrations of several LRP ligands in AD patients, including ApoE, α1 anti-chymotrypsin and urokinase (Aoyagi et al. 1992; Licastro et al. 1995; Taddei et al. 1997) suggesting that LRP may be either overwhelmed, downregulated or faulty in AD. More recently, this has been confirmed by data showing that LRP expression at the BBB is decreased in AD patients and mouse models (Deane et al. 2004a). As discussed above (see section on ECs and protein disposal in the brain), this is consistent with the results from mouse models where antibody-mediated blockade of megalin and LRP significantly prolonged the time taken to clear Aβ from the brain (Shibata et al. 2000; Bell et al. 2007).

CONCLUSION AND FUTURE DIRECTIONS

In recent years there has been considerable progress in our understanding of protein deposition disorders. In particular our understanding of the nature and significance of amyloid formation and deposition and the role that these play in disease has taken dramatic leaps forward. Further advances in understanding of the mechanisms which control extracellular protein folding are likely to identify new strategies for effective disease therapies. This chapter focused on what little is known about quality control of the folding of amyloid forming proteins in the extracellular space of the brain. Since the EC field is still in its infancy, this chapter is necessarily speculative in a number of areas. However, it is quite clear that amyloid forming proteins such as Aβ and prion proteins are "chaperoned" in the brain by what have become termed extracellular chaperones. It is proposed that once bound to misfolded proteins, extracellular chaperones guide them to specific receptors that function to direct proteins into lysosomes for degradation. In this scenario, specific receptors such as LRP and megalin are an integral and critical part of a quality control system which provides protection against dangerously hydrophobic proteins/peptides in the brain. Since relatively little is known about ECs and their role in protein deposition diseases many future directions are possible. Initially, it will be important to better understand the mechanism by which, under some conditions, ECs promote amyloid formation, as this has clear relevance to the development of any EC-based therapies. Certainly, it will be critical to evaluate the roles of ECs in a variety of in vivo models of protein deposition diseases. It may be possible to accelerate the pace of advances by utilizing innovative new disease models such as those developed using the fruit fly *Drosophila*. The short generation times and ease of genetic manipulation in these models create opportunities not accessible with mammalian systems. Much remains to be discovered, ensuring that exciting times lie ahead.

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PART IV

AGING, CONTROL OF LIFE SPAN AND EXPRESSION OF HEAT SHOCK PROTEINS

CHAPTER 16

NEURAL EXPRESSION OF SMALL HEAT SHOCK PROTEINS INFLUENCES LONGEVITY AND RESISTANCE TO OXIDATIVE STRESS

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Abstract: Aging is a complex process determined by a combination of genetics and environmental factors and is accompanied by a decreased capacity of organisms to cope with various forms of stress. *Drosophila melanogaster* is a powerful genetic system to study aging mechanisms using either genetics or conditional tissue-specific gene expression approaches. Over expression of ROS scavenging enzymes or of various members of the small heat shock proteins family increase *Drosophila* lifespan and resistance to oxidative stress confirming the importance of ROS and protein homeostasis in aging and in age-related diseases. Using cell-specific promoters, these beneficial effects are shown to be particularly striking when over expression is targeted to neural cells suggesting the importance of these cells in aging. The effects of over expressing small HSPs in different compartments of the cell (i.e. mitochondria, nucleus, cytosol) on lifespan and resistance to the ROS generator paraquat suggest a pleiotropic effect of small molecular chaperones on the aging process

Keywords: Small heat shock proteins; molecular chaperones; scavenging enzymes; ROS; protein aggregation; Drosophila

CHAPERONES AND AGING

Aging is a complex process involving genetics and epigenetics factors (Sun and Tower, 1999; Vieira et al., 2000; Martin et al., 2007; Muller et al., 2007) which is generally accompanied by a decreased capacity of organisms to cope with various forms of stress. Among these, oxidative stress and accumulation of protein

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aggregates have been associated with aging and with many age-related protein conformational diseases such as Huntington, various ataxia, Parkinson, Alzheimer and a number of neuropathies (reviewed in Sun and MacRae, 2005a; Carra and Landry, 2006, 2008; Chaudhuri and Paul, 2006; Chiti and Dobson, 2006; Ding et al., 2006; Hinault et al., 2006; Hipkiss, 2006; Humphries et al., 2006; Morrow and Tanguay, 2006; Orejuela et al., 2007).

Reactive oxygen species (ROS) are known to induce damages to many macromolecules including DNA, lipids and proteins and have been proposed over 50 years ago by Harman to be major determinants of lifespan (Harman, 1956). Oxidation of proteins by ROS leads to their irreversible aggregation and the resulting protein aggregates can in turn lead to the generation of higher levels of ROS that will induce more damages and have a negative impact on cell quality and lifespan (Bulteau et al., 2006; Chakravarti and Chakravarti, 2007) (Figure 1). The analysis of gene expression by microarrays during aging or after oxidative stress is consistent with such a tight relation between oxidative stress, protein aggregation and aging as many genes are similarly regulated under both situations (Landis et al., 2004). Given this close relation between ROS, protein aggregates and aging, it is logical to consider that intervening in either the cell REDOX state or its capacity to deal with protein damages through repair or degradation processes could both have beneficial effects on lifespan (Hinault et al., 2006). Accordingly, reducing the generation of ROS by intervening with cell scavenging enzymes such as superoxide dismutases (SOD) has shown beneficial effects on lifespan in *Drosophila* (Parkes et al., 1998; Sun et al., 2004b; Landis and Tower, 2005). Moreover, small heat shock proteins (sHSP), which help in preventing protein aggregation by either facilitating proper refolding of proteins or targeting damaged proteins to the proteasome or the autophagy degradation pathways can also have a beneficial effect on lifespan (Morrow et al., 2004b, Wang et al., 2004). The natural pattern of expression of sHSP during aging is also consistent with some of these chaperones being important determinants of lifespan and cell quality.

Amongst the various HSP families, the sHSP have attracted particular attention in recent years as they have been found to be associated with various cell bodies containing aggregated proteins (aggresomes), which are present in many age-related protein conformational degenerative disorders. In addition, mutations in specific *shsp* genes have been associated with various neuropathies (Der Perng and Quinlan, 2004; Dierick et al., 2005; Carra and Landry, 2006, 2008; Morrow and Tanguay, 2006; Orejuela et al., 2007) suggesting cell-specific functions. Moreover, sHSP show a clear cell-specific pattern of expression during differentiation and normal development (Davidson et al., 2002; Michaud et al., 2002) consistent again with cell-specific functions. The role of sHSP in aging will be illustrated using the fruitfly *Drosophila melanogaster* as a model paradigm. The importance of cell specific expression for resistance to oxidative stress and in aging will also be discussed.

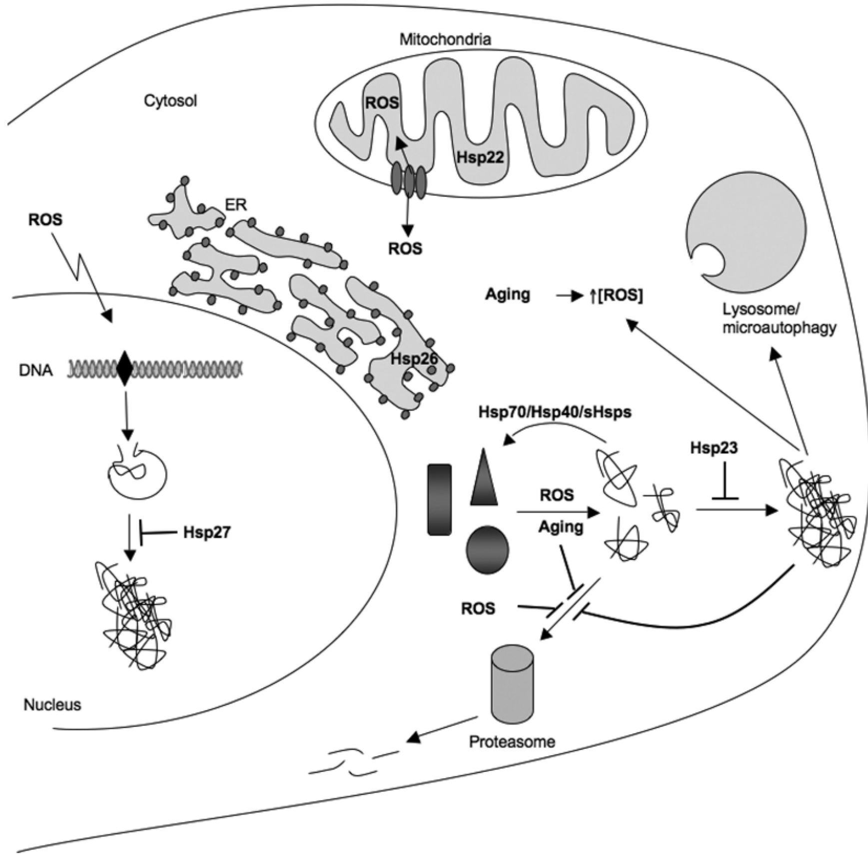


Figure 1. Small heat shock proteins help in preventing aging- and ROS-induced damages Aging and ROS give rise to DNA, protein and lipid damages which in turn results in the accumulation of mutated proteins and protein aggregates. These abnormal proteins can have negative effects on cellular homeostasis and contributes to ROS production. As molecular chaperones, sHsps can protect cells from the deleterious effect of such proteins. Their presence in each major organelles may be important to keep cellular homeostasis

THE SMALL HSP FAMILY AND AGING

The sHSP family comprises many members, which all share the C-terminus structural motif alpha-crystallin-like domain but have variable N-termini. Small HSP are molecular chaperones as shown by their capacity to prevent protein aggregation induced by heat or by other stress agents (Haslbeck et al., 2005; Sun and MacRae, 2005b). This chaperone activity has been mainly documented by in vitro aggregation assays using well-defined protein substrates subjected to heat or chemical stress. Recently their chaperone potential in vivo has also been shown

in cultured cells (Carra et al., 2005; Bryantsev et al., 2007). The sHSP have been suggested to act in concert with the HSP70 and HSP40 chaperones and to bind to damaged proteins to prevent their irreversible aggregation and subsequent negative effects on cellular homeostasis (Figure 1).

In addition to their role as molecular chaperones, sHSP have been reported to have other functions such as interacting with and regulating cytoskeletal elements (Lavoie et al., 1993, 1995). Moreover, many sHSP have been reported to counteract the apoptotic process through their interaction with various partners of the cell death machinery (Reviewed in Arrigo 2007). Over expression of small HSP has also been reported to protect mammalian cells from oxidative stress (reviewed in Arrigo et al., 2005).

In *Drosophila melanogaster*, there are 12 putative *shsp* genes but four of these have been the subject of more intensive research because of their distribution in distinct intracellular compartments including the nucleus, mitochondria and the cytosol (Michaud et al., 2002). The implication of these sHSP in the aging process was suggested by observations reporting that the levels of *hsp22* and *hsp23* mRNA were higher in aged flies (King and Tower, 1999). Conversely it was also observed that other small HSP like Hsp26 showed a down regulation during the same period (Zou et al., 2000). Interestingly flies selected for an increased longevity phenotype also had a higher level of *hsp22* transcripts as compared to shorter lived flies (Kurapati et al., 2000).

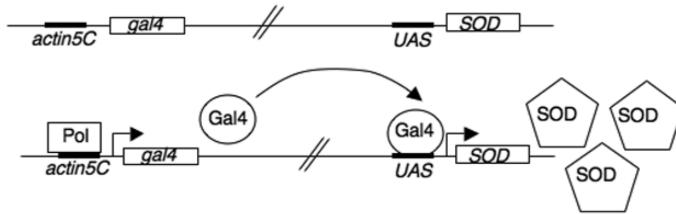
Given the link between aging, ROS, and abnormal proteins and the fact that sHsps are involved in preventing damages due to these abnormal proteins by favoring either their renaturation or their degradation, it was of interest to test if the small chaperones of *Drosophila* were also able to elicit a protective effect against aging and oxidative stress in vivo.

OVER EXPRESSION SYSTEMS IN *DROSOPHILA MELANOGASTER*

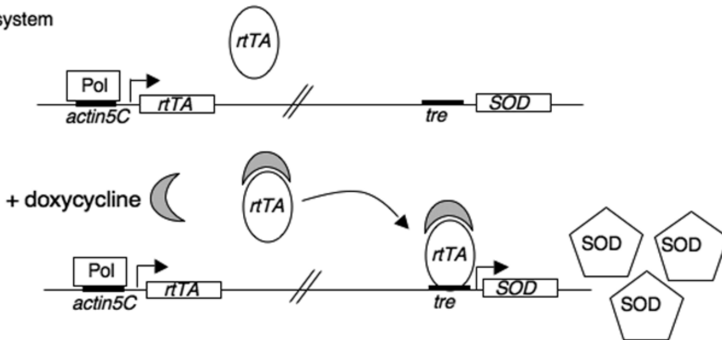
There are several ways to over express proteins in *Drosophila*, all using the advantages of the transposable p-elements. The easiest way is to obtain flies carrying a p-element containing a promoter X followed by a coding sequence Y. This system allows expression of the target protein Y according to the specifications of the promoter X but requires the making of new transgenic strains each time a new tissue-localization is investigated.

The development of the GAL4-UAS system in *Drosophila* has opened the way to directly assess the effects of protein over expression in a tissue-specific manner (Brand and Perrimon, 1993). In the Gal4/UAS system, a fly carrying the first transgene (p-element) containing a specific promoter (driver) and the yeast Gal4 coding sequence (Gal4-drivers) is crossed with another fly that carries a UAS sequence upstream of the coding sequence of the protein to be expressed. Activation of the promoter leads to Gal4 expression, which in turn binds to UAS sequence to direct the expression of the protein of interest in a promoter-specific manner (Figure 2A). Multiple Gal4-drivers are available and expression of the protein of

A Gal4/UAS system



B Tet-ON system



C FLP-OUT system

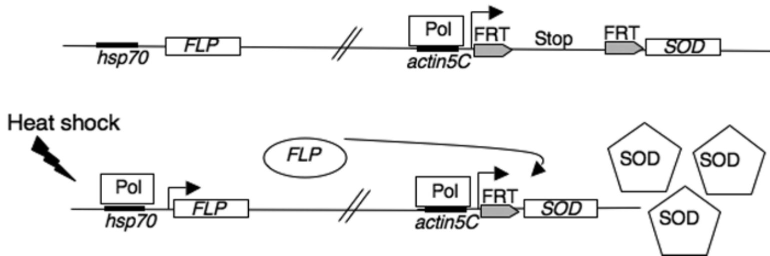


Figure 2. Over expression systems in *Drosophila melanogaster*. The Gal4/UAS system (**A**), the Tet-On system (**B**) and the FLP-OUT system (**C**) have been used in studies aimed at investigating the impact of ROS scavenging enzymes and sHsps in aging and resistance to stress. See section "over expression systems in *Drosophila melanogaster*" for a description of each system. UAS: upstream activating sequence, SOD: superoxide dismutase, pol: polymerase, rtTA: reverse tetracyclin-controlled transactivator, tre: tetracyclin response element, stop: transcriptional stop sequence

interest in different tissues only requires new crossings. As the genetic background of flies is important in these experiments, a variant of this system has been developed and is known as the GeneSwitch system. In this system, RU486/mifepristone serves as an inducing agent for the expression of Gal4 adding a time-specificity to the system as well as allowing to compare flies of the exact same genotype (±RU486/mifepristone).

Other powerful tools for over expression of proteins in specific cell-types and/or in a conditional manner are the Tet-ON (Figure 2B) and the FLP-OUT systems (Figure 2C).

In the Tet-ON system (Bieschke et al., 1998) the first transgene contains the rtTA transactivator downstream of the specific promoter and the second transgene carries the tetracycline response element upstream of the coding sequence of the protein of interest. In absence of doxycycline (a tetracycline analogue), the rtTA protein is produced according to the specificity of the promoter but cannot bind to the tetracycline response element while in presence of doxycycline, the rtTA binds to tetracycline response element and directs the expression of the protein (Figure 2B).

In the FLP-OUT system (Figure 2C), one transgene contains the *hsp70* promoter upstream of the yeast FLP recombinase while the other transgene carries the *actin5C* promoter and the coding sequence of the protein of interest separated by a stretch of DNA containing a transcriptional stop sequence flanked by two FRT sites. Therefore, under normal conditions, activation of the *actin5C* promoter does not give rise to protein expression due to the presence of stop codons in the transcriptional stop sequence. Upon heat-shock, the FLP recombinase is expressed due to the *hsp70* promoter and governs the precise excision of the transcriptional stop sequence by using the FRT flanking sites (Sun and Tower, 1999) and therefore allows the expression of the protein of interest under the specificity of the *actin5C* promoter (Figure 2A).

In all these systems the tissue-specificity of the expression is directed by the promoter used. They are powerful tools for protein over expression in specific cell-types and in defined time-frames. However, each of these systems has some limits and unfortunately they do not always give identical results for the same gene (see Ford et al., 2007 for example). Still these techniques are well suited for aging studies as controls and experimental animals are genetically identical. They have thus been used for studies aimed at determining the effects of over expressing ROS scavenging enzymes and protein folding chaperones on aging and resistance to different forms of stress with a particular emphasis on oxidative stress.

CELL-SPECIFIC EFFECTS ON AGING

Importance of the Expression Site of ROS Scavenging Enzymes on Longevity

Enzymes of the ROS scavenging pathway such as superoxide dismutases 1 and 2 (SOD1, SOD2) and catalase were obvious candidates for testing the effects of their over expression on longevity and resistance to oxidative stress. The results obtained using the different expression systems are summarized in Table 1.

Ubiquitous over expression of SOD1 using the *actin5C* driver results in a 10%–20% increase in lifespan depending on the over expression method (Reveillaud et al., 1991; Sun and Tower, 1999; Sun et al., 2002) while no increase in lifespan has been obtained using central nervous system (*elav*-Gal4) and skeletal muscle

Table 1. Effect of over expression of ROS scavenging enzymes on *Drosophila* lifespan

Protein	Over expression system/cell localization	Effect on lifespan	Ref.
Catalase	FLP-OUT system[§] /Ubiquitous (*actin5C*)	≤ control	3, 5
	Gal4/UAS system/Motoneurons (D42)	→ no change	4
	Mitochondrial targetted transgene/ ND	→ no change	6
SOD1	Gal4/UAS system/Motoneurons (D42)	↑ 40% (HS1) ↑ 16% (HS2)	1
	Gal4/UAS system/Central nervous system (*elav*)	→ no change (HS1) → no change (HS2)	1
	Gal4/UAS system/Ubiquitous (*hsp70*)	→ no change (HS1) → no change (HS2)	1
	Gal4/UAS system/Skeletal muscle (*myosin heavy chain*)	→ no change	4
	Transgene/Ubiquitous (*actin5C*)	↑ 10%	2
	FLP-OUT system[§] /Ubiquitous (*actin5C*)	↑ 10% – 14% (SOD^{3A1})^F ↑ 16% – 20% (SOD^{3B2})^F ↑ 7% – 14% (SOD^{3A1})^M ↑ 48% (SOD^{3A1})^M ↑ 14% ($SOD^{2A;\,3A1}$)^M	3, 5
SOD1 + Catalase	FLP-OUT system[§] /Ubiquitous (*actin5C*)	≤ control^F	3
	Gal4/UAS system/Motoneurons (D42)	→ no change	4
SOD2	Gal4/UAS system/Motoneurons (D42)	↑ 30%	4
	FLP-OUT system[§] /Ubiquitous (*actin5C*)	↑ 16%^{M}	5
SOD2 + Catalase	FLP-OUT system[§] /Ubiquitous (*actin5C*)	≤ control^M	5
	MnSOD transgene + Mitochondrial targetted Catalase transgene/ ND	↓ 27% ($Dm4_{SOD/OCAT}$)	7
SOD1 + SOD2	FLP-OUT system[§] /Ubiquitous (*actin5C*)	↑ 10%-14%^{F} ↑ 5%-14%^{M}	8

^{F} Females, ^{M} Males, SOD1: CuZn superoxide dismutase, SOD2: Mn superoxide dismutase, 1: Parkes et al. (1998), 2: Reveillaud et al. (1991), 3: Sun and Tower (1999), 4: Phillips et al. (2000), 5: Sun et al. (2002), 6: Mockett et al. (2003), 7: Bayne et al. (2005), 8: Sun et al. (2004b).

[§] 5 Days-old adults.

(*mhc*-Gal4) specific promoters (Parkes et al., 1998, Phillips et al., 2000). Ubiquitous expression of SOD2 using the FLP-OUT system also resulted in a 16% increase in lifespan. Simultaneous expression of the cytosolic SOD1 (Cu/ZnSOD) and the mitochondrial SOD2 (MnSOD) with the FLP-OUT approach was found to have an additive positive effect on lifespan (Sun et al., 2004b). Intriguingly, the targeted over expression of either SOD1 or SOD2 in motoneurons yielded larger increase in lifespan (16%–40% and 30% respectively) (Parkes et al., 1998, Phillips et al., 2000). These results argue for the sensitivity of motoneurons to aging damages. This selective vulnerability of motoneurons would be in part due to their high energy requirement, their reliance upon axonal transport to accomplish their function and their increased exposure to toxic environmental conditions due to their large cell-surface area (reviewed in Mattson and Magnus, 2006).

Surprisingly, flies over expressing catalase alone did not show any increase in lifespan and even had shorter lifespan than controls (Sun and Tower, 1999; Phillips et al., 2000; Sun et al., 2002). Expressing a modified version of catalase targeted to the mitochondria did not show any effect (Mockett et al., 2003) but increased lifespan when expressed jointly with the mitochondrial SOD2 (Bayne et al., 2005). However expressing unmodified catalase (peroxysomal) at the same time as SOD1 or SOD2 did not affect lifespan either (Sun and Tower, 1999; Phillips et al., 2000; Mockett et al., 2003), which supports the idea that adult flies already have an excess of catalase activity at least with regards to its action on longevity (Sun et al., 2002).

Overall it can be seen that in addition to the over expression approach used, the cell-type targeted as well as the intracellular compartment where the protein is targeted are important factors. Moreover as discussed below for Hsp22, the timing of expression during development is also likely to be important for the phenotypic effect on aging.

Small HSP Also Affect Lifespan in a Cell-Specific Way

As an alternative mean to alleviate age-related damages to proteins by ROS, one could consider molecular chaperones as they are important elements for the repair or elimination of damaged proteins. Results obtained with Hsp70 were rather disappointing (Reviewed in Morrow and Tanguay, 2003). The effects of the small HSP were therefore tested using the same genetic approaches. The importance of the site of expression of sHSP could be inferred by the early observation of King and Tower that the levels of transcripts from the *hsp22* gene were higher in certain tissues than others; thus at 35 days the level of *hsp22* was 60 fold higher in brain and 16 fold higher in thorax (muscle) than in 6-day old flies (King and Tower, 1999). Table 2 summarizes the results of over expression of the different sHSP of *Drosophila* using different approaches.

Initially the mitochondrial small Hsp22 was chosen on the basis of its localization in the organelle mainly responsible for the production of cellular ROS. Over expression of the endogenous Hsp22 increased lifespan by over 30% (Morrow et al., 2004b). It also had a beneficial effect on resistance to oxidative stress but

Table 2. Effect of over expression sHsps on *Drosophila* lifespan

Proteins	Over expression system/cell localization (promoter)	Effects of sHsp over expression on					Ref.
		Longevity[§] 25°C	Locomotor activity	Oxidative stress resistance (20 mM paraquat)	Longevity[§] 29–30°C	Longevity[§] 36–37°C	
Hsp22	Gal4/UAS system/ Motoneurons (D42)	↑ 32%	40d: →no change 60d:↑20%	2d, 24h exposure ↑ 35% 20d, 24h exposure ↑ 19%	↑ 39%	↑ 23%	2, 3
	Gal4/UAS system/ Central nervous system (*elav*)	↓ 6%	40 d: →no change 60 d: →no change	20d, 24h exposure ↑ 20%	→no change	→ no change	2, 3
	Gal4/UAS system/ Nervous system (*scabrous*)	↑ 15%	40 d: →no change 60 d: →no change	20d, 24h exposure ↑ 40%	→no change	→ no change	2, 3
	Gal4/UAS system/ Ubiquitous (*actin5C*)	↑ 32%	40 d: ↓20% 60 d: →no change	20d, 24h exposure ↓ 30%	→no change	→ no change	2, 3
	Tet-on system*/ Ubiquitous (*actin*)	↓ 5% (23) →no change (20A)	ND	11d: ↓15% (23) 11d: ↓20% (20A)	↓ 15% (23) ↓ 21% (20A)	↓ 10% (23) ↓ 13% (20A)	1
Hsp23	Gal4/UAS system/ Motoneurons (D42)	→no change (5/7) ↑ 15% (5/14)	40 d: ↓31% (5/7) ↓45% (5/14) 60 d: →no change (5/7) ↓18% (5/14)	20d, 24h exposure ↑ 43% (5/7) ↑ 21% (5/14)	→no change	→ no change	6

(*Continued*)

Table 2. (*Continued*)

Proteins	Over expression system/cell localization (promoter)	Effects of sHsp over expression on					Ref.
		Longevity§ 25°C	Locomotor activity	Oxidative stress resistance (20 mM paraquat)	Longevity§ 29–30°C	Longevity§ 36–37°C	
	Gal4/UAS system/Nervous system (*scabrous*)	↑ 10% (5/7) ↑ 7% (5/14)	40 d: ↓29% (5/7) ↓43 (5/14) 60 d: ↑13.5% (5/7) →no change (5/14)	20d, 24h exposure ↑ 54% (5/7) ↑ 50% (5/14)	→no change (5/7) ↓4% (5/14)	→ no change	6
	Gal4/UAS system/Ubiquitous (*actin5c*)	→no change	40 d: →no change (5/7) ↓ 47.9% (5/14) 60 d: →no change (5/7) ↓ 22% (5/14)	20d, 24h exposure ↓ 16% (5/7) →no change (5/14)	→no change	→ no change	6
Hsp26	Gal4/UAS system/Ubiquitous (*hsp70*)	↑ 30% (II) ↑ 31% (III)	ND	3d, 42h exposure ↑ 21% (II) ↑ 18% (III)	↑ 13%	↑ 30% (II) ↑ 25% (III)	4, 5
Hsp27	Gal4/UAS system/Ubiquitous (*hsp70*)	↑ 27% (II) ↑ 31% (III)	ND	3d, 42h exposure ↑ 23% (II) ↑ 21% (III)	↑ not shown	↑ 25% (II) ↑ 35% (III)	5

ND: not determined, 1: Bhole et al. (2004), 2: Morrow et al. (2004b), 3: Morrow and Tanguay (unpublished), 4: Seong et al. (2001), 5: Wang et al. (2004), 6: Samson et al. (unpublished).

*Doxycycline added at 4 days-old.

§ Comparison at mean survival.

in a cell-specific manner (see below). The beneficial effect of Hsp22 on longevity was confirmed by a decrease in lifespan of 50% in flies lines not expressing Hsp22 (Morrow et al., 2004a). In contrast ubiquitous over expression of Hsp22 using the Tet-ON system (Figure 2B) gave different results. Indeed, Bhole et al., reported that over expression of Hsp22 using this approach gave a decreased lifespan (Bhole et al., 2004). One reason that could explain the discrepancy between both approaches is the fact that with the Gal4/UAS system, Hsp22 was over expressed since embryogenesis while in the Tet-ON system, Hsp22 expression was induced in adults of 4 days-old and upward. These data suggest that up-regulation of Hsp22 in early stages of life is important to its effect on lifespan. Consistent with this, microarray experiments aimed at studying the age-dependent changes in gene transcription in *Drosophila* have shown that most changes occur during the early phase of aging (before middle age) and remain constant thereafter (Kim et al., 2005).

As Hsp23 was also increased in aged flies, its effects on lifespan and resistance to oxidative stress were also tested in the UAS-Gal4 system. Over expression of transgenic Hsp23 extended lifespan by 10%–15% depending on the site of insertion of the transgene (unpublished). Interestingly a fly line in which the normal pattern of expression of Hsp23 was misregulated showed a 50% reduction in lifespan consistent with an effect of this cytosolic HSP on aging as Hsp22 (unpublished). In addition, ubiquitous over expression of Hsp26 and Hsp27 using the Gal4/UAS system and a basal Hsp70 promoter was also reported to increase lifespan and resistance to oxidative and thermal stress by up to 30% (Wang et al., 2004). Thus all sHSP of *Drosophila melanogaster* tested up to now have an effect on lifespan. In some cases the effect has been shown to be dependent on the site of expression and/or in specific stages of development. Analysis of survival curves suggests that the different sHSP act on the aging process by distinct pathways. Although it would be interesting to over express more than one or even all four sHsp at a time and create a "super fly", nothing as such has been reported in the literature so far. Engineering this "super fly" may not be easy due to a possible titration effect of the transactivator protein inherent to the use of multiple target constructs in all the binary over expression system described. Indeed data on the FLP-OUT system have shown a reduced and more variable over expression level when more than one target construct is present at the same time (Sun et al., 2004b).

The effect of Hsp22 and Hsp23 on lifespan and resistance to stress has been tested with different drivers. In the case of Hsp22, targeting expression to motoneurons was sufficient to lead to a 32% increase in lifespan and in resistance to oxidative and thermal stress. Experiments on resistance to paraquat using different drivers clearly show that Hsp22 must be expressed in neurons either with scabrous (all neurons) or D42 (motoneurons) drivers to have a beneficial effect (Figure 3).

In the case of Hsp23, targeting the expression in the nervous system using the scabrous driver was more beneficial (Figure 4). In fact, while Hsp22 and Hsp23 had a somewhat similar effect on lifespan and stress resistance when expressed in the nervous system with scabrous-Gal4, the effect of Hsp23 was more restricted when over expressed only in motoneurons. Since Hsp22 is localized in the mitochondria

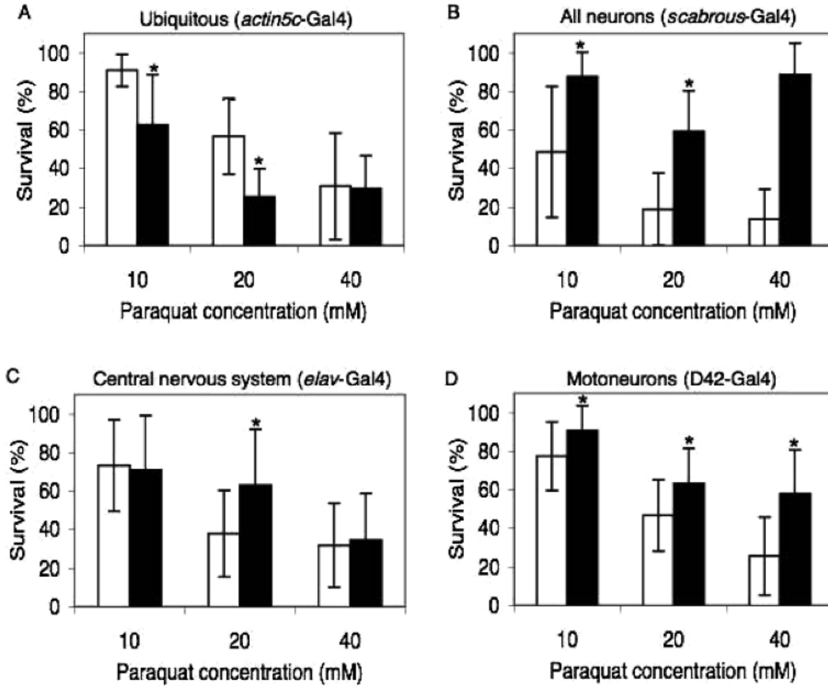


Figure 3. Different levels of oxidative stress protection according to the type of cells where Hsp22 is expressed. Survival to paraquat exposure of 20 days-old adult males over expressing Hsp22 (black) (**A**) ubiquitously (*actin5c*-Gal4), (**B**) in all neurons (*scabrous*-Gal4), (**C**) in the central nervous system (*elav*-Gal4) or (**D**) in motoneurons (D42-Gal4) and of their matched controls (white). *: $p<0.05$.

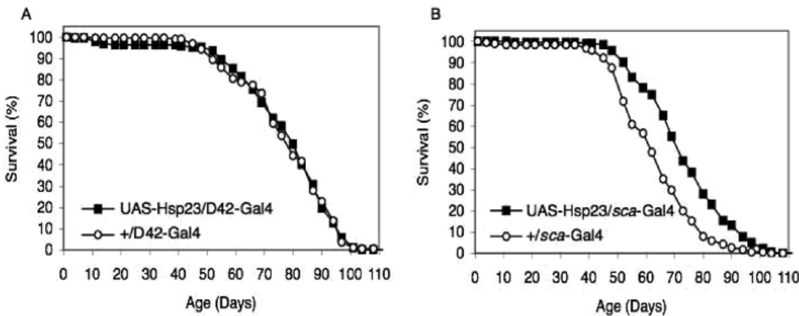


Figure 4. Over expression of Hsp23 in all neuronal cells increases lifespan. Longevity curves of adult males expressing Hsp23 (closed squares) (**A**) in motoneurons (D42-GAL4) or (**B**) in all neuronal cells (*scabrous*-GAL4) and of their matched control (opened circles). Results are expressed as percentage of flies alive at each days of age.

and Hsp23 is in the cytosol (Michaud et al., 2002), these results suggest a higher sensitivity of mitochondria motoneurons. This could be in part due to the extreme physical dimension of motoneurons and the need to actively recruit mitochondria to nerve terminals (reviewed in Chan, 2006).

INTRACELLULAR COMPARTMENTALIZATION OF SHSP AND MECHANISMS OF AGING

How the four sHSP of *Drosophila*, which are localized in distinct intracellular compartments, act on longevity remains a mystery at this time. In mammals it has been reported that the small HSP can form both homo- and hetero-oligomers (Benndorf et al., 2001; Sun et al., 2004a). Since most members of this HspB family are found in the cytoplasmic compartment, it is conceivable that the different members could influence one another for example by sequestration of other family members (See Carra and Landry this book for discussion). However in the case of *Drosophila*, individual sHSP are localized in specific intracellular compartments (Hsp22 in mitochondria, Hsp23 in the cytosol, Hsp27 in the nucleus) making interactions between the different sHsps less likely. Another possibility is that their action as molecular chaperones in different organelles contributes to longevity by protecting distinct proteins within these compartments. The protection of such proteins might be particularly critical for prevention of age-related damages. It will be important to define the preferred substrates of the individual sHsp within their respective compartments and in specific cells of the brain for example as the action of sHSP on longevity is cell-type specific. This is consistent with the natural differential pattern of expression of sHSP in different tissues in the absence of stress (Michaud et al., 2002).

PERSPECTIVES

The exact mechanisms by which the small mitochondrial Hsp22 can extend longevity are presently unknown. One possibility is that Hsp22, alone or in combination with other mitochondrial chaperones, could act as a powerful chaperone to prevent age- or ROS-induced damages to mitochondrial proteins in general. In the cyanobacterium *Synechocystis*, sHSP have been shown to interact with proteins involved in multiple cellular functions (Basha et al., 2004). The observation that over expression of the sHSPs of *Drosophila* in different cell compartments can extend lifespan is certainly compatible with such an unspecific chaperone function protecting numerous proteins involved in distinct functions. However whether the chaperone activity of the sHSP is essential in vivo, remains to be shown. It cannot be excluded either that Hsp22 has specific substrates within mitochondria to protect, for example, OXPHOS complexes. Interestingly the over expression of another mitochondrial chaperone, mortalin, also known as Grp75/mtHsp70, has

been associated with longevity in *C. elegans* (Yokoyama et al., 2002, Kimura et al., 2007). One of the known functions of mortalin in mitochondria is to help in import of mitochondrial protein (reviewed in Kaul et al., 2007). Thus it remains to be seen if its effects on aging are related to this import function or rather to its chaperone role on damaged proteins or as a regulator of oxidative stress.

Whether drug-induced over expression of chaperones can be applied to prevent age-associated damages in neurodegenerative diseases for example, remains an open question. Heat shock proteins are important in the process of protein folding and/or prevention of protein aggregation, but their levels must be carefully controlled as suggested by earlier studies on Hsp70 by Lindquist's laboratory, which showed that high levels of Hsp70 became detrimental rather than beneficial (Petersen and Lindquist, 1988, 1989, Dellavalle et al., 1994). Thus there seems to be a critical balance for chaperones and this may prove difficult to rigorously control in vivo.

Another question raised by the studies in *Drosophila* deals with the issues of cell-and developmental-specificity of expression of the sHSP. As mentioned above, expression of Hsp22 in motoneurons is particularly effective in extending lifespan and protecting flies from oxidative stress damages while expression in all cells using the *actin5c* driver has an effect on lifespan but not on protection from oxidative stress. This may reflect an optimal concentration range for chaperones in different cell types. Finally, there are likely different mechanisms of extension of lifespan that may or may not be conserved in different taxa. The effects of sHSP on protein homeostasis might explain their effect on longevity as ROS have been suggested to have an impact on lifespan through protein oxidation in various organisms. However due to their involvement in different cellular processes like stabilization of the cytoskeleton or in autophagy, it will be interesting to test if these small chaperones are involved in lifespan extension and/or in resistance to oxidative stress through a general chaperone function or rather through such specific processes.

When compared to a classical heat shock which rapidly activates some cell signaling pathways like protein B-AKT and the MAPK pathways to protect cells against these extreme stress and favor a return to normal cellular homeostasis (Nadeau and Landry, 2007), aging is a slower long-term adaptation process and the crosstalk linking the heat shock response and other signaling pathways involved in longevity such as the insulin/insulin growth factor pathway are clearly of interest. Secondly as pointed out, the heat shock (HS) response is a transient one triggered to resist extreme stress while aging is a more progressive and slower but still regulated process as shown by hormesis experiments (Le Bourg et al., 2001; Hercus et al., 2003; Arumugam et al., 2006).

Thus given the protective and life extension effects of over expression of the small HSP of *Drosophila* and the documented life-extension effects of regulation of the DAF-2 pathway in *C. elegans* (dlnr in *Drosophila*), it will also be imperative to investigate the crosstalks between this pathway, the sHSPs and the aging process.

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CHAPTER 17

MECHANISTIC LINKS BETWEEN AGING AND AGGREGATION-MEDIATED PROTEOTOXICITY: ROLE OF HSF-1 AND DAF-16

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Abstract: Aberrant protein aggregation is mechanistically linked to late onset human neurodegenerations such as Parkinson's, Huntington's and Alzheimer's diseases. Why these disorders emerge late in life is a principal enigma, however, recent results indicate that the aging process plays an active role in enabling their emergence. Perhaps the best characterized regulator of the aging process in worms, flies and mice is the Insulin/IGF-1 Signaling (IIS) pathway. In worms, IIS reduction results in activation of the FOXO-like transcription factor, DAF-16, resulting in enhanced stress resistance and longevity. The benefits associated with reduced IIS are also dependent upon another transcription factor, heat shock factor 1 (HSF-1). Reduced IIS also protects worms from proteotoxicity by regulating opposing activities: HSF-1 promotes disaggregation, while DAF-16 mediates protective active aggregation. The exploration of these mechanisms argues that the IIS enables the emergence of neurodegeneration late in life by reducing cellular capabilities to counter toxic protein aggregation. Here we review the recent studies and discuss the current themes and therapeutic potential of the IIS as a link between the aging process and late onset neurodegenerations

Keywords: Aging; insulin signaling; aggregation; proteotoxicity; neurodegeneration

INTRODUCTION

Aging is the major risk factor for the development of human neurodegenerative disorders such as prion maladies, Huntington's (HD), Parkinson's (PD) and Alzheimer (AD) diseases (Amaducci and Tesco 1994), all tightly linked to the aggregation, accumulation and deposition of aberrantly folded proteins (Kopito and Ron 2000; Selkoe 2003). In AD, a dual proteolytic digestion of the Amyloid Precursor Protein (APP) releases aggregation-prone peptides, termed Aβ,

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which form fibrillar structures of various sizes, and initiate disease (Haass and Selkoe 2007). Similarly, α-synuclein aggregation is associated with the emergence of PD (Lee and Trojanowski 2006) and the aggregation of mutant huntingtin, bearing abnormally long polyglutamine (polyQ) stretches, initiate HD (Bates 2003). The detailed mechanisms that lead to the development of these disorders are unknown, however a large body of data suggests that high-molecular weight aggregates are not causative, but rather, small, intermediate aggregating structures are the major toxic species which initiate neurodegenerative disorders (Reviewed by (Caughey and Lansbury 2003)).

Neurodegenerations appear in three distinct fashions: sporadically, as mutation-linked familial diseases or, uniquely to prion maladies, as transmissible disorders (Gajdusek and Gibbs 1968; Aguzzi and Polymenidou 2004). AD and PD mostly appear sporadically but rarely as familial diseases (Selkoe 2003), while HD is solely inherited (Bates 2003). The majority of all sporadic cases appear during the seventh decade of the patients life or later, while most individuals who carry disease-linked mutations develop clinical signs during their fifth decade (Amaducci and Tesco 1994). Why different neurodegenerations emerge late in life, in a relatively narrow age range, are key unsolved enigmas. Two basic models can explain the late onset phenomenon; one suggests that the buildup of protein aggregation-mediated toxicity is a very slow process that requires many years to initiate disease. The other model predicts that the aging process plays an active role in enabling these syndromes to onset. Recently the latter model was strongly supported as a few studies suggest that aggregation mediated proteotoxicity is not a stochastic process but highly depends upon the aging process (Morley et al. 2002; Hsu et al. 2003; Cohen et al. 2006). Aging is suspected to negatively regulate cellular counter-proteotoxicity mechanisms, a process which enables constitutive aggregation to become toxic late in life.

THE REGULATION OF AGING

At least three distinguishable pathways are known to regulate lifespan and aging; dietary intake, mitochondrial respiration and the insulin/IGF-1 signaling (IIS) pathway (reviewed by Kenyon (2005)).

The IIS, perhaps the most prominently studied aging regulating mechanism, controls lifespan and stress resistance of worms, flies and mice (Kenyon 2005). In the nematode *Caenorhabditis elegans* (*C. elegans*), the sole insulin/IGF-1 receptor, DAF-2, mediates the phosphorylation of its downstream forkhead-like transcription factor, DAF-16, prevents its nuclear localization, compromises its target genes expression, shortens lifespan and elevates stress sensitivity (Henderson and Johnson 2001; Lee et al. 2001). Thus, *daf-2* knockdown hyper-activates DAF-16, creating long-lived, youthful, stress-resistant worms (Kenyon et al. 1993; Kenyon 2005). The ability of reduced IIS to promote longevity in worms, is completely dependent upon the single FOXO family member in *C. elegans*, DAF-16, as all long-lived *daf-2* mutated strains exhibit short lifespan when *daf-16* is either

mutated or reduced by RNAi (Kenyon et al. 1993; Tissenbaum and Ruvkun 1998; Lee et al. 2001). Similarly, in mice, deletion of one copy of *igf1-R*, the murine *daf-2* ortholog, increases longevity and stress resistance (Holzenberger et al. 2003). Additionally, tissue specific knockout of the insulin receptor in adipose tissue (Bluher et al. 2003), as well as growth hormone knockout mice (Coschigano et al. 2003), have an increased lifespan.

The Heat Shock Factor 1 (HSF-1), a highly conserved (Liu et al. 1997), leucine zipper containing (Rabindran et al. 1993) transcription factor, plays critical roles in stress response (Sarge et al. 1993) and innate immunity (Singh and Aballay 2006) is also vital for worm lifespan extension facilitated by compromised IIS. First, long-lived worms, expressing mutated, weak *daf-2* allele and wild-type worms had similar, exceptionally short, life spans when developed and grown on bacteria expressing *hsf-1* dsRNA (In worms, gene knockdown can be achieved feeding worms bacteria expressing dsRNA of the gene of interest (Timmons and Fire 1998)). Secondly, worms that express an additional *hsf-1* gene live longer and are more stress resistant compared to their wild-type counterparts (Hsu et al. 2003; Morley and Morimoto 2004). Thus, although it is not yet clear how HSF-1 is mechanistically linked to the IIS, it is required for the IIS regulation of longevity, stress resistance and protection from proteotoxicity.

THE IIS AND TOXIC PROTEIN AGGREGATION

Studies in worm models indicate that manipulation of the IIS affects the onset of aggregation-mediated proteotoxicity. Researchers from Richard Morimoto's lab (Morley et al. 2002), created a series of transgenic worm strains each expressing a polyQ stretch of different length, fused to the Yellow Fluorescent Protein (YFP). Exploiting the transparency of *C. elegans*, the researchers visualized the aggregative fluorescent polypeptides within living worms and found that at least 40 glutamine repeats are required for efficient aggregation in young (day 2 of adulthood) worms. Interestingly, the threshold number of repeats needed for aggregation decreased as the animals aged. In worms expressing polyQ lengths of 35, polyQ35-YFP, aggregates were observeable by day 4 of adulthood while polyQ29-YFP aggregates could not be detected earlier than day 9 of adulthood. Since in this model polyQ-YFP aggregation impairs motility, Morley and colleagues examined this toxic effect. In accordance with aggregation, worms expressing 33–35 glutamine repeats exhibited no motility impairment at young ages but succumbed to toxicity later in life. The researchers also presented data indicating that the RNAi mediated reduction of *age-1* (a component of the IIS, that when inactivated, results in long-lived animals comparable to *daf-2* reduction) protects worm embryos from the aggregation of polyQ82-YFP. Accordingly, *age-1* RNAi reduced the motility impairment of young polyQ82-YFP worms. These protective effects were *daf-16* dependent as RNAi towards *daf-16* abolished the protective effect. Analogous observations were reported by Parker et al. (2005) who found that in worms, neuronal polyQ mediated toxicity is mitigated by Resveratrol, a Sirtuin

activator known to extend lifespan of yeast (Howitz et al. 2003), worms, flies (Wood et al. 2004), and fish (Valenzano et al. 2006), in a *daf-16* dependent fashion. *sir-2* is thought to act immediately upstream of *daf-16* to help regulate expression of DAF-16 longevity genes (Tissenbaum and Guarente 2001). Importantly, this study indicated that reduced IIS plays a role in protecting neurons from proteotoxicity.

The link between aging and the onset of polyQ aggregation was further established by Hsu et al. (2003) who found that in worms, the Heat Shock Factor 1 (HSF-1) is essential not only for lifespan extension by reduced IIS but also for the mitigation of polyQ40-YFP aggregation. This aggregation was elevated as well when the expression of *daf-16* was compromised. This study also points to small heat shock proteins, members of the crystalline family that are transcriptionally regulated by HSF-1, as important players in the counter aggregation effect of reduced IIS. Similar conclusions were suggested by Morley and Morimoto in a following study (Morley and Morimoto 2004) which confirmed the critical role of HSF-1 and of chaperones in the compromised IIS-mediated lifespan extension of worms. Interestingly, in this study, additional chaperones, members of the hsp70 family, were found to be vital for the reduced IIS mediated lifespan extension.

The results described above established the idea that reduction of the IIS pathway can protect worms from polyQ proteotoxicity; however, key questions were left unanswered. First, can reduced IIS counter proteotoxicity of other disease-linked aggregation-prone proteins? What are the mechanistic details of this reduced IIS mediated protective effect? To address these and other questions we employed model worms that express the AD-linked human $A\beta_{1-42}$ in their body wall muscles. This expression and the Aβ subsequent aggregation result in progressive paralysis of the animals (Link 1995). Analogous to its effect on polyQ-YFP toxicity, decreased IIS significantly reduced the $A\beta_{1-42}$ mediated proteotoxicity (Cohen et al. 2006). Using genetic, biochemical, in-vitro and microscopic techniques (Figure 1) we found that the amounts of high molecular weight (High-MW) $A\beta_{1-42}$ aggregates do not correlate with toxicity as: (i) reduction of the IIS (*daf-2* RNAi) resulted in reduced toxicity but in no reduction of High-MW $A\beta_{1-42}$ aggregates. (ii) Reduction of DAF-16 (*daf-16* RNAi) reduced the amount of High-MW aggregates but enhanced toxicity and (iii) *hsf-1* RNAi treatment led to elevation of both toxicity and amount of High-MW $A\beta_{1-42}$ aggregates. Similar to the polyQ-based studies, our observations support the apparent mechanistic link between aging and the onset of proteotoxicity in general and the involvement of the IIS in regulating protective activities in particular. Based on these findings we proposed a model suggesting that the IIS negatively regulates both *hsf-1* and *daf-16* and subsequently compromise both counter proteotoxic mechanisms, disaggregation and active aggregation (Figure 2).

In contrast to the current polyQ based studies discussed above, our data argues that there is no direct correlation between high-MW aggregates and toxicity. This idea is consistent with data of others showing that in different neurodegenerations

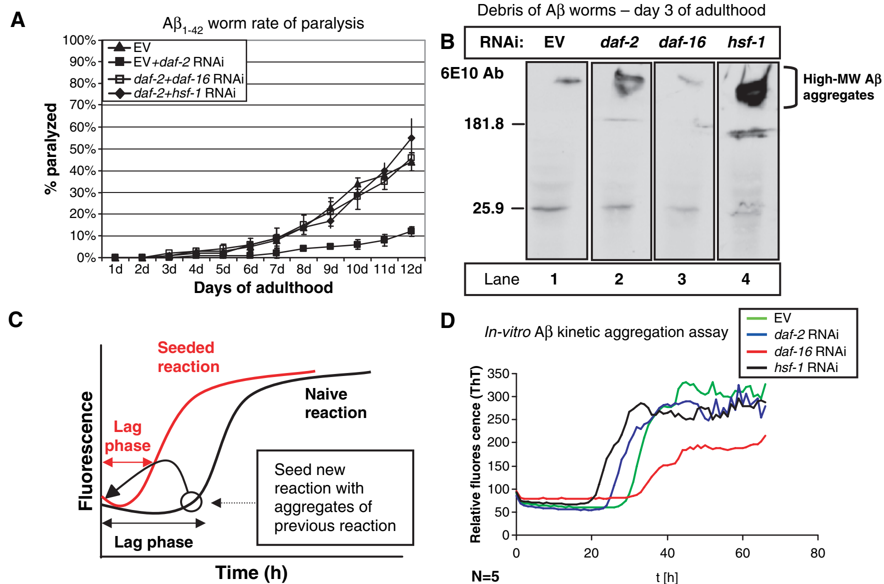

Figure 1. Lack of correlation between high molecular weight Aβ aggregates and toxicity. (**A**) *daf-2* RNAi protects Aβ worms from the paralysis phenotype associated with Aβ expression. RNAi towards either *daf-16* or *hsf-1* completely abolish the *daf-2* RNAi counter toxic effect (EV, empty RNAi vector bacteria). (**B**) Aβ worms were grown on RNAi bacteria as indicated. At day 1 of adulthood the worms were homogenized, spun and debris were separated from soluble fraction. Aβ contents in worm debris were analyzed using Western blot (with 6E10 antibody). (**C**) In-vitro kinetic aggregation assay. The typical lag phase that is associated with an in-vitro aggregation of Aβ can shortened by seeding of the reaction with previously aggregated Aβ. This technique has been exploited to measure Aβ aggregate content in worm samples. (**D**) Aβ seed contents of worms grown on RNAi bacteria (as indicated) were evaluated using kinetic aggregation assay. Reproduction from Cohen et al. (2006)

it is not high-MW aggregates but small aggregative structures are the major toxic species (Caughey and Lansbury 2003; Chesebro et al. 2005; Silveira et al. 2005). This apparent contradiction might be a result of the possibility that hyper aggregation is protective and an animal forms large aggregates before succumbing to toxicity. Thus, the observed large polyQ aggregates might be a marker of proteotoxicity, but not its source. Toxicity plausibly stems from small aggregative structures, often termed protofibrils. An alternative explanation suggests that Aβ and polyQ differ in their aggregation and toxicity properties. Importantly, all worm-based studies described above indicate that IIS reduction protects worms from proteotoxicity.

Cell culture-based reports and studies performed in rodent models appear to disagree with the idea that counter proteotoxicity activities are mediated by reduced IIS. Humbert et al (2002) reported that activation of IGF/AKT pathway promotes neuroprotection from specific polyQ mediated toxicity. This protection involved the phosphorylation of huntingtin by AKT, a kinase positively regulated by the IIS, and its clearance by autophagy. Consistent with this observation, Yamamoto

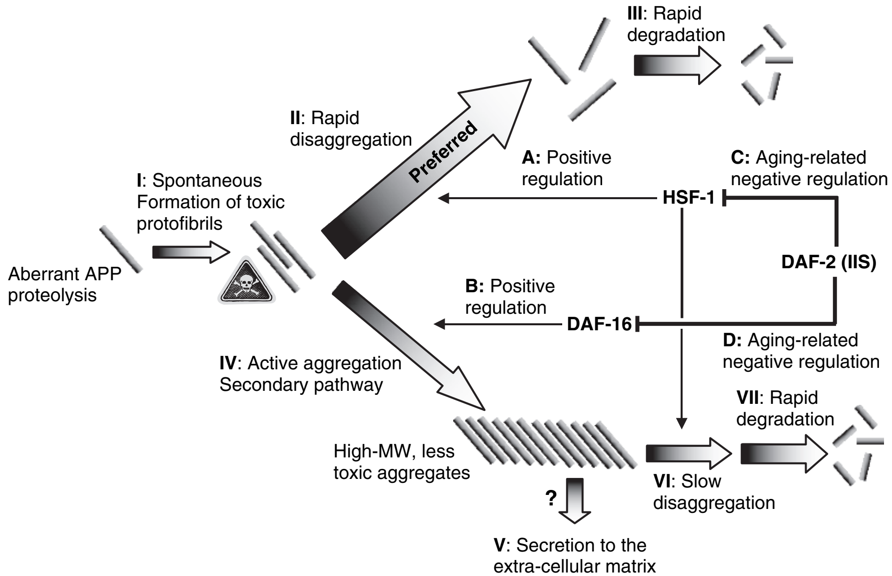

Figure 2. The Insulin / IGF-1 Signaling pathway links aging and proteotoxicity. (**I**) Serial digestion of the Amyloid Precursor Protein (APP) releases the aggregative-prone peptides Aβ which spontaneously form small toxic aggregates. (**II**) HSF-1 regulated (**A**) disaggregation machinery disrupts the Aβ aggregates and prepares them for (**III**) degradation. (**IV**) When the disaggregation pathway is overtaxed a DAF-16 regulated (**B**) active aggregation apparatus creates high molecular weight (high-MW) aggregates of lower toxicity which are possibly secreted from the cell (**V**). (**VI**) The high-MW aggregates undergo slow disaggregation and subsequent degradation (**VII**). DAF-2 compromise the activity of both protective mechanism in an age dependent manner by negatively regulating HSF-1 (**C**) and DAF-16 (**D**). Reproduction from Cohen et al. (2006)

and colleagues (Yamamoto et al. 2006) found that autophagy-mediated clearance of huntingtin is triggered by the activation of insulin receptor substrate 2 (IRS-2), a protein that mediates the signaling of growth factors such as insulin and insulin-like growth factor 1 (IGF-1). However, unlike Humbert et al., Yamamoto and colleagues reported that the counter polyQ toxicity effect is AKT independent, suggesting that signals downstream of IRS-2 must diverge to counter proteotoxicity in this setting.

Similarly, Carro et al (2002) reported that increased levels of circulating IGF-1 induces the clearance of Aβ in rat brains. They infused IGF-1 to old rats and found that Aβ levels in the animals brains were reduced to these observed in young rat brains. The researchers concluded that hyper-activation of the IIS is neuroprotective. Recently, the same group reported that IGF-1 injection to AD-model mice reduced the typical behavioral impairments associated with increased Aβ and reduced the Aβ in their brains (Carro et al. 2006). Apparently, these results, generated in cell cultures and rodent models, contradict the data obtained from *C. elegans* polyQ and Aβ proteotoxicity models. However, these observations do not necessarily contradict the worm based studies as the possibility that IGF-1,

AKT and IRS-2 play complex roles in signaling pathways other than their roles in worms' IIS should not be excluded. One possible explanation to reconcile the results from mammalian and worm experiments is that under conditions of local, acute upregulation of the IIS pathway actually results in a prolonged dampening of IIS signaling. In this model, spike activation of IIS signaling, by injecting larges amounts of insulin or IGF-1, results in future compensation of the pathway to reduce further stimulation of the IIS pathway. At least two key experiments need to be performed to resolve this discrepancy. One, reduction of IIS in a bona fide mammalian model of AD needs evaluation. Two, does ectopic, acute stimulation of the IIS pathway result in later down regulation and compensation of the IIS pathway in these mammalian models of AD. Nonetheless, the fact that aging is the major risk factor for the development of neurodegenerations and that reduced IIS slows aging of mice (Holzenberger et al. 2003), seriously challenge the idea that activated IIS protects from proteotoxicity

BIOLOGICAL COUNTER PROTEOTOXICITY ACTIVITIES

Countering proteotoxicity involves the detoxification and clearance of harmful protein aggregates by biological activities such as disaggregation (Bosl et al. 2006), degradation (Iwata et al. 2001; Bandhyopadhyay and Cuervo 2007) and perhaps active protein aggregation (Shorter and Lindquist 2004; Behrends et al. 2006). The involvement of the IIS in regulating proteotoxicity raise several key questions: do protective activities decline with age? What are the cellular components which promote these activities? Does the IIS play roles in regulating the expression and stability of these components and subsequently these protective activities?

The disassembly of monomeric polypeptides and their separation from large aggregates appears to be required to enable their efficient degradation. In yeast, the chaperone HSP104 was shown to mediate disaggregation activity in concert with other heat shock proteins (Glover and Lindquist 1998). No obvious *hsp104* orthologs can be identified in mammalian systems or *C. elegans*, however, disaggregation activity is present in these systems, as disaggregation has been observed in mammalian cells (Nollen et al. 2001) and in worm homogenates (Cohen et al. 2006).

To date it is unclear what cellular components promote disaggregation activity, however HSF-1 is involved in the regulation of this activity (Cohen et al. 2006). This finding suggests that disaggregation might be compromised by the aging process. Perhaps the apparent involvement of the crystalline chaperones in countering polyQ aggregation and their regulation by HSF-1 and/or DAF-16 (Hsu et al. 2003) points to these chaperones as possible mediators of disaggregation.

Proteolysis is an additional critical counter proteotoxicity activity. Two cellular degradation mechanisms are known to digest abnormally processed, unfolded and damaged proteins: the ubiquitin proteasome system (UPS) and lysosomes. Proteasomes possess the capability to degrade poly-ubiquitin tagged proteins (reviewed in Ciechanover 2003). Proteasomes were found within the vicinity of potentially toxic

protein aggregates (Cohen and Taraboulos 2003) and proposed to be involved in polyQ aggregate clearance (Bennett et al. 2005). However, several studies indicate that proteases, not proteasomes, mediate the degradation of misfolded aggregative proteins, perhaps through a mechanism termed chaperone-mediated autophagy (CMA) (Cuervo et al. 2004). Proteases including Neprilysin (Iwata et al. 2001) and the Insulin Degrading Enzyme (IDE) (Leissring et al. 2003) have been also shown to be directly involved in the degradation of Aβ. It was suggested that the aging process in general and the IIS in particular play roles in the regulation of proteolysis (Massey et al. 2006). The third protective activity to be discussed here is active aggregation. Traditionally, protein aggregation was thought to be an uncontrolled, sporadic, toxic process which living cells sought to prevent. This view has been recently challenged with indications that cells actively aggregate proteins under certain conditions as large aggregates have been proposed to bear lower toxicity compared to their smaller counterparts. Shorter and Lindquist (2004) reported that the yeast chaperone Hsp104 can catalyze the aggregation of the yeast prion-like protein, Sup35. This chaperone activity was observed when Sup35 was present in high concentration and was abolished upon dilution. It is also shown in this study that Hsp104 disrupts large aggregates, therefore the authors suggest that the Hsp104 machinery possess opposing activities in an aggregate concentration dependent manner. This raises the intriguing possibility that in higher organisms, the distinct and differentially regulated disaggregation and aggregation activities evolved from the primordial Hsp104 functional ortholog.

Similarly, the cytosolic chaperonin TRiC promotes the aggregation of polyQ stretches (Behrends et al. 2006). This activity was found to be associated with the activity of the Hsp70/Hsp40 machinery and to be cell-protective as the larger aggregates were less toxic than their smaller counterparts. Analogous to Hsp104, TRiC active aggregation activity is concentration dependent, as overexpression of TRiC resulted in the reduction of PolyQ aggregation.

The idea that active aggregation is protective was supported by our study (Cohen et al. 2006) which indicated that this activity is regulated by the transcription factor DAF-16 and thus, most likely declines with age.

Collectively the studies reviewed herein argue that the aging process compromises counter proteotoxicity mechanisms. This aging-associated decline enables constitutive protein aggregation to become toxic and to initiate neurodegeneration late in life.

WHAT MIGHT INFLUENCE THE AGE OF ONSET OF NEURODEGENERATION?

When will a certain individual succumb to accumulating toxic aggregates and develop neurodegeneration? The answer to this essential question probably depends upon the balance between toxic protein aggregation and the efficiency of detoxification activities. Therefore, it is plausible that a threshold amount of toxic species is required to initiate disease. Early in life the aggregate clearance capabilities

exceed the rate of aggregation and toxic species do not accumulate to reach the disease initiation threshold. As the individual ages, the aging-associated decline in protective activities (disaggreation, active aggregation, proteolysis, export) enables the accumulation of toxic structures to the levels required to commence neurodegeneration. In this regard, an interesting study suggest that a single perturbation of the proteome by an uncontrolled aggregation can initiate disease by affecting the folding of other proteins (Gidalevitz et al. 2006). Apparently, as a population, human beings age at similar rates, thus, it is plausible that their capabilities to clear proteotoxic species declines at similar paces. This might explain why distinct neurodegenerations emerge at similar ages. The rates of potentially toxic aggregate formation probably vary among individuals depending on their specific genetic background and environmental conditions. Nevertheless, it is clear that patients harboring neurodegeneration-linked mutated genes produce relatively high amounts of aggregation-prone toxic polypeptides and are more likely to develop disease early in life. Individuals who do not carry neurodegeneration-linked mutated genes produce less toxic aggregates and therefore develop disease later in life if at all (Figure 3).

Many aspects of the mechanisms that link aging and neurodegenerations are still obscure, nevertheless, the current understanding point to the manipulation of aging, and the consequent maintenance of counter aggregation activities, as a possible exciting avenue towards the development of future counter neurodegeneration treatments.

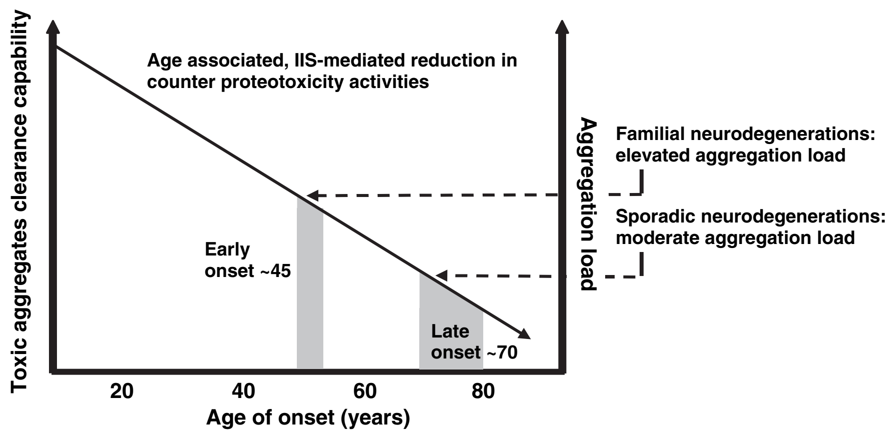

Figure 3. The balance between the production of toxic protein aggregates and the aging-associated reduction in counter proteotoxicity activities determines the age in which the amount of toxic aggregates will cross the threshold level required for disease onset. Higher aggregation load and lower protective activities will lead to early onset while lower aggregation load and higher protective capabilities will postpone the disease age of onset. This model proposes that the similar ages of onset of distinct neurodegenerations stem from one phenomenon, the age-related decline in the natural counter proteotoxicity activities

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CHAPTER 18

PROTEIN QUALITY CONTROL AND HEAT SHOCK GENE EXPRESSION IN THE NERVOUS SYSTEM

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Abstract: Protein damage accumulates in neuronal cells due to oxidative stress, transduction with prion particles with dominant conformations or due to genetic alterations in proteins that lead to formation of insoluble aggregates. Failure to repair such protein damage can lead to the formation of lethal protein aggregates and ultimately cell death All prokaryotic and eukaryotic cells deploy strategies to counteract these changes and avoid the accumulation of protein aggregates. These pathways for protein quality control include: (1) the protein chaperone and refolding systems and (2) targeted proteolysis of the malfolding protein. In mammalian cells, the molecular chaperones heat shock proteins 70 and 90 (HSP70 and 90) appear to play key regulatory roles in protein triage after damage. These molecular chaperones bind to malfolded proteins, deter the aggregation cascade and then target the protein substrates towards either: (1) the pathways of refolding by chaperonin-containing folding structures or (2) can promote ubiquination of its target through mechanisms involving the ubiquitin ligase CHIP and deliver the ubiquinated protein to the proteasome for degradation. Dysregulation of this system occurs during aging and is amplified during a range of degenerative disease states. Failure of this defense system may occur at many levels and decreased expression of proteins that mediate pathways 1 and 2 appears to be involved in aging, particularly of neuronal cells

Keywords: Heat shock protein; aging; aggregation; molecular chaperone; CHIP; ubiquitin; proteasome; heat shock factor 1

INTRODUCTION: HEAT SHOCK PROTEINS

The heat shock proteins (HSP) are products of a number of distinct gene families required for cell survival during stress, named for the approximate Mr of their products and include HSP10, 27, 40, 60, 70, 90, and 110 (Table 1) (Lindquist and Craig, 1988; Bukau and Horwich, 1998; Easton et al., 2000; Tang et al., 2005). The cytoprotective properties of the HSPs are closely linked to their primary functions

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Table 1. The major heat shock protein families in human cells

Protein	Function	Co-chaperones	References
HSP27	Molecular Chaperone	None	Arrigo (2005)
HSP60	Chaperonin	HSP10	Spiess et al. (2004); Young et al. (2004)
HSP70	Molecular Chaperone	HSP40, GrpE, BAG, HSPBP1, Hip, Hop, CHIP	Bukau and Horwich (1998) and Mayer and Bukau (2005)
HSP90	Molecular Chaperone	p23, HOP, FKBP51, FKBP52, Cyp40, cdc37	Bukau and Horwich (1998); Pratt and Toft (2003) and Wegele et al. (2004)
HSP110	Molecular Chaperone	None	Easton et al. (2000)

as molecular chaperones (Lindquist and Craig, 1988; Nylandsted et al., 2000; Beere, 2004). The intracellular reactions catalyzed by the HSP, which led to their designation as molecular chaperones, are divided into two main categories which have been described as (1) “protein holding” and (2) “protein folding” (Buchner, 1999; Wegele et al., 2004). The principle holding proteins belong to the HSP70 and HSP90 families which bind to unfolded sequences in polypeptide substrates, showing preference for hydrophobic regions (Wegele et al., 2004; Mayer and Bukau, 2005). Such holding interactions occur during: (1) mRNA translation when HSP70 binds to the elongating polypeptide chain in order to prevent premature self-associations within the nascent protein, (2) during heat shock when proteins partially unfold and expose hydrophobic sequences which are bound by HSP and (3) constitutively when HSP90 binds to proteins with unstable tertiary structures (Pratt and Toft, 2003; Wegele et al., 2004). HSP70 and HSP90 function in large complexes or chaperone machines which also contain a number of accessory proteins or co-chaperones, that bind the primary chaperone in order to mediate substrate selection, and cycles of association with and disassociation from the substrate (Table 1) (Pratt and Toft, 2003; Mayer and Bukau, 2005). After completion of their molecular chaperone function, HSP 70 and 90 are actively released from protein substrates using intrinsic ATPase domains (Bukau and Horwich, 1998).

The co-chaperones HSP40, p23, FKBP51, 52, Cyp40 and cdc37 facilitate interactions with the substrate of the primary chaperone. Others including GrpE, BAG-1 and HSPBP1 function as nucleotide exchange factors that permit efficient ATP binding and hydrolysis. A third class of co-chaperones permit the molecular chaperones to interact with other proteins: HOP bridges the HSP70 and HSP90 systems and CHIP links HSP70 to the ubiquitin-proteasome system.

Throughout the chapter, where the broad properties of individual HSP families are discussed, abbreviations for the hsp are capitalized, as in “HSP70”. Where individual family members are referred to, as with the *heat shock cognate 70* protein, the symbol is Hsc70.

Protein folding (2), involves the HSP60 “chaperonin” family as well as a number of other related chaperonin proteins (Spiess et al., 2004; Young et al., 2004). Chaperonins self-associate in order to form large folding chambers in which the substrate protein can undergo the appropriate intramolecular interactions required to attain its correct tertiary structure in an ATP-dependent process (Spiess et al., 2004; Young et al., 2004). These distinctions are however not absolute and HSP70 can mediate protein folding (Mayer and Bukau, 2005). Molecular chaperone substrates have been carefully evaluated in *Escherichia coli*, in which at least 340 cytosolic proteins have been shown to require the HSP70 homolog DnaK for folding, while a distinct group of proteins is folded by the HSP60 homolog GroEL (Tomoyasu et al., 2001). Protein folding also involves the HSP27 family; the small HSP assemble into large aggregates that mediate holding and folding in an ATP independent manner (Arrigo, 2005). In addition to this humble molecular chaperone role the HSPs also play key parts in the control of cellular metabolism (Pratt and Toft, 2003). Cell regulation is effected largely by HSP70 and HSP90, each of which can bind stably to a number of regulatory molecules (Pratt and Toft, 2003). HSP90 plays a major role in regulation of mitogenesis and cell cycle progression through such association and HSP70 is closely involved in protection from programmed cell death (PCD) each through interaction with a number of key regulatory proteins.

Of central importance to the regulation of the HSP70 and HSP90 co-chaperone systems is the presence of acceptor sites at the extreme carboxy-terminus for proteins that contain the tetratricopeptide repeat (TPR) domain (D’Andrea and Regan, 2003; Pratt and Toft, 2003; Mayer and Bukau, 2005). The TPR domain is formed of a number of helical structures arrayed in such a way as to forms binding region for TPR acceptor sites in interacting proteins (D’Andrea and Regan, 2003). TPR domain proteins involved in molecular chaperone function include the scaffold protein Hop with at least 2 TPR domains (D’Andrea and Regan, 2003). Hop is thus able to bind simultaneously to both hsp70 and hsp90 and by stabilizing their interactions, permit their coordinated activity in protein folding (Pratt and Toft, 2003; Mayer and Bukau, 2005). In addition, another TPR domain protein Hip aids to the ATPase cycle of HSP70 while the immunophilins Cyp40, FKBP51 and FKBP52 bind to HSP90 through its C-terminal TPR acceptor site and catalyze further steps in protein folding (Cheung-Flynn et al., 2003; Pratt and Toft, 2003; Mayer and Bukau, 2005). Association of the primary molecular chaperones HSP70 and HSP90 with these TPR domain proteins is thus essential for many of the properties of HSP70 and HSP90 required for protein folding.

The molecular chaperone properties of HSPs are harnessed during heat shock when a large number of cellular proteins undergo synchronous unfolding due to the chaotropic effects of heat and threaten the cellular catastrophe of protein aggregation (Schlesinger, 1994). A similar process occurs in aging when proteins damaged by oxidative stress or undergoing aggregation due to dominant conformations that tend towards aggregation begin to accumulate (Liu et al., 1996; Lee et al., 2002).

Such protein aggregation is deterred by engagement of the heat shock response and the accompanying abundant expression of the HSP cohort which recognizes denatured proteins through the holding properties of HSP27, 70 and 90 and refold such denatured proteins with the aid of the chaperonins (Hut et al., 2005). Interestingly studies in *E. coli* have shown that aggregation during heat shock largely involves a group of unstable proteins whose aggregation can be inhibited by upregulation of the *E. coli* HSP70 DnaK (Tomoyasu et al., 2001). In addition, as protein denaturation and aggregation are powerful triggers of PCD, HSP have developed powerful anti-apoptotic properties that deter PCD and thus permit a time window for subsequent repair of the proteome (Beere, 2004; Nylandsted et al., 2004). The massive HSP expression that occurs during the heat shock response involves facilitation of expression at each level, including the activation of the potent heat shock transcription factor 1 (HSF1), HSP mRNA stabilization, selective HSP translation and HSP stabilization at the protein level (Lindquist and Craig, 1988; Calderwood, 2005).

MOLECULAR CHAPERONES AND PROTEASES COMBINE TO MEDIATE PROTEIN QUALITY CONTROL

In all organisms there are proteins of differing degrees of stability which tend to become denatured and aggregated and this process occurs progressively during aging. Two major strategies exist for dealing with protein aggregates and these include (1) protein folding by molecular chaperones and (2) protein degradation by proteases. In *E. coli*, the processes are tightly coupled and the genes encoding the major cytosolic chaperones and proteases are under common control by the transcription factor σ^{32} which controls the heat shock regulon (Tomoyasu et al., 2001). At the functional level however, the molecular chaperones and proteases operate individually to resolve protein aggregates and each can protect *E.coli* against heat shock.

Unlike in *E.coli*, in mammalian cells, proteases are not prominent members of the heat shock family (Lindquist and Craig, 1988; Tomoyasu et al., 2001). However numerous functional links between molecular chaperones and proteases are emerging (Agarraberes and Dice, 2001). It was shown a number of years ago that HSP70 family member Hsc70 plays a role in recruiting substrates to the lysosome and recent studies indicate that HSC70 mediates the entry of such substrates into the lysosomal lumen prior to degradation (Agarraberes and Dice, 2001). However, the most compelling connection between these two systems involves the protein CHIP (carboxyl terminus of HSP70 interacting protein) which bridges the molecular chaperone and ubiquitin-proteasome systems (Ballinger et al., 1999). CHIP contains a U box domain that permits it to conjugate ubiquitin to its substrates and a TPR domain that permits it to bind to molecular chaperones and other TPR domain proteins (Demand et al., 2001; Jiang et al., 2001).

FUNCTIONAL AND STRUCTURAL COUPLING OF MOLECULAR CHAPERONES AND THE UBIQUITIN PROTEASOME SYSTEM THROUGH CHIP

The detailed features of the ubiquitin-proteasome system are reviewed in other chapters of this volume and will not be repeated here. In brief, the ubiquitin-proteasome system permits the specific covalent tagging of proteins that are selected for degradation through a protein degradation machine called the proteasome (Ciechanover, 2005; Hershko, 2005). The proteasome contains multiple proteins including a number of proteases arranged in the form of a degradation chamber which permits rapid degradation of substrates to small polypeptides (Ciechanover, 2005; Hershko, 2005). Proteins selected for degradation by the proteasome are first tagged by the addition of a chain of low molecular proteins called ubiquitin (Ciechanover, 2005; Hershko, 2005). Ubiquitin residues are added sequentially to specific lysine residues in the protein to be degraded (Ciechanover, 2005; Hershko, 2005). This process, known as "ubiquilation" is catalyzed by a three enzyme cascade: Enzymes in the cascade include E1 which recruits ubiquitin, E2 which receives ubiquitin from E1 and E3 the ubiquitin ligase that receives ubiquitin from E2 and couples it to the substrate (Ciechanover, 2005; Hershko, 2005). While there is only one E1 enzyme and a limited number of E2s, there are many E3 ligases which reflect the varied number of substrates in the cell. The E3 ligases which are members of a number of families (the single subunit RING finger type, the multi-subunit RING-finger type and the HECT domain type, UFD2 homology (U box) proteins) permit the selection of a wide variety of motifs in the substrate in order to select the target for ubiquilation; In some cases for instance relative degrees of phosphorylation differentially regulate E3 binding permitting the regulation of protein degradation through protein kinase cascades (Cardozo and Pagano, 2004). This is exemplified by the widely studied the multi-subunit RING-finger type Fbw1 containing E3 ligases that catalyze the ubiquilation of phosphorylated IκBα, β-catenin and Cdc25 and thus control key regulatory pathways (Cardozo and Pagano, 2004). CHIP is a member of the U-box domain family (Connell et al., 2001; Jiang et al., 2001). Significantly, its target specificity is provided by its TPR domain which can bind to the molecular chaperones HSP70 and HSP90 (Connell et al., 2001; Jiang et al., 2001). Thus by binding to molecular chaperones, CHIP can target denatured proteins bound to HSP70 or HSP90 for degradation by catalyzing the poly-ubiquilation of the bound denatured protein (Demand et al., 2001).

MOLECULAR CHAPERONES AND PROTEASOMAL DEGRADATION

When denatured proteins and protein aggregates accumulate within the aging cell, at least three scenarios can be envisaged based on the known interactions between molecular chaperones and CHIP, as depicted in (Figure 1). Aggregates can continue to form as in pathway 1 and lead to cell inactivation and death (Figure 1). However,

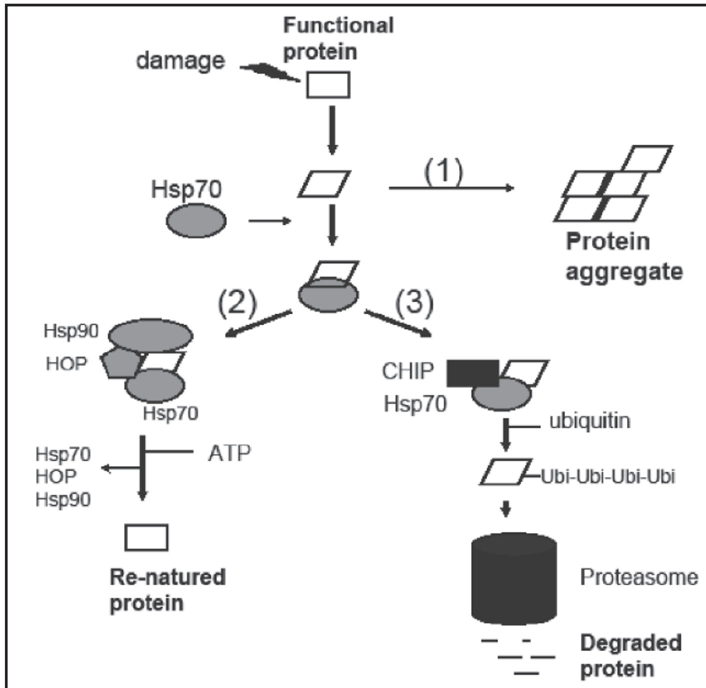

Figure 1. Quality control and salvage pathways for aggregated proteins. Functional proteins may become denatured and aggregated through damage, mutation or interaction with proteins with dominant conformations that lead to aggregation. If not arrested such aggregation (pathway (1)) may lead to cell death. Denatured and aggregated proteins are however recognized by molecular chaperones such as HSP70 and HSP90, which form complexes with them. Binding to the HSP may lead to salvage of the denatured protein through pathway (2) in which the denatured protein is refolded through a pathway involving sequential interactions with molecular chaperones HSP70 and HSP90 which are bridged through binding to the TPR domains of HOP. However, the proteins may enter a third pathway (3) through binding of the HSP70 to the TPR domain of the ubiquitin E3 ligase CHIP. Recruitment of CHIP leads to polyubiquilation of the denatured protein and its degradation through the proteasome pathway. These two pathways (2 and 3) thus compete for the denatured proteins through the binding of key TPR domain proteins in each pathway to the TPR acceptor motifs on HSP70 and HSP90

denatured / aggregated proteins are recognized by the denatured protein binding domains of HSP70 (and HSP90) and form complexes with such denatured proteins. Such complexes can next be assembled into protein refolding chaperone machines as in pathway 2 (Figure 1). The HSP use the TPR acceptor sites in their C-terminal domains to bind to the TPR domain-containing scaffold protein HOP as well as co-chaperones Hip, Cyp40, FKB51 and FKB52 in order to initiate an ATP dependent cycle of reactions that leads to refolding of proteins towards native, functional conformations (Figure 1). However, the proteins can take an alternative pathway (3) when the TPR acceptor site of HSP70 is bound to the TPR domain of CHIP instead of the other co-chaperones; in this case, the substrate protein is brought

close to the U box domain of CHIP, becomes polyubiquilated in cooperation with adjacent E1 and E2 enzymes, and finally targeted to the proteasome and is rapidly degraded (Figure 1). Thus cells have at least two potentially competing pathways for the resolution of protein aggregates. By competing with co-chaperones such as HOP for the TPR acceptor sites on HSP70 and HSP90, CHIP can inhibit the refolding pathway while other TPR domain proteins can, in turn inhibit the CHIP pathway (Connell et al., 2001).

There are in addition, further levels of regulation of the pathways in Figure 1 which are mediated through additional co-chaperones that bind to HSP70. For example, when HSP70 binds to the co-chaperone BAG-1, there is enhanced interaction of the HSP70-CHIP1 complex with the proteasome and increased degradation of HSP70-bound substrates such as glucocorticoid receptor (Demand et al., 2001). By contrast, another member of the BAG family, BAG-2 which also binds to HSP70 inhibits ubiquilation by CHIP and enhances the activity of the refolding pathway (Arndt et al., 2005). Likewise another HSP70 co-chaperone, HSPBP1 inhibits substrate ubiquilation and enhances the refolding pathway (Alberti et al., 2004). These proteins, each of which interact with the ATPase domain of HSP70 are thus plausible regulatory molecules for the competing pathways of protein aggregate resolution by refolding or degradation and provide further layers of regulation.

CHANGES IN HSP EXPRESSION AND PROTEIN QUALITY CONTROL WITH AGING

Aging is associated with the degeneration of HSP expression with time and the loss of resistance to cellular oxidants: elevated HSF1 leads to significant increase in lifespan in *c elegans* and *Drosophila* (Lee et al., 1996; Liu et al., 1996; Gutsmann-Conrad et al., 1998; Bonelli et al., 2001; Verbeke et al., 2001; Hsu et al., 2003). The effects of HSF1 and HSP on longevity appear to be particularly mediated through their ability to protect motor neurones and for instance HSP knockdown in the motor neurones of *Drosophila* causes an equivalent effect on longevity compared to a whole body knockdown (Morrow et al., 2004b,c). The already fragile heat shock response of neuronal cells becomes additionally burdened during exposure to a range of neurodegenerative diseases that lead to the increasing accumulation of insoluble, aggregated proteins and inclusion bodies and the inactivation of components of the proteasomal degradation pathway (Helfand, 2002; Berke and Paulson, 2003; Cowan et al., 2003). We will discuss here the mechanisms underlying the loss of heat shock response in aging with particular emphasis on neuronal cells.

Many of the processes in neurodegeneration are accompanied by decreased expression of molecular chaperones with time and the accumulation of tangled and aggregated proteins which are toxic to neurones (Harrison et al., 1993; Muchowski et al., 2000; Zatloukal et al., 2002). Studies on mechanisms of aging have implicated HSF1 as an important factor in longevity (Garigan et al., 2002; Hsu et al., 2003). As mentioned, inhibition of HSF1 expression or function decreases lifespan while extra copies of *HSF1* increase lifespan in c-*elegans* (Garigan et al., 2002; Hsu et al.,

2003). This increase appears to be due to the transcriptional activation of small heat shock proteins (sHSP: *HSP16.1, HSP16.49; HSP12.6*) in c-*elegans* (Hsu et al., 2003). This increase in longevity due to elevated HSP expression appears to be related to protection of neuronal function as RNAi antagonism of HSF1 expression led to the accelerated onset of polyglutamine aggregates in *c-elegans* (Hsu et al., 2003). The sHSP (as well as HSP70) also play a key role in longevity in *Drosophila* (Tatar et al., 1997; Kurapati et al., 2000). Inactivation of the sHSP family HSP22 gene in *Drosophila* markedly decreases lifespan and, more significantly, a similar decrease in lifespan is seen if only motor neurones are targeted, strongly implicating a critical role for these tissues in aging and dependence on HSP (Morrow et al., 2004a,c). The effects of the sHSP may be related to ability to resist the toxicity associated with oxidative stress. A similar role for sHSP in human disease is provided by recent findings showing protective effects for HSP27 in Huntingdon's disease (Wyttenbach et al., 2002). Naturally occurring polymorphisms in HSP22 and HSP27 are also associated with motor neurone neuropathy (Benndorf and Welsh, 2004). Thus the decreased expression of sHSP or the occurrence of inactivating mutations in sHSP are associated with neuronal cell death particularly in motor neurones (Benndorf and Welsh, 2004).

Heat shock transcription factor 1 (HSF1) is the mammalian regulator of the response to protein stress (including heat, oxidative, ischemic stress) and activates the transcription of heat shock protein (*HSP*) genes (Voellmy, 1994; Wu, 1995). Aggregated, denatured and damaged proteins are the common proximal inducers of HSF1 activity which can be observed acutely during the response to heat shock, a potent protein denaturant (Ananthan et al., 1986; Kampinga et al., 1995; Kampinga et al., 1995). Disruption of the *hsf1* gene in mouse embryonic fibroblasts leads to a profound loss of thermotolerance in such (*hsf1–/–)* cells and markedly increased susceptibility to heat-induced apoptosis (McMillan et al., 1998). Aging is also accompanied by degeneration of the heat shock response and the thermostability of DNA binding activity of HSF1 was significantly reduced with age in a cell-free system as well as in isolated hepatocytes (Heydari et al., 2000). Two additional members of the mammalian HSF family HSF2 and HSF4 have also been discovered (reviewed by (Wu, 1995)). Although their physiological function is not entirely clear, one isoform of HSF2 (HSF2A) cooperates with HSF1 in *HSP* gene transcription (He et al., 2003). One consistent finding in neuronal cells and tissues is that while glial cells express abundant HSF1 and HSF2, and mount a sturdy heat shock response, the heat shock response is deficient in aging neurones (Marcuccilli et al., 1996; Brown and Rush, 1999; Kaarniranta et al., 2002). Similar findings were made by Batalan et al. who showed that HSF1 fails to be activated in motor neurones even when microinjected with plasmids encoding HSF1 expression vector, suggesting a block to the signal transduction pathways leading to HSF1 expression in these cells (Batulan et al., 2003).

In understanding the deficit in expression of HSP during aging, it is important to consider what we know about the regulation of HSF1. Under non-stress conditions, HSF1 is transcriptionally repressed and HSP transcription is minimal (Hensold

et al., 1990; Price and Calderwood, 1991). In the inactive complex, HSF1 is monomer that is constitutively phosphorylated and lacks the ability to bind the *cis*-acting heat shock elements (HSE) located in the promoters of *HSP* genes (Figure 2) (Baler et al., 1993; Rabindran et al., 1993; Wu, 1995).

Increased levels of denatured and aggregated proteins result in conversion of HSF1 from inactive monomer to DNA-binding trimer (Baler et al., 1993; Rabindran et al., 1993; Sarge et al., 1993; Westwood and Wu, 1993). Activation of HSF1 is a multi-step process, involving trimerization, acquisition of HSE-binding activity and inducible phosphorylation, which results in the transcription of HSP genes (Hensold et al., 1990; Price and Calderwood, 1991; Voellmy, 1994; Wu, 1995). HSP90 is the principal cellular repressor of HSF1 in unstressed cells playing a major role in retaining HSF1 in an inactive state; HSF1 trimerization is accompanied by sequestration of HSP90 in protein aggregates and escape from HSP90-containing HSF1 complexes (Zou et al., 1998; Guo et al., 2001). However, little is known regarding the potential role of HSP90 in the decline of HSF1during aging. Other important levels of regulation also control HSF1 activity and it was shown recently that 14-3-3 mediated nuclear exclusion represses HSF1 after activation by the protein kinases ERK1 and GSK3 and subsequent recruitment of 14-3-3 (He et al., 1998; Wang et al., 2003, 2004). HSF1 thus resembles a number of factors in being subject

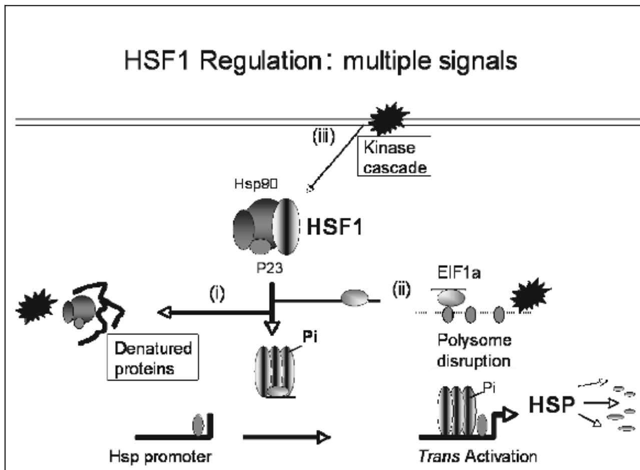

Figure 2. In non-stress conditions, HSF1 is maintained in an inactive complex containing Hsp90, immunophilins such as Cyp40, FKBP1, FKBP2 and P23. Heat shock (i) leads to activation, which involves the escape from the Hsp90 complex, sequestration Hsp90 by denatured proteins, and HSF1 trimerization. HSF1 trimers then bind HSE in Hsp genes. Additional signals also lead to activation. These include: (ii) the binding To HSF1 trimers of the EIF1a/HR1 complex released from heat damaged polysomes and (iii) the induction of a kinase cascade that involves upstream tyrosine kinases and downstream HSF1 phosphorylation on serine residues. Active trimers bind to *hsp loci* that are already in an open conformation with bound RNA polymerase II. Additional on-chromatin events involving ATP dependent remodeling proteins and histone acetylation are also involved

to regulation by phosphorylation and 14-3-3 dependent alterations in nucleocytoplasmic shuttling (Brunet et al., 2002).

POTENTIAL MECHANISMS FOR DECREASED HSF ACTIVITY AND HSP EXPRESSION IN AGING

The increased levels of protein aggregation with increasing age of the organism may be due to (i) progressive accumulation of insoluble protein products with time, (ii) the decreased ability of the molecular chaperone/protein degradation system to deal with increased concentrations of aggregated proteins or, more likely (iii) a combination of both processes. Most studies show a decrease in *hsp* gene transcription and HSF1 activity in neurones and degeneration of HSF1 activity during aging seems a feature of most tissues (Walker et al., 2003; Shamovsky and Gershon, 2004). The decrease in stress-induced HSP expression with age was first ascribed to a decrease in HSF1 expression at the protein level and a reduced ability of HSF1 to form DNA binding trimers (Marcuccilli et al., 1996; Brown and Rush, 1999). HSF1 concentration is at critical levels in most cells and activation requires trimerization, a kinetically rare event favored by higher HSF concentrations – reviewed in (Wu, 1995). Decreases in HSF1 levels thus place the response beneath a key threshold for activation. Subsequent studies also indicated defect in HSF regulation in cultured motor neurones and Batalan et al. showed that, while HSF1 was not activated in cultured neuronal cells even under conditions of HSF1 overexpression, a construct lacking the regulatory domain could be activated (Figure 1) (Batulan et al., 2003). The regulatory domain of HSF1 is a major protein interaction region and phosphorylation of this domain by the protein kinases ERK1 and GSK3 leads to HSF1 repression through recruitment of the adaptor protein 14-3-3 and nuclear exclusion (Figure 2) (Chu et al., 1996, 1998; Holmberg et al., 2001; Wang et al., 2003). Nuclear exclusion leads to loss of HSF1 from *HSP* gene promoters and repression of *HSP* transcription (Wang et al., 2003, 2004). In addition, our recent studies indicate that this domain may also represent a phosphodegron site for ubiquitin E3 ligase binding and modulation of GSK3 may up-and down-regulate HSF1 levels (Khaleque et al., 2005). Interestingly abnormal increases in GSK3 levels occur in some neuronal disorders such as Alzheimer's disease, an effect which may lead to HSF1 repression (Bhat et al., 2004). 14-3-3 levels also become altered in areas of the brain subjected to prion diseases and the appearance of 14-3-3 proteins in the cerebrospinal fluid is characteristic of some neurodegenerative diseases (Van Everbroeck et al., 2005). It thus seems likely that age-related changes in GSK3 and 14-3-3 levels and activities are involved in the progressive loss of capacity in the heat shock response with time particularly in the CNS. The studies of Batalan also showed that increased expression of HSF2, as opposed to HSF1 in cells exposed to inhibitors of the proteosome leads to activation of HSP expression (Batulan et al., 2003). HSF2 can cooperate with HSF1 in the activation of *HSP* promoters, and our studies showed that increased expression of the active isoform of HSF2, (HSF2A) causes a marked increase in stress-induced *HSP* transcription

(He et al., 2003). Altered regulation of both HSF1 and HSF2 may therefore mediate the high threshold for induction of the stress response in motor neurones. HSF1 has also been shown to be regulated by CHIP which mediates the ubiquination of denatured proteins, targeting them for degradation through the proteasomal pathway (Dai et al., 2003; Sahara et al., 2005; Zhang et al., 2005). CHIP is essential for the transcriptional activation of HSF1 (Dai et al., 2003). It may be significant that CHIP and Hsc70 cooperate with another ubiquitin E3 ligase, Parkin in the degradation of the receptor Pael-R and that defects in this system mediate the changes involved in Parkinson's disease (Takahashi and Imai, 2003). It would thus be instructive to examine age related CHIP activity in relation to Parkin inactivation in neuronal cells (Dai et al., 2003; Takahashi and Imai, 2003). In addition, it has been shown that when CHIP associates with HSP70 and HSP90 through its TPR domains it can lead to polyubiquilation of the molecular chaperones themselves in addition to their protein cargo (Jiang et al., 2001). Dysregulation of HSP-CHIP interactions and altered HSP degradation may thus also underlie some of the changes in HSP levels that accompany aging (Jiang et al., 2001). It may also be significant that CHIP associates with expanded polyglutamine repeats that accumulate in cells over time and sequestration of CHIP by high concentrations of proteins bearing polyglutamine repeats may mediate the inhibition of HSF1 in neurodegeneration and aging (Jana et al., 2005). Finally changes in the transcription of HSP genes could occur on chromatin, at steps after the signaling stages described above, and alterations in DNA methylation and histone modification may mediate some of the changes in activity of HSF1 and heat shock promoters during aging.

As discussed earlier, our understanding both of HSF1 regulation and *HSP* promoter function is increasing. The reduced levels of HSP during aging, a decrease which makes neuronal tissues particularly vulnerable to protein damage and cell death, is due to age-related alterations in expression at a number of regulatory levels. Alterations in HSF1 levels, modulations in the functional regulation of HSF1 and changes in the properties of *HSP* promoters have been observed. Although it is known that *HSP* promoters are coordinately regulated by HSF, stress induction of such promoters also involves other independent effects that influence RNA polymerase II activity and chromatin structure (Smith et al., 2004; Thomson et al., 2004). In addition other transcription factors target HSP genes including the FOXO factor DAF 16 and STAT1 and changes in the activity of these factors are likely to make an impact on age-related gene expression (Stephanou and Latchman, 1999; Hsu et al., 2003).

CONCLUSIONS

Mammalian cells possess powerful mechanisms for protein quality control which permit (1) the resolution of aggregated proteins by molecular chaperones and (2) the degradation of proteins that fail to be salvaged by the molecular chaperone pathway through the ubiquitin-proteasome system. Cross talk between these pathways is regulated by TPR domain proteins such as the scaffold protein HOP that permits

assembly of molecular chaperone complexes and CHIP a ubiquitin E3 ligase that mediates the ubiquination and degradation of denatured proteins. These TPR domain proteins compete for HSP-denatured protein complexes and the outcome of this competition determines the fate of the damaged protein. During aging of neuronal cells, the rate of transcription of the HSP genes decreases and the HSP as well the co-chaperones become sequestered in protein aggregates. This leads to a decline in activity of the protein quality control pathways, loss of vigor and the symptoms of aging.

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CHAPTER 19

SERUM HSP70 LEVEL AS A BIOMARKER OF EXCEPTIONAL LONGEVITY

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Abstract: When overexpressed intracellularly, heat shock proteins protect cells exposed to a wide variety of stressful stimuli. In contrast, extracellular heat shock protein 70 (Hsp70) has both protective and deleterious effects. We recently assessed Hsp70 for its potential role in human longevity. Due to the importance of HSP in disease processes, cellular protection, and inflammation, we hypothesized that Hsp70 levels in centenarians and centenarian offspring are different from controls and that alleles in genes associated with Hsp70 explain these differences. In this cross-sectional study, we assessed serum Hsp70 levels from participants enrolled in either the New England Centenarian Study (NECS) or the Longevity Genes Project (LGP). In addition, we examined genotypic and allelic frequencies of polymorphisms in HSP70-A1A and HSP70-A1B. We demonstrated that low serum Hsp70 level is associated with longevity; however, in this study no genetic associations were found with two SNPs within two *hsp70* genes

Keywords: Aging; centenarians; genetics; heat shock proteins; longevity

Abbreviations: HSF-1, heat shock factor-1; Hsp70, seventy kilo Dalton heat shock protein; LGP, Longevity Genes Project; NECS, New England Centenarian Study

INTRODUCTION

Heat shock proteins are proteins that specifically help cells respond to stress. Numerous heat shock proteins have been identified and they are categorized based upon their size. With regard to exceptional longevity, researchers have focused much

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of their work on heat shock protein 70 (Hsp70), the 70-kd heat shock protein, since Hsp70 plays an important role in a wide array of diseases and disorders including neurodegeneration, wound healing, trauma, exercise, development, cancer, cardiovascular disease, and stroke, to name a few (for review see Calderwood et al., 2007). As such, the focus of this chapter will be on Hsp70 and not the other heat shock proteins.

Under normal conditions, Hsp70 expression occurs at low levels within all known cells. However, during stressful conditions, additional intracellular HSPs are synthesized in order to chaperone, transport, and fold damaged proteins (Lindquist and Craig, 1988; Villar et al., 1993). By performing these functions, HSPs prevent and/or limit cellular damage and preserve cell viability (Georgopoulos and Welch, 1993; Hartl, 1996).

In contrast to intracellular Hsp70, which has been extensively studied, less is known about extracellular Hsp70 (also referred to as serum Hsp70). Recent evidence suggests that extracellular Hsp70 activates the immune system resulting in an inflammatory cascade (for review see Asea and DeMaio, 2007). In the context of cellular insult, this cellular response can be protective; however, an excessive response can be detrimental to the cell and the organism.

HSP70 AND AGING

Various cross-sectional human studies have examined serum Hsp70 levels at different ages. Rea et al. demonstrated a progressive decline in serum Hsp70 levels at older ages in 60 individuals ranging in age from 20 to 96 years (Rea et al., 2001). Similarly, Jin et al. noted a decline in serum Hsp70 at older ages in a study of healthy male donors aged 15–50 years (Jin et al., 2004).

Studies examining serum Hsp70 in centenarians and their offspring are consistent with these findings. In a nationwide sample of 87 centenarians, 93 centenarian offspring, and 126 controls enrolled in either the New England Centenarian Study (NECS), or the Longevity Genes Project (LGP) directed by Nir Barzilai at the Albert Einstein College of Medicine, the median sex-adjusted serum Hsp70 levels (ng/mL) were lowest for centenarians and centenarian offspring compared to controls. For the LGP centenarians, NECS centenarian offspring, LGP spousal controls, and NECS controls, Hsp-70 extracellular levels were 1.13, 1.05, 3.05 and 6.93, respectively. After adjustment for covariates including age, sex, smoking status, presence and/or history of hypertension, and myocardial infarction, Hsp70 was significantly lower in NECS offspring than in NECS controls (sex-adjusted $p < 0.001$). Similarly, a trend towards lower Hsp70 was demonstrated among LGP centenarians compared to LGP controls (age-adjusted $p = 0.058$). These results suggest that low serum Hsp70 is associated with longevity even after covariate adjustment (Terry et al., 2006). Of course, this study was unable to account for a host of other covariates that may play a role in serum Hsp70 levels and longevity.

All of the studies referenced above suggest that serum Hsp70 levels are lower at older ages, although they do not indicate whether levels decline within an individual

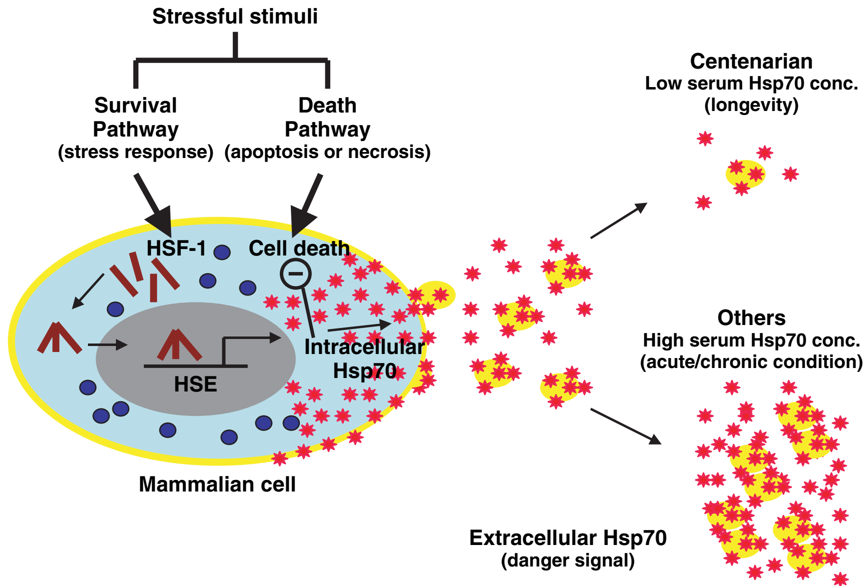

Figure 1. Schematic representation of the working hypotheses. All mammalian cells are composed of an outer plasma membrane a cytoplasm (light grey area) that contains various organelles (circles) and a nucleus (dark grey sphere). Under normal physiological conditions low levels of heat shock proteins are expressed. However, in response to various kinds of stressful stimuli including environmental stimuli (U. V. radiation, heat shock, heavy metals and amino acids), pathological stimuli (viral, bacterial, parasitic infections or fever, inflammation, malignancy or autoimmunity) or physiological stimuli (growth factors, cell differentiation, hormonal stimulation or tissue development), two pathways can be activated. Depending on the intensity of the stressful stimuli the death pathway could be activated and the cell dies via apoptosis or necrosis. Alternatively, the survival pathway could be activated in which the cell protects itself by producing HSPs; a process known as the stress response. During the stress response heat shock factor-1 (HSF-1; rods) found in the cytoplasm of all cells trimerizes and migrates from the cytoplasm to the nucleus. Once inside the nucleus the trimerized HSF-1 binds to a specific region of the DNA known as the heat shock element (HSE). This binding initiates the synthesis of many heat shock proteins including Hsp70 (stars). The increased expression of Hsp70 (stars) within the cell (intracellular Hsp70) protects it from stress-induced cell death (–). During the stress response, Hsp70 is released into the extracellular milieu (extracellular Hsp70). If the extracellular Hsp70 comes from a stressed or damaged self-protein and is taken up by professional antigen presenting cells (e.g., macrophages, dendritic cells and B cells) it will act as a danger signal. This implies that it will stimulate a potent inflammatory response including the release of pro-inflammatory cytokines, chemokines, nitric oxide production and maturation of dendritic cells, a process known as the chaperokine activity of Hsp70. In this study, we show that centenarians have low extracellular Hsp70 concentrations. High concentrations of serum Hsp70 have been reported in individuals with acute and/or chronic conditions including arthritis, acute myocardial infarction, arthrosclerosis, cancer and acute infection

over time. To our knowledge, no study has examined this very important question as of yet. Other studies have examined whether there is a decline in Hsp70 function over time. Cell lines from centenarians have been used to examine this question. When exposed to heat stress, cell lines from centenarians demonstrated a similar transcriptional response to the *hsp70* gene (Ambra et al., 2004). In addition, they had similar Hsp70 synthesis (Marini et al., 2004), and were less prone to apoptosis, or cell death. These findings suggest that centenarians have preserved intracellular Hsp70 function; however, to our knowledge, extracellular Hsp70 function has not been examined.

There are several proposed explanations as to why serum Hsp70 levels are low at very old age (see Figure 1). It may be that cells in long-lived individuals are exposed to similar stress but do not mount the same response from heat shock factor-1 (Hsf-1), the transcription factor that regulates Hsp70 synthesis. Data from both rat hepatocytes (Wu et al., 1993) and human T-lympocyte (Effros et al., 1994) cultures that demonstrate a decrease in Hsp70 synthesis with age are consistent with this notion. However, a study of *Caenorhabditis elegans* makes this notion less plausible since an overexpression of the *hsf-1* gene in this organism leads to a 40% greater life-span compared to wild type animals (Hsu et al., 2003).

Another possible explanation for the low serum Hsp70 level in very old individuals and their offspring is that their cells synthesize similar amounts of intracellular Hsp70 but the cell walls are less permeable resulting in less leakage into the extracellular space. Consistent with this, recent evidence indicates that there is a direct relationship between intact cell membranes and the release of Hsp70 (Broquet et al., 2003; Bausero et al., 2005).

Lastly, it may be that survivors to very old age have always had low serum Hsp70. This could be attributed to a lack of exposure to cellular stress or it could be because of decreased exposure to inflammation. A lack of exposure to inflammation may, in part, explain why centenarian offspring, who have significantly lower serum Hsp70 levels, also have lower rates of cardiovascular disease, and fewer cardiovascular risk factors (Terry et al., 2003) compared to similarly aged controls.

GENETIC FINDINGS

The role of genetics in the relationship between Hsp70 and survival to very old age has only begun to be examined. Altomare et al. (2003) in a study of Italian centenarians, determined an association between longevity and a polymorphism within the *hsp70* gene promoter in females only. Marini et al. subsequently reported that when compared to the C/C genotype, the A/A genotype of the promoter region was associated with a reduced expression of Hsp70 protein in response to heat stress (Marini et al., 2004).

In contrast to the positive associations noted above, genotypic and allele frequencies of two functional SNPs, rs1043618 in HSP70-A1A and rs6457452 in HSP70-A1B, that were examined in centenarians, their offspring and controls enrolled in the NECS or the LGP, did not reveal any associations with longevity

(Terry et al., 2006). There are several potential explanations for this negative finding: (a) it is a true finding, (b) the sample sizes were not robust enough to determine an association, and (c) the differences in serum Hsp70 levels are the consequence of genetic differences further upstream. Future genetic and proteomic studies may help to further elucidate the role of Hsp70 levels, genetics and longevity.

CONCLUSIONS

In conclusion, serum Hsp70 levels are lower in those individuals that reach an advanced age. In addition, low serum Hsp70 levels are associated with longevity independent of other covariates such as age, gender, race, income, alcohol, cardiovascular disease, and a variety of other age-related diseases. No statistically significant associations were found with two tagSNPs within *hsp70* genes. The reason for this is unknown. Likely, further investigation is needed to determine why centenarians and centenarian offspring have low levels of serum Hsp70 and to better understand the role of genetic polymorphisms in this observation.

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Errata

The first author name is N. K. Singh.
It is unfortunate that the name appears as Al K. Singh.

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