Heat Shock Proteins 6
Series Editors: Alexzander A. A. Asea · Stuart K. Calderwood

Brian Henderson
A. Graham Pockley *Editors*

Cellular Trafficking of Cell Stress Proteins in Health and Disease

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HEAT SHOCK PROTEINS

Volume 6

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Preface

The establishment that cells responded to stress by the induction of specific gene expression led to the identification, in the 1980s, of heat shock or cell stress proteins and the realisation that these proteins are involved in the folding, re-folding, and prevention of aggregation, of client proteins. Such protein-folding proteins are now known as molecular chaperones, with the term protein-folding catalysts (PFCs) being applied to proteins such as thioredoxin and peptidyl prolyl isomerases that involve an enzymic step in the protein folding mechanism. Initially, these proteins were thought to be exclusively intracellular. However, in 1988 evidence was presented for the secretion and uptake of Hsp70 proteins by cultured cells suggesting that at least one molecular chaperone underwent aberrant cellular trafficking. Some years later the aberrant cytoplasmic and cell surface location of the mitochondrial Hsp60 protein began to be defined. These finding suggested a potentially wider remit for the function of molecular chaperones within the cell than had previously been considered.

A growing number of reports in the 1990s established that a number of molecular chaperones and PFCs had cell signalling actions when applied externally to cultured cells, and this realisation has prompted a rapid expansion of work in this field. Despite being dogged by suggestions that the biological and immunological properties of extracellular stress proteins results from contaminants in the preparations used, it is now becoming accepted that cell stress proteins can indeed be released from cells and that, in the extracellular environment, they elicit a number of regulatory functions. This line of work has culminated in the finding that a number of molecular chaperones and PFCs are present in the circulation and that levels of these proteins may reflect tissue and organismal pathology.

Since the beginning of the twenty-first century there has been a rapid increase in our understanding of the cellular trafficking mechanisms of molecular chaperones both in eukaryotes and in prokaryotes. In the former, molecular chaperone trafficking can occur between the various cellular compartments, with concomitant movement of other proteins and in some instances at least, the release of molecular chaperones from cells. In bacteria, molecular chaperones are involved in the trafficking of other proteins and are themselves released into the external milieu. There is an increasing appreciation of the role of molecular chaperones and PFCs in the interplay between bacteria and the cells of their hosts and this is now an important area of research for understanding the mechanisms of infectious diseases.

This volume brings together experts in the biochemistry, cellular biology, immunology and molecular biology of molecular chaperones and PFCs with a focus on the mechanisms of cellular trafficking of these proteins and the role of these variegated trafficking mechanisms in both human and animal health and disease. To guide readers who may be unfamiliar with this, now voluminous, field of research, this book starts with a number of introductory chapters which provide a historical background to the key aspects of molecular chaperone biology. The second section focuses on intracellular trafficking of molecular chaperones and their interactions with different cellular compartments and cellular components and the roles that such trafficking plays in the maintenance of cell health and in controlling the death of the cell. The third section deals with the roles played by molecular chaperones in the control of selected receptors that can play roles in immunological homeostasis. Section 4 deals with the unexpected finding that molecular chaperones can actually exist in the extracellular milieu and the consequences of such release for health and disease.

This book should be of interest to a wide range of biomedical scientists.

Brian Henderson
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Note on Nomenclature

Understanding the biology of molecular chaperones and protein folding catalysts is complicated by the fact that there are several nomenclatures for these proteins, often with different names being given to prokaryotic and eukaryotic homologues. In addition, a new nomenclature has been introduced for some of the major human molecular chaperones (Kampinga et al. 2009). In this nomenclature the following names are given for particular molecular chaperones:

Old Name	New Nomenclature
Hsp10	HSPE1
Hsp27	HSPB3 (plus 10 other designations for human small HSPs)
Hsp40	DNAJB1 (plus 49 other designations for the human proteins)
Hsp60	HSPD1
Hsp70	HSPA1A (plus 12 other designations for the remaining human Hsp70 proteins)
Hsp90	HSPC1 (plus 4 other designations for the Hsp90 proteins)

Kampinga HH, Hageman J, Vos MJ et al (2009) Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 14:105–111

Chapter 1
Discovery of the Cellular Secretion of Cell Stress Proteins

Lawrence E. Hightower and Emily J. Noonan

Abstract This chapter describes the discovery of extracellular Hsps or molecular chaperones. The first part, written by LEH, provides the historical context and a personal account of this discovery. The second part, written by EJN, brings these findings up to date by describing new discoveries that support and extend the original observations.

1.1 Introduction

A number of the major discoveries in the field known historically as the heat shock response, and more broadly as the cellular stress response, initially were met with quizzical expressions, if not outright disbelief. And no wonder, since several of these discoveries caused paradigm shifts in our view of how cells, tissues and, ultimately, animals function. This was one of the themes of a recent review article by Antonio De Maio (De Maio 2011) that he dedicated to the pioneer of the heat shock response Ferruccio Ritossa with the statement "It is never known how far a controversial finding will go!". In this article, Dr. De Maio pointed out that Ritossa's manuscript was rejected by the editor of a prestigious journal for insufficient relevance to the scientific community. Eventually it was accepted and published in the Swiss journal *Experientia* (Ritossa 1962). He also noted that my observation that heat shock proteins (Hsp) are released from mammalian cells "was initially deemed irrelevant and impossible". Our objectives in the present chapter are to provide the background on how this discovery came about and to link it to more recent studies.

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1.2 The Path to a New Discovery

In the early 1980s researchers in my laboratory and others were searching for functions of the Hsps (particularly Hsp70 now termed HSPA1A—see nomenclature section) inside of cultured animal cells and tissues as well as carrying out biochemical studies on purified Hsp70 in an effort to obtain clues to its functions based on its molecular properties. Two events led us to look for extracellular Hsps. The first was our collaboration with Fredric P. White. Fred had found evidence for a protein translocation system in incubated rat brain slices. This system was inhibited by vinblastine, colchicine and low concentrations of calcium ions (White 1979). Hsp70, which he called SP71, was among the proteins that rode this translocation pathway away from blood capillaries (White 1980). The mechanism was not known but he suggested two possibilities: synthesis of Hsp70 in the end feet of astrocytes that abut microvessels with intracellular transport away from the capillary bed, and synthesis of Hsp70 in vascular endothelial cells followed by extracellular transfer to astrocyte glial cells. This latter proposal was clearly heretical at the time, when Hsp70 was considered by almost all investigators to spend its entire useful lifetime inside of cells. The second stimulus was a localization study from the Lindquist lab. Susan and I were graduate students in the same program at Harvard University and even though we did not meet each other then, I knew her mentor Matthew Meselson. In fact, we had even managed to lunch together at the Hotel Continental in Saigon during the Vietnam War, when I was a soldier drafted out of graduate school into the US Army and Professor Meselson was on a USAID mission to evaluate the effects of Agent Orange on the civilian population. We discussed his plans and a possible collaboration. By the time I returned to Cambridge, MA, he was already presenting a dramatic talk on the effects of Agent Orange on the rural families and their crops in South Vietnam. I had a rough idea what was going on in his laboratory, which was supplemented by my friend and fellow graduate student Thomas Cline who worked just down the hall in Woody Hastings' laboratory at the Biological Laboratories. When I moved to the University of Connecticut to begin my faculty career, it was Tom who suggested to me over Thanksgiving turkey that I may have stumbled into the heat shock field, which was known only in *Drosophila* at that time. My description of the proteins induced by the amino acid canavanine in culture chicken embryo cells as a kind of stress protein, triggered Tom's brain to make the association with *Drosophila* Hsps. Interestingly, Susan and Tom, the two fellow graduate students who helped me the most in trying to understand what I was studying, both went on to win the US National Medal of Science!

I promptly ordered Susan's Ph.D. thesis on interlibrary loan. My summer student and I read it cover to cover. After that, I sought out every paper that subsequently flowed from the Lindquist lab. In a 1980 paper, they showed that Hsps were not only concentrated in the nuclei of *Drosophila* salivary gland tissues, but also at the cell boundaries, especially over the lumen of the gland (Velazquez 1980). This latter point had been virtually ignored and certainly neglected by investigators in the field. It suggested membrane associations and possibly secretion into an extracellular compartment. Peter Guidon, Jr., one of my Ph.D. students, had already found fatty

acids noncovalently associated with Hsp70 and its cognate Hsc70 purified from tissues of heat shocked rats (Guidon and Hightower 1986). We had begun to think about these Hsps as lipoproteins. The ideas of membrane associations and release into the extracellular space fitted this hypothesis and we started to look. In 1985 we published our first two-dimensional gel patterns of proteins released into the medium over cultured rat embryo cells using a radioisotopic pulse-chase protocol (Hightower et al. 1985). The newly made radioactive proteins that chased into the culture medium were mostly the same from control and heat shocked cells, based on molecular masses and isoelectric points. For example, the rat heat shock cognate protein Hsc73, which we had identified previously, was present in both patterns. However, medium from heat shocked cells contained two additional inducible proteins, Hsp71 and Hsp110, not detected in medium from control cells. Rat Hsp71 was the major inducible protein generally known as Hsp70 and the other protein was later studied in great detail by John Subjeck and his co-workers and shown to be a phylogenetic relative of Hsp70 with some of the same activities that made it even better at molecular chaperoning (Leeyoon et al. 1995). It was still early for us in this line of investigation so we could not yet rule out the possibility of release of these proteins by necrotic cells in the cultures. But already it was clear to us that the overall gel patterns of released proteins were different than those from cytoplasmic extracts prepared by detergent lysis and that both the sodium ionophore, monensin and the antimicrotubule drug, colchicine did not block this release. We began thinking about the possibility of a new release mechanism different from the ER-Golgi pathway.

Later that same year, Fred White called me to discuss a poster from the laboratory of Michael Tytell that he had seen at a neuroscience meeting. On the poster were gel patterns of proteins isolated from squid giant axons known as glia-axon transfer proteins. Within these patterns, Fred had noticed polypeptides that looked a lot like those he had seen in patterns of stressed rat tissues in his work. This was intriguing and hinted at the possibility of tissue level functions and cell to cell transfer, if indeed they were Hsps. Approximately a year later, Tytell and co-workers published their work done in the squid giant axon model and titled "Heat shock-like protein is transferred from glia to axon" (Tytell et al. 1986). They noted the similarity in molecular mass and isoelectric point between traversin, the most abundant protein transferred from glia to axons, and Hsp70. In addition, they had found the publications from the Lasek laboratory showing that a traversin-like protein was produced in the heat shocked nerves of *Aplysia californica.* The excitement about the Tytell paper for us was the fact that here was a new tissue level property of Hsp70 that suggested a general function which we began calling 'altruistic cytoprotection'. Two heat-inducible proteins were transferred into the axon, traversin (Hsp70) and Hsp95. They carried out careful controls to show that these Hsps were not produced by the axoplasm and must have been transferred in. The transfer mechanism was not known but Tytell and coworkers speculated that it might involve the engulfment by the axon of cell surface membrane protrusions of the glial cells. This mechanism would still find favor today. But transfer for what purpose? They suggested that in the squid the transfer of these proteins might help the axon cope with the acute effects of injury or stress. They also used Fred White's favorite word for the response trigger in mammalian tissue, 'trauma'. They

then did a very clever thing. They cited the work by White and Currie describing these proteins in the mammalian central nervous system and generalized their conclusions. Such cell-to-cell transfer could be a mechanism for distribution of Hsps throughout a tissue or entire organism. All cells need not make Hsps, particularly cells with limited protein synthesis capacity or unusual processes like nerve cells that required a long time to get newly made proteins from cell body to deep into the axon. At the time, one could practically count on the fingers of one hand the number of published papers analyzing the heat shock response as a tissue level response.

1.3 Hsps are Released from Mammalian Cells by an Active Process Other than the ER-Golgi Pathway: The Odyssey of Peer Review

In the same year that our book chapter appeared, I attended a heat shock meeting in Santa Fe, NM. I met at the poster session one of the first cellular immunologists whom I had seen in the field, and of course he was interested in the possibility of extracellular Hsps as potential cytokines. I told him that I had found several Hsps in the culture medium over mammalian cells but I did not know the mechanism of release. He said that as an immunologist, he did not care how the Hsps were released, he was interested in what they did afterward. This point of view was in stark contrast to that of a prominent biochemist and cell biologist who told me at the same meeting that I would never convince colleagues of the biological relevance of extracellular Hsps until I had determined the molecular mechanism of their release from cells. This should have been a big clue to what would lie ahead when we tried to publish our observations, and indeed, we worked to include controls to rule out release from dead and dying cells.

1.3.1 Hightower and Guidon 1989

We then proceeded to develop our preliminary study described above into a full length paper. My approach to studying the release of Hsps from late stage rat embryo cells was based on years of work with chicken embryo fibroblasts infected by the avian paramyxovirus, Newcastle Disease Virus. We had investigated the synthesis and processing of viral glycoproteins in the ER using radioisotopic pulse-chase protocols and inhibitors of glycoprotein biosynthesis (Schwalbe and Hightower 1982). This protocol allowed us to follow the migration of viral glycoproteins to the cell surface where they were incorporated into budding virus particles at the plasma membrane. Essentially the paramyxoviral particles can be viewed as specialized vesicles so we were keyed into thinking about these pathways as well. Several modifications of the viral protocols were necessary for the Hsp release protocol. Confluent cultures of rat embryo cells were heat shocked at 45 °C for ten minutes followed by a 2 h recovery at 37 °C to allow protein synthesis to recover. It became important that

these initial manipulations were done without medium changes. Following recovery, the cultures were washed and placed in label medium containing ^{35}S-methionine at high specific activity for ten minutes at 37 °C. The cultures were washed again and placed in chase medium containing 10-fold excess unlabeled methionine for further incubation at 37 °C. A key modification of the chase medium was the omission of serum. Albumin with a molecular of mass of about 68,000 had to be eliminated because it overloaded the two dimensional gels and distorted the region containing Hsp71 and Hsc73. In addition, ovalbumin at 43,000 Da was used as a carrier in the medium precipitation step instead of albumin. The pH was carefully controlled throughout.

Quite unexpectedly, we found that the medium washes stimulated the release of Hsp71 (inducible Hsp70), Hsc73 (constitutive Hsc70), Hsp110 and nonmuscle actin from heat shocked cells and Hsc73 along with nonmuscle actin from control cultures. This confirmed a previous observation from Peter Rubenstein's lab, coincidentally another classmate from Harvard, about the release of nonmuscle actin from cultured embryonic skeletal muscle cells (Rubenstein 1982). A small amount of Hsp110 or possibly a cognate form of this protein was also detected among the proteins released from control cells. Elimination of the medium washes also eliminated the release of these extracellular proteins. We were encouraged by the highly reproducible nature of the gel patterns from independent experiments that immediately suggested that we were not dealing with the release of cytosolic protein from a variable number of necrotic cells in these cultures. The release was not dependent on heat shock: similar proteins were released from control cells as well. Further, the release was not continuous but appeared to be stimulated by a simple medium change, i.e. a change in the extracellular environment of the cultured cells. A thumbnail calculation indicated that, only a small amount of the newly synthesized Hsps were released from cells, roughly 1–2 %. The release was very rapid, on the order of seconds not minutes and thus it was unlikely to involve the 'classical' pathway of ER-Golgi-plasma membrane migration.

In order to formally remove the possibility that the release involved the 'classical' pathway, we showed that the carboxylic ionophore monensin, which inhibits protein secretion by vesiculation of the Golgi complex, and colchicine, which blocks transport vesicle movement by inhibiting microtubule assembly, did not block these rapidly released Hsps and actin. They did however block the extracellular appearance of Grp78, which became our positive control protein, since it was known to be produced and to function in the lumen of the ER. Small amounts of these ER proteins were known to escape the ER retention mechanisms so it was not surprising to find small amounts of extracellular Grp78, another member of the Hsp70 family. Other treatments were tested including chasing at 46 °C to block a possible thermolabile component, but to no avail. Also, neither cytochalasin E or the Ca^{++} ionophore A23187 added to the chase medium failed to inhibit the rapid release Hsps and nonmuscle actin. This would become the Achilles heal of the first manuscript submitted to the Journal of Cellular Physiology. We were proposing that the release was specific for the detected set of proteins described above but we had not identified a specific inhibitor of the process, which would have at least operationally defined a new release pathway. The reviewers balked and the editor declined to publish the work until this issue was addressed.

Our original submission did include several experiments aimed at the possibility that the extracellular proteins were released by lysis of a small number of necrotic cells in the cultures. Cells labeled under our standard conditions with ^{35}S-methionine were either treated with 1 % Triton X-100, a nonionic detergent, or dounce homogenized in a low salt buffer. The patterns of proteins released under these conditions were more complex and dominated by the tubulins and vimentin, both known to be released readily from necrotic cells. Neither of these proteins were found under our conditions of rapid release. Therefore, we confidently concluded that the proteins in our gel patterns were not the result of release from necrotic cells in our cultures. After our manuscript was returned from the journal, we spent months considering how to address the reviewers' concerns experimentally. Ultimately the answer had been sitting in front of us the entire time in the guise of small bottles of amino acid analogs that we used to produce analog-substituted proteins to study the induction of Hsps under conditions that did not involve the use of heat as a stressor (Hightower 1980). Analog-substituted proteins often did not fold properly and as a result, they might compete for the rapid release machinery thus blocking release. Alternatively they might not achieve the proper cellular location to engage the release mechanism. We simply added the lysine analog aminoethylcysteine to the radioactive methionine-containing label medium and performed our standard pulse-chase protocol. Synthesis of the ^{35}S-methionine labeled analog-substituted proteins could only occur in metabolically active cells. However, release of these analog-substituted radioactive proteins was blocked, consistent with the idea that the release was from living cells via a specific release pathway that depended on the three-dimensional conformation of the released proteins. We added the new experiment to our manuscript and it was promptly accepted.

The nature of the release mechanism remained elusive. We were well aware from the prior work of both Fred White and the Tytell group that vesicles might be involved. Using ultracentrifugation methods, we found that the Hsps and most of the actin in the releasate remained in the supernatant following centrifugation at 65,000 g (4 °C) for 30 min. We also layered the releasate onto 10–65 % sucrose gradients and subjected them to contrifugation at 65,000 g (4 °C) for 16 h. Again, the rapidly released radioactive proteins remained in the supernatant on the top of the gradient. We did not find evidence of vesicles or large aggregates but such a negative result cannot rule out the possibility that the extracellular Hsps may have been released into labile vesicles that did not survive in our buffers. The presence among the rapidly released proteins of nonmuscle actin, some of which is located in the cellular cortex, suggested the enticing possibility of an active release mechanism involving microfilaments underlying regions of plasma membrane. We cited an immunocytological study (LaThangue 1984) reporting that proteins analogous to Hsc73 and Hsp71 colocalize with actin-containing microfilaments in the ruffling membrane at the leading edge of motile fibroblasts. We proposed that 'perturbations of the cell surface of cultured cells cause the selective release of proteins from such dynamic regions of the cell periphery'. Writing today, we would add exosomes, ectosomes and microparticles to the short list of possibilities.

The specific stimulus associated with the medium washes that triggered release was also frustratingly elusive. Some of the likely possibilities included pH

fluctuations, changes in gas tension, disruption of the diffusion boundary at the cell surface, or mechanical stresses such as the flux of media over the cell surface. We recognized at the time that these same factors were under consideration as part of tissue level responses to trauma and we included as a possible *in vivo* correlate the phrase "cellular responses to breaks in tissue homeostasis such as wounding". Recently, I had the pleasure of writing a meeting review with two colleagues describing some of the ongoing research that links cellular stress responses to wound healing (Doshi 2008). It is clear now that the release of Hsps from cells is part of the cellular stress response and that it is a normal release pathway that has been co-opted evolutionarily by Hsps as part of cellular stress responses.

This work is right up there among the most satisfying research endeavors of my professional career from the standpoint of intellectual stimulation and creativity at the bench. In retrospect and if I could magically go back in time to the writing of this paper, I would add a sentence or two to cover the possibility that the released proteins might function as cytokines, or as Calderwood, Asea and colleagues termed them, chaperokines (Asea et al. 2000).

1.4 Extracellular Hsps-Still Hot

In a new age of high throughput and *in silico* research methods we have now firmly established that extracellular detection of Hsps is not restricted to a certain Hsp, cell type or physiological context. Moreover they clearly lack the hydrophobic signal peptide for ER/Golgi release or any uniformly encoded secretory signal sequence. The Stress Observation System (SOS), or the ability of extracellular Hsps to function as cellular communicators in response to stress, was a termed conceived by Antonio De Maio in his aforementioned review (De Maio 2011). Indeed this is a hot area in the chaperone field, and one that has kept researchers busy over the past few decades uncovering these important findings. Key issues still remain surrounding the mechanism of Hsp trafficking from the cytosol including the working destination of these molecules, be it the cell membrane, cell surface or into the extracellular space, and more mysteriously, the function of the extracellular Hsps in mediating cellular responses to stress. Central to their function we must ask if their signaling is intended to be autocrine, paracrine or endocrine. Given the functionally rich history of Hsps in response to stressors and altered physiological states, the answers are most likely not very restrictive.

1.5 Mechanism of Extracellular Hsp Release

The non-classical (non-ER/Golgi) putative secretion mechanisms of Hsps probably occur by a series of events which help them reach their target destination. In a paper by Mambula and Calderwood in 2006, the authors showed that Hsp70 is released from certain tumor cells through a mechanism using the endo-lysosomal compartment (Mambula and Calderwood 2006). Interestingly their data provided

evidence that ABC transporter proteins helped facilitate this process as secretion was blocked with their inhibition. In a later review of the subject, Mambula and Calderwood speculated that Hsps are released through this pathway by merging of the endo-lysosomal compartment with the plasma membrane, and subsequent release at the cell surface (Mambula et al. 2007). There are other studies reporting detection of Hsps in the lysosomal compartment following stress in fish, rodents and humans (Mayer et al. 1991; Jethmalani et al. 1997; Polla et al. 2007; Yabu et al. 2011).

Release of Hsps by vesiculation is one of the more commonly proposed mechanisms of release. Heat responsive chaperones may have a higher cortical cell concentration following stress aiding in their release by vesiculation. Vesiculation is typically described as the release of the Hsps through extracellular vesicles. In general, these vesicles originate from the plasma membrane through different mechanisms and would include, exosomes, ectosomes and microparticles. Exosomes are formed through an endocytic process in which the plasma membrane invaginates inward engulfing not only components of the membrane itself (like surface receptors and ligands) but additional cytosolic content (including Hsps). This results in the formation of early endosomes which mature into late endosomes by removal of specific membrane proteins and lipids that are returned to the cell membrane. The late endosomes undergo a secondary invagination resulting in the formation of multivesicular bodies (MVB). These organelles are then able to fuse with the plasma membrane moving their contents into the extracellular microenvironment (Mathivanan et al. 2010). Formation of ectosomes and microparticles occur by way of membrane evagination and involves the help of actin polymerization. Similarly to exosomes, these vesicles also contain both membrane and cytosolic components. What is interesting about both of these processes in extracellular release is that they appear to have a filter for their contents, not just capturing whatever is in the neighborhood but a more specific array of molecules. In a number of different species and physiological conditions, different Hsps have been shown to be released through exosomes. These include the constitutively expressed Hsc70 (Hegmans et al. 2004; Buschow et al. 2010), heat inducible Hsp72 (Bausero et al. 2005; Asea et al. 2008; Chalmin et al. 2010; Hurwitz et al. 2010), and Hsp90 (Cheng et al. 2008; Buschow et al. 2010; Mccready et al. 2010), Hsp60 (Gupta and Knowlton 2007), and the small chaperones Hsp27 (Clayton et al. 2005) and AlphaB crystallin (Gangalum et al. 2011). Mitochondrial Hsp70 has been detected in ectosomes or microparticles, although its exact role has not been established (Moskovich and Fishelson 2007). Lastly, secretory-like granules were found to play a role in release of Hsp70 following heat shock in A431 (human epithelial carcinoma) cells (Evdonin et al. 2006).

1.6 Hsps on the Cell Surface

When considering the presence of Hsps on the cell surface, translocation is another potential mechanism of transporting these molecules extracellularly. Hsp70 has been found in association with lipid rafts in human colon cancer cell lines. Translocation

is enhanced by heat shock and cannot be blocked with secretory inhibitors but can be inhibited with lipid raft disrupting agents (Broquet et al. 2003). This parallels with reports of Hsp70 being able to incorporate into artificial lipid bilayers and play a role in channel conductance (Arispe and De Maio 2000; Vega et al. 2008). The ability of Hsp70 to incorporate into the lipid membrane is thought to be dependent on membrane fluidity and composition (Arispe et al. 2004; Gehrmann et al. 2008; Horvath et al. 2008; Sugawara et al. 2009). As Hsps are also involved in shuttling other transmembrane proteins across these lipid membranes, it seems logical that these chaperones could then remain at the cell surface essentially having piggybacked their substrate. It is now well established that a number of different Hsps can be found on the surface of tumor cells in both stressed and unstressed conditions. For example, high levels of cytosolic Hsp72 accumulate in response to certain therapies in tumor cells and can then become expressed on the surface of these tumor cells (Gehrmann et al. 2005). Hsp72 is expressed on the surface of many cancer cells lines in response to stress (Multhoff et al. 1995). Hsp72 is also expressed on the surface of tumor cells under physiological conditions (Botzler et al. 1996; Multhoff et al. 1997). Hsp70B', an Hsp70 family member expressed in response to extreme stress, is only found on the surface of tumor cells in response to proteasome inhibitors and not mild heat shock (Noonan et al. 2008). Interestingly, in normal cells, Hsp70 is associated with cell surface receptors while in tumors it is integrated into the cell membrane. This distinction becomes important in terms of technical approaches to distinguishing between these different associations. For details see a recent review (Multhoff and Hightower 2011). In conclusion, we have only scratched the surface in our explorations of the exciting lives of extracellular Hsps, a research area that may well yield new therapies for major human diseases and new insights into human physiology.

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Chapter 2
Discovery of the Extracellular Agonist Actions of Molecular Chaperones and Protein-Folding Catalysts

Brian Henderson

Abstract Surprisingly, the history of the agonist actions of extracellular molecular chaperones can be traced back to the 1970s, with the cytokine macrophage migration inhibitory factor (MIF) and chaperonin (Hsp)10. The next cell stress protein to be identified as a molecular chaperone was the peptidylprolyl isomerase, cyclophilin A, in 1992. It is only later in the 1990s that the major signalling cell stress proteins—chaperonin (Hsp)60 and Hsp70 are found to have agonist activities. There are still ongoing discoveries of stress proteins with agonist actions and the latest such proteins are a new group of molecular chaperones—the extracellular/circulating molecular chaperones which include clusterin and α-acid1-glycoprotein.

2.1 Introduction

The phenomenon of the human system called science is endlessly fascinating for the paradoxes it encompasses. Science, at its heart, is the creation of the story of the Universe/Multiverse we live in, with its various disciplines focused on different parts of this larger picture. The basic unit of science is the testable hypothesis and the discoveries that the hypothesis machine provides us with are never complete and always need to be altered or even radically changed. Starting with the initial discovery of the heat shock response (Ritossa et al. 1962), the evolution of the phenomenon of the cell stress response and the discovery of the process of protein chaperoning (Laskey et al. 1978), it was the basic assumption that the proteins involved in the stress response were only active within the cell. This paradigm of molecular chaperones and protein-folding catalysts (PFCs—collectively cell stress proteins) being found only within the cell, has seriously, and negatively, influenced the new paradigm that has emerged since the 1990s, that molecular chaperones and PFCs can be secreted from the cell interior and function as cell surface receptors or cell signalling soluble agonists (See Chap. 1 for a personal history of the discovery of the secretion of Hsp70). This failure, by the cell stress protein community, largely to ignore the biology of extracellular molecular chaperones and PFCs, is curious, as in 1977, 1 year before Laskey's coining of the term chaperone for the function

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of nucleoplasmin, a group of Australian scientists identified an immunosuppressive factor in the serum of women in the first trimester of pregnancy (Morton et al. 1977; Noonan et al. 1979). Unfortunately, the molecular nature of this factor was not identified until 1994, when it was shown to be human chaperonin (Cpn)10 (Cavanagh and Morton 1994). However, this result seems to have been overlooked and the growing numbers of publications, arising from around the early 1990s, on the cell signalling actions of recombinant cell stress proteins, which would have been strongly supported by the physiological actions of Cpn10, were largely ignored or criticised on the grounds that they were due to bacterial contaminants (e.g. Tsan and Gao 2009). This criticism is still extant at the time of writing, even though there are a growing number of reports of the actions of extracellular cell stress proteins that could not possibly be due to bacterial contamination—either because the actions have nothing to do with the activity of pro-inflammatory bacterial contaminants or because the proteins under study are made in eukaryotic systems or are, indeed, totally synthetic proteins/peptides (Henderson and Pockley 2010; Henderson et al. 2010). It is hoped that reviews like this one, which provide a historical perspective, will encourage a fairer response to the study of the extracellular actions of molecular chaperones.

2.2 Secretion of Molecular Chaperones

Cell stress proteins are soluble signalling mediators—is a proposition that can only be accepted if there is evidence that these proteins are capable of being secreted. Again, the cell stress community has been guilty of ignoring key information, such as the early work from Tytel and Hightower that specific cell stress proteins are released from viable cells (Tytell et al. 1986; Hightower and Guidon 1989—see Chap. 1 for full details). The major problem has been a lack of understanding that both in bacteria (Holland 2010) and in eukaryotic cells (Nickel and Rabouille 2009), there are a plethora of protein secretion pathways in addition to the classical signal peptide secretion mechanism. Good evidence now exists that eukaryotic molecular chaperones can be secreted via one or other of these newly discovered secretion pathways (Table 2.1). In contrast, we know almost nothing about the release pathways that are involved in the secretion of bacterial molecular chaperones. Clearly much more work is needed to determine if the secretion of the many cell stress proteins found in the body fluids is due to a novel system for maintaining homeostasis, or if it is involved in tissue and cell pathology. However, it is now perfectly clear that a number of the major cell stress proteins are normally secreted and therefore their presence in body fluids and their actions on cells is not some artefact of the scientific process but is a manifestation of normal biological processes in both bacteria and eukaryotic cells.

Both bacteria and eukaryotes have a common set of cell stress proteins. It would appear, from the current literature, that eukaryotic cells have evolved to secrete numerically more of these proteins than bacteria have (Fig. 2.1). However, this may only represent the particular personal focus on the release of these proteins by both of these cell Kingdoms, with less attention being paid to bacterial cell stress proteins.

Table 2.1 The known secretion pathways for cell stress proteins

Protein	Secretion pathway	Reference
Thioredoxin	Novel pathway with some similarities to that of IL-1β	Rubartelli et al. 1990, 1992; Tassi et al. 2009
HSPB5	Exosomal secretion pathway	Gangalum et al. 2010
Peroxiredoxin	Brefeldin-insensitive non-classical pathway	Chang et al. 2006
PPIs	Unique vesicle-associated process	Suzuki et al. 2006
Cpn/Hsp60	Exosomal secretion pathway	Gupta and Knowlton 2007
Hsp70	Exosomal or vesicle-dependent secretion pathway	Lancaset and Febbraio 2005; Zhan et al. 2009
Hsp70	Non-classical pathway involving lysosomes	Mambula and Calderwood 2006
HspB1	Classic secretion pathway	Evdonin et al. 2009
BiP	Brefeldin-inhibited secretion pathway	Xiao et al. 1999
Hsp90	Possible exosomal pathway	Cheng et al. 2008

PPIs peptidyprolyl isomerases

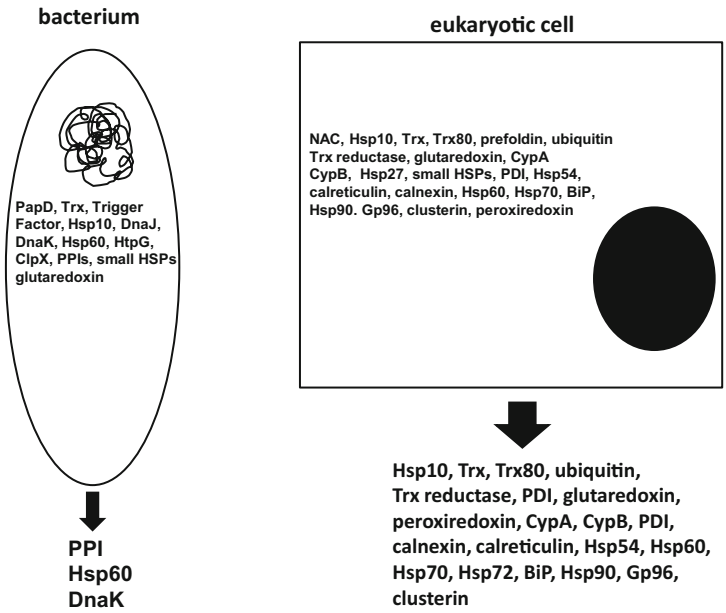

Fig. 2.1 The secretion of the total population of molecular chaperones and PFCs by bacteria and eukaryotic cells

2.3 Identification of the Cell Signalling Agonist Actions of Cell Stress Proteins

2.3.1 Chaperonin 10 (HSPE1)

As has already been discussed, the first evidence that cell stress proteins have the ability to act as cell signalling agonists was the report that a serum factor found in early pregnancy, and in consequence termed, early pregnancy factor (EPF), was an immunosuppressive factor (Morton et al. 1977). The nature of this factor was not clarified in the initial studies (e.g. Clarke et al. 1978) and it was only in 1979 that the immunosuppressive actions of this protein were defined (Noonan et al. 1979). However, it took until 1994 before the identity of EPF was confirmed. Using platelets as the source of EPF, led to the isolation of a pure form of this protein and its unequivocal demonstration as chaperonin (Cpn) 10, the co-chaperone for chaperonin (Cpn)60 (Cavanagh and Morton 1994). About a decade later it was shown that administration of recombinant human Cpn10 to rats could inhibit experimental inflammation (e.g. Zhang et al. 2003). Perhaps not surprisingly, this has led on to the Australian biopharmaceutical company AC Bio testing recombinant human Cpn10 for the treatment of a variety of human diseases including rheumatoid arthritis (Vanags et al. 2006) and multiple sclerosis (Broadley et al. 2009). One of the curiosities of the agonist actions of cell stress proteins is that it is impossible to predict what the next moonlighting action of these proteins will be. Proteins with more than one unique function are termed moonlighting proteins (Jeffery et al. 1999). It has recently been reported that Cpn10 is an erythropoietin-inducible secreted protein with effects on endothelial cell differentiation (Dobocan et al. 2009). Another interesting facet of cell stress proteins is the role that bacterial protein homologues play in disease. Thus with human Cpn10, the protein is immunosuppressive and anti-inflammatory. A similar situation is seen with the homologue from *Mycobacterium tuberculosis* (e.g. Ragno et al. 1996). In contrast the Cpn10 protein from the *Chlamydiae* appear to be pro-inflammatory (Zhou et al. 2011; Jha et al. 2011).

2.3.2 Macrophage Migration Inhibitory Factor (MIF)

While Cpn10 was the first molecular chaperone and cell stress protein to be discovered as a signalling agonist, the exact identification of the nature of EPF was not made until 1994. Thus, it can be argued that Cpn10 is not the signalling cell stress protein prototype. If this argument is heeded, then which cell stress protein takes the prize of being the first to be found with cell signalling activity? The surprising answer is that we can now take the history of cell stress proteins as immunomodulators back to 1971, and the discovery of the cytokine—macrophage migration inhibitory factor (MIF—e.g. Bartfield and Atoynatan 1971). This is one of the earliest and most confusing of cytokine molecules, which has been implicated in the pathology

of diseases including: sepsis, pneumonia, diabetes, rheumatoid arthritis, inflammatory bowel disease, psoriasis and cancer and for which novel low molecular mass inhibitors have been developed (Al-abed and Van Patten 2011). Whilst being around since the early 1970s, it was only in the late 1990s that the other guise of this protein was identified with the discovery that MIF is a thiol protein oxidoreductase (Kleeman et al. 1998; Potolicchio et al. 2003). A later study confirmed the influence MIF has on protein folding (Cherepkova et al. 2006). MIF is a secreted protein and it appears to induce its myriad effects by binding to CD74 on target cells, and such binding is now known to play a role in cancer (Shachar and Haran 2011). Unfortunately, nothing is really known about the connection between the protein-folding action of MIF and its myriad extracellular agonist functions. However, MIF serves to remind the reader that a potent cytokine can also function as a protein folding protein and should reinforce the hypothesis that molecular chaperones and PFCs can also function as cell signalling agonists with cytokine activity.

2.3.3 Thioredoxin

Again, the reader could accuse the writer that both examples of cell stress proteins so far discussed were only found to be molecular chaperones many years after their initial discovery as cytokine-like factors and that the earliest discovery of a molecular chaperone as a cell signalling agonist has not yet been defined. The example of the first molecular chaperone to be defined as a cell signalling agonist must then be human thioredoxin (Trx). This story can be said to start with the finding that lymphocyte activation is dependent on cell surface thiol oxidation status (Noelle and Lawrence 1981). Some years later it was reported that HTLV-1-transformed T lymphocytes secreted a factor, termed adult T cell leukaemia (**A**TL)-**d**erived **f**actor (ADF) that enhanced the expression of the p55 subunit of the IL-2 receptor. ADF synthesis was increased by classic T cell activators such as mitogens and phorbol ester. ADF was then shown to be human Trx (Tagaya et al. 1989). ADF was shown to be an autocrine growth factor for lymphocytes and to synergise with both IL-1 and IL-2 (Wakasugi et al. 1990). As has been shown in Table 2.1, Trx was the first protein folding catalyst to be shown to be secreted by a novel secretion pathway whose complete elucidation still eludes us. Interestingly, the secretion of key inflammatory cytokines such as IL-1 (Tassi et al. 2009) and the chemokine MCP-1 (Chen et al. 2010) is controlled by Trx. In addition to functioning to enhance the growth of T and B lymphocytes, Trx has also been shown to be secreted in greater amounts from T regulatory cells (T regs) and this is associated with a decreased level of T reg apoptosis (Mougiakakos et al. 2011).

Since the initial discovery of ADF/thioredoxin it has been established that Trx is a naturally secreted protein whose levels in the circulation are regulatable and with a wide range of important biological function (Holmgren and Lu 2010). This chapter is not the place to review the actions of thioredoxin and while elevated levels can be associated with poor outcome in patients with AIDS (Nakamura et al. 2001) it is