Integrating the Cell Stress Response: A New View of Molecular Chaperones as Immunological and Physiological Homeostatic Regulators

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Abstract:	The response of cells to stress was first documented in the 1960s and 1970s and the molecular nature of the families of proteins that subserve this vital response, the molecular chaperones, were identified and subjected to critical study in the period from the late 1980s. This resulted in the rapidly advancing new field of protein folding and its role in cellular function. Emerging at the same time, but initially largely ignored, were reports that molecular chaperones could be released by cells and exist on the outer plasma membrane or in the body fluids. These secreted molecular chaperones were found to have intercellular signalling functions. There is now a growing body of evidence to support the hypothesis that molecular chaperones have properties ascribed to the Roman god Janus, the god of gates, doors, beginnings and endings, whose two faces point in different directions. Molecular chaperones appear to have one set of key functions within the cell and, potentially, a separate set of functions when they exist on the cell surface or in the various fluid phases of the body. Thus it is a likely hypothesis that secreted molecular chaperones act as an additional level of homeostatic control possibly linking cellular stress to physiological systems such as the immune system. This review concentrates on three key molecular chaperones: Hsp10, Hsp60 and the Hsp70 family for which most information is available. An important consideration is the role that these proteins may play in human disease and in the treatment of human disease.

Review for Cell Biochemistry and Function

Integrating the Cell Stress Response: A New View of Molecular Chaperones as Immunological and Physiological Homeostatic Regulators.

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Abstract

The response of cells to stress was first documented in the 1960s and 1970s and the molecular nature of the families of proteins that subserve this vital response, the molecular chaperones, were identified and subjected to critical study in the period from the late 1980s. This resulted in the rapidly advancing new field of protein folding and its role in cellular function. Emerging at the same time, but initially largely ignored, were reports that molecular chaperones could be released by cells and exist on the outer plasma membrane or in the body fluids. These secreted molecular chaperones were found to have intercellular signalling functions. There is now a growing body of evidence to support the hypothesis that molecular chaperones have properties ascribed to the Roman god Janus, the god of gates, doors, beginnings and endings, whose two faces point in different directions. Molecular chaperones appear to have one set of key functions within the cell and, potentially, a separate set of functions when they exist on the cell surface or in the various fluid phases of the body. Thus it is a likely hypothesis that secreted molecular chaperones act as an additional level of homeostatic control possibly linking cellular stress to physiological systems such as the immune system. This review concentrates on three key molecular chaperones: Hsp10, Hsp60 and the Hsp70 family for which most information is available. An important consideration is the role that these proteins may play in human disease and in the treatment of human disease.

INTRODUCTION

The founder of Cell Biochemistry and Function, Joe Chayen, was a pioneer systems biologist who attempted to integrate the methodologies of biochemistry and cytology to generate what he termed 'multiphase biochemistry' [1]. Chayen's idea was to integrate the biochemistry of the individual cell within the framework of the whole organ in which the cell was present. Using quantitative cytochemistry to probe the biochemical activity of cells within sections of a tissue, allowed region-specific biochemistry to be practised, and the relationships between individual cells within a cellular complex to be defined. In 2009 integrative/integrated biology is now a key buzzword with the term 'integrated biology' producing 10,400,000 hits on a Google search.

This review focuses on a specific aspect of 'multiphase biology', specifically the multiphase biology of molecular chaperones. These are variously known as heat shock proteins (Hsps) or cell stress proteins, the latter being a more correct term as many stresses can induce the synthesis of these proteins, which are essential proteins involved in maintaining cellular homeostasis. This review will focus on the emerging findings that molecular chaperones, which were initially believed to be purely intracellular proteins with purely intracellular functions, are now seen to be 'multiphase proteins' present on the extracellular membranes of cells and in the extracellular fluids, including the blood. These proteins are emerging with a multitude of extracellular functions from involvement as receptors for key signalling molecules to acting as insect toxins. This review will provide an overview of the emerging evidence of the multiphasic biology of molecular chaperones.

AN INTRODUCTION TO MOLECULAR CHAPERONES

Ron Laskey, currently the Charles Darwin Professor of Embryology, and a guitar-toting lecturer, was the originator of the term 'molecular chaperone'. Laskey's group at the University of Cambridge was studying the packaging of DNA into nucleosomes. These are oligomeric 'particles' containing 146bp of DNA wrapped around an octamer of the basic nuclear proteins known as histones. Nucleosome formation occurs rapidly in amphibian eggs once they become fertilised, with the basically charged histones binding to the negatively charged DNA. The nucleosomes can be dissociated with buffers containing high salt concentrations and it was therefore expected that DNA and histones should self-assemble into nucleosomes. Experimentally, this is not the case. Removal of the salt resulted in the formation of non-specific aggregates, but no nucleosomes [2]. Laskey's group showed that homogenates of amphibian eggs (they used *Xenopus*) added to the histone/DNA mixture would promote nucleosome formation. They purified what turned out to be an abundant active component and identified it as an acidic nuclear protein that they called nucleoplasmin. This protein alters the interaction between the histones and the DNA such that nucleosome formation is favoured over aggregate formation. Nucleoplasmin has two important properties which shaped the subsequent development of the concept of molecular chaperones and chaperoning. The first is that the final product, the nucleosome, does not

contain nucleoplasmin. The second, is that if the conditions are right, nucleosomes can form in the absence of nucleoplasmin. Gentle dialysis of dissociated nucleosomes, which slowly lowers the salt concentration, allows them to reform naturally. This means that the nucleoplasmin does not provide steric information for the generation of the nucleosome, but simply provides a means of enabling the interactions of the DNA and histones to be modified such that the natural self assembly is favoured over the formation of non-productive complexes (Fig 1). Now, in prior centuries and still in some parts of the world, the men and women cannot meet alone and chaperones are required to prevent untoward interactions. Laskey used this analogy to describe nucleoplasmin as a 'molecular' chaperone which prevented 'unhealthy' interactions between the histones and the DNA [2].

The discovery of the best studied molecular chaperone, chaperonin (Cpn)60 (also known as Hsp60), can be traced back to two distinct areas of research: (i) the synthesis of the major chloroplast protein, Rubisco and (ii) the genetics of phage synthesis in *E. coli.* Without going into detail, it was shown that these two proteins were evolutionarily related and that they were involved in protein folding. This led John Ellis, who has been a tireless exponent of the science of molecular chaperones [3], to define these proteins as 'a large and diverse group of proteins that share the property of assisting the non-covalent assembly/disassembly of other macromolecular structures but which are not permanent components of these structures when these are performing their normal biological functions' [4] (Fig 1). To aid the reader Table 1 provides details of some of the molecular chaperones relevant to this review and their cellular locations.

Thus by the late 1980s/early 1990s, and with the beginnings of the identification of the 30-odd protein families that are now defined as molecular chaperones, it was believed that these were essential intracellular proteins that solved the problem caused by the enormous amount of protein that exists in each cell. It is this so-called 'protein crowding' that is believed to promote protein misfolding and to be the evolutionary pressure for the development of chaperoning [5,6]. However, at exactly the same time as this was happening, individuals in the bacteriological and immunological communities were also identifying chaperonin (Cpn)60 as a powerful immunogen in bacterial and other microbial infections and this protein was entitled 'common antigen' as it was present in all bacteria examined [7,8]. Other molecular chaperones have also been found to exert powerful immunological responses. An excellent example are the peptidylprolyl isomerases, a number of which are, unexpectedly, targets for the major immunosuppressive drugs such as cyclosporine, tacrolimus and rapamycin [9]. Such drugs are essential for allowing organ transplantation to take place.

The finding that molecular chaperones are powerful immunogens may have suggested that these proteins were secreted or existed on the cell surface. However unbeknown to the scientific community a molecular chaperone had been found to be present in the blood one year prior to Laskey's coining of the term molecular chaperone.

Early pregnancy factor (EPF) [10] is an immunosuppressive protein [11] that appears in the first trimester. However, it took until 1994 to verify that EPF was the chaperonin, chaperonin (Hsp)10 [12]. Now Hsp10 is more usually known as the heptameric complex which caps the annulus in chaperonin 60 allowing this protein to fold proteins [13]. Thus Hsp10 was actually defined as a homeostatic immunosuppressive factor before being found to be an essential part of the intracellular protein folding process.

MOLECULAR CHAPERONES: A PARADIGM REVOLUTION

With the introduction of the concept of protein chaperoning there was an exponential increase in the numbers of papers dealing with this process and with the identification of new molecular chaperones. Tucked away in the literature were the beginnings of a paradigm revolution in molecular chaperone biology. In the original paradigm molecular chaperones were intracellular proteins, because it was within cells that their actions were required. They had no extracellular functions and therefore were not expected to be found outside of cells. Thus in 1989 when Hightower and Guidon revealed that members of the Hsp70 family of proteins were released by cultured rat embryonic cells [14] it came as a surprise. This work supported earlier studies from Tytell and co-workers who had had previously shown similar proteins being transferred from squid glial cells to neurons [15].

Not surprisingly, the concept that molecular chaperones could be released from cells and have additional, perhaps non-folding, functions was largely ignored as it did not fit in with the then powerfully growing paradigm of molecular chaperone biology. An obvious reason for ignoring the non-folding actions of molecular chaperones was the fact that biological science was still working within another potent paradigm.

ONE GENE → ONE PROTEIN → ONE FUNCTION

MOONLIGHTING PROTEINS

Many proteins have enzymic activity and it is sensible to assume that the enzymic active site, which may take up only a fraction of the protein (in terms of volume or surface area) is the only part of the protein with biological activity. The rest of the protein has evolved to generate the circumstances (topology) of the active site and no more. The concept of protein moonlighting was introduced into the scientific literature by Campbell and Scanes in 1995 in an article in which they discussed the immune modulating activity of endocrine peptides [16]. Constance Jeffery has been most active in bringing this concept to the attention of the protein world [17,18]. As an example of the unexpected nature of moonlighting, the enzymes of the glycolytic pathway, beloved of all undergraduate students in the biological sciences, with a few exceptions, have moonlighting functions [19]. For example, phosphoglucose isomerase (PGI), which functions as its name suggests, is also a neuroleukin [20], an autocrine motility factor, important in tumour metastasis [21], a differentiation and maturation mediator for myeloid cells [22] and an implantation factor [23]. To compound this discussion of protein moonlighting – the receptor for autocrine mobility factor/PGI also exhibits ubiquitin E3 ligase activity in the endoplasmic reticulum

[24]. Enolase, glyceraldehyde 3 phosphate dehydrogenase, triose phosphate isomerase, pyruvate dehydrogenase, all have additional activities over and above their enzymic ones [20]. The emergence of this finding, that individual proteins can have multiple functions, is opening up a whole new view of evolution and its effects on protein structure and function. Perhaps this is one explanation for the small number of genes that mammals have. If each gene product has multiple functions then it provides a much richer protein-function landscape than if each gene only provides one biological activity. It now appears that one of the major groups of moonlighting proteins are the molecular chaperones, which are increasingly being shown to exhibit unexpected biological functions. It is these functions that will be discussed in the remainder of this review. Given the evolved function of molecular chaperones to interact with other proteins it is perhaps not surprising that they have evolved this capacity to 'molecularly multi-task'.

MOLECULAR CHAPERONE SEQUENCE CONSERVATION AND BIOLOGICAL FUNCTION

Before discussing the novel roles that molecular chaperones play, it is important to clarify the relationship between the homology of molecular chaperones and their extracellular biological actions. Molecular chaperones have extremely conserved sequences and the chaperonin 60 protein from mammals and from bacteria will share 50% sequence conservation. This is sufficient for workers on the folding aspects of these chaperones to assume that these proteins are functionally identical and to talk about Hsp60 proteins as if they have universal actions no matter how much their sequences may differ. Now, it is well known to biologists that single residue changes in a protein can dramatically modify its biological activity. The classic example of this is the substitution of valine for glutamic acid in haemoglobin which results in the precipitation of the deoxygenated form of the protein thus leading to the sickling of erythrocytes. It is beginning to appear that molecular chaperones from different sources can have widely different biological actions in spite of an appreciable sequence conservation. Examples of this include the Hsp70 proteins Hsp70a (now HSPA1) and BiP (now HSPA5). Thus HSPA1 is well recognised as a proinflammatory ligand causing activation of human and murine monocytes [25]. In contrast, HSPA5, which has 64% sequence identity to HSPA1, is a potent monocyte inhibitor and anti-inflammatory protein which is starting trials as a therapy for the chronic inflammatory disease, rheumatoid arthritis [26,27]. *Mycobacterium tuberculosis* is one of a proportion of bacteria that have more than one gene coding for chaperonin 60 proteins and in this organism the proteins are termed chaperonin 60.1 and 60.2 (Hsp65). These proteins exhibit around 61% sequence identity. Attempts to inactivate both chaperonin 60 genes reveals that, only the gene encoding the 60.1 protein can be inactivated [28]. Surprisingly, the chaperonin 60.1 protein of *M. tuberculosis* turns out not to be a molecular chaperone (it does not fold proteins), but to be a potent virulence factor [28]. Moreover both proteins differ markedly in their ability to modulate the formation of the major cell population, the osteoclast, which is responsible for homeostatic bone resorption.

Thus the chaperonin 60.1 protein is a potent inhibitor of osteoclast formation while the chaperonin 60.2 protein neither promotes nor inhibits osteoclastogenesis [29].

MOLECULAR CHAPERONES: CELL SIGNALLING PROTEINS AND RECEPTORS FOR CELL SIGNALLING PROTEINS

The thesis being developed in this review is that molecular chaperones (and related proteins such as protein folding catalysts – proteins such as thioredoxin and protein disulphide isomerase) have moonlighting functions and the nature of these functions depends on where these proteins exist in the organism. Within the cell it is assumed that these proteins largely act as molecular chaperones – although this is open for discussion. Molecular chaperones are also found on the surfaces of bacteria and of eukaryotic cells where they can functions as receptors for a diverse, and sometimes bizarre, set of client ligands. In other examples the presence of a molecular chaperone on the surface of a cell has some other, as yet, undefined function. The best example of this is the role of certain molecular chaperones in sperm capacitation in the mouse [30]. The third site for molecular chaperones is in the extracellular fluids of the body where, it is assumed, they function as intercellular signals for a variety of cells. Another complication to our understanding of the general biology of the non-folding aspects of molecular chaperones is the significant differences in the numbers of selected molecular chaperones that different species have and in the use to which they put them. For example humans have only one functional Hsp60 (HSPD1) gene. In contrast, a not inconsiderable proportion of bacteria have more than one Hsp60 gene [31]. The differences in the activities of the *M. tuberculosis* Cpn60 proteins has been described [28,29] and an even more distinct difference in folding function [32] and signalling activity [33] is seen with the three Cpn60 proteins of the soil bacterium *Rhizobium leguminosarum*. *Drosophila melaogaster* has four Hsp60 proteins and each seems to serve a distinct function [34]. We must bring this information under the one umbrella to fully appreciate the systems biology of the molecular chaperone/cell stress protein fraternity. In this review, attention will focus on the non-folding actions of the molecular chaperones and protein folding catalysts of eukaryotes with only selected examples being taken from the literature on bacterial chaperones. For more detail on the signalling and other actions of bacterial molecular chaperones the reader is referred to [35].

Extracellular Signalling Actions of Eukaryotic Molecular Chaperones

Most of these studies focus on the ability of recombinant molecular chaperones to interact with immune cells and activate or inhibit them. The literature suggests that molecular chaperones have a major role to play in immune homeostasis. The review of the literature will be restricted to Hsp10, Hsp60 and Hsp70 as these are the proteins which have been most closely studied and for which most reliable information is available. This review will not deal with the functioning of intracellular molecular chaperones.

Hsp (Chaperonin)10

The first reported signalling function of a molecular chaperone was not recognised as such. This was the actions of early pregnancy factor (EPF) [10,11] which occurred in the 1970s and suggested that this moiety was immunosuppressive. It was not until 1994 that EPF was identified as chaperonin 10 or Hsp10 [12] and since then it has been shown that recombinant human Hsp10 has immunosuppressive activity *in vitro* and in animal models [36-38]. The Cpn10 protein from *M. tuberculosis* is also able to inhibit the induction of adjuvant arthritis in the rat [39]. These findings have persuaded the Australian Biotech company CBio Ltd to develop human Hsp10 (renamed XToll) for the treatment of rheumatoid arthritis. Preliminary clinical trial data shows some efficacy in patients with rheumatoid arthritis [40,41]. A phase II clinical trial of Hsp10 in a small group of patients with multiple sclerosis has shown that this protein is well tolerated but did not produce statistically significant effects [42]. A larger trial is now called for. Of relevance is the finding that circulating levels of Hsp10 in patients with periodontal disease are lower than in matched, disease-free, controls and blood levels come back to normal only after effective therapy [43]. These findings suggest that circulating Hsp10 levels are controlled by local levels of inflammation and support the hypothesis that this molecular chaperone is a homeostatic controller of inflammation. In a small study of patients with ovarian cancer it was found that HSP10 was present in the sera and ascites of patients, but was not detectable in controls. Ovarian tumour cells in culture released Hsp10. The sera which contained Hsp10 was able to inhibit the expression of a key T lymphocyte signalling protein CD3-zeta and specific removal of this protein resulted in the loss of this T cell suppressive activity [44].

Expanding the biological roles of Hsp10 is the report that erythropoietin stimulated human umbilical vein endothelial cells (HUVECs) to release Hsp10. To determine if this release was biologically relevant, the effect of Hsp10 was examined on erythroid cell differentiation. It was shown that Hsp10 decreased the proliferation of the erythroleukemia cell line K562 and increased the amounts of the erythroid differentiation markers glycophorin A and haemoglobin in TF-1 cells. Such changes were associated with specific alterations in intracellular signalling [45].

In addition to having a role in early pregnancy, potentially by acting as an immunosuppressive factor to help in preventing immune responsiveness to the early embryo, it has recently been reported that Hsp10 is present on the surface of the mouse spermatozoa where it may aid in sperm capacitation [46]. Thus in addition to having biological actions in the body fluids, Hsp10 also may have functions when on the outer cell membrane.

Hsp (Chaperonin)60

The second molecular chaperone to come to the attention of the scientific community because of its alleged intercellular signalling activity was Hsp60. It was initially reported that the chaperonin 60 protein (specifically the

Cpn60.2 or Hsp65 protein) of *M. tuberculosis* stimulated human peripheral blood-derived monocytes to release a variety of pro-inflammatory cytokines [47]. This finding started off a search for the mechanism by which this, and other, chaperonin 60 proteins stimulated monocyte activation and to discriminate the effects from that of the key Gram-negative cell wall component, lipopolysaccharide (LPS). While this article focuses on mammalian (mainly human) Hsp60 proteins, much of our information of the signalling properties of Hsp60 proteins have come from the study of bacterial proteins; as this may have relevance to the properties of the human protein it will be briefly discussed. A major area of current research in immunology and infectious diseases research is the activation status of human or rodent monocytes [48]. Macrophages exposed to interferon gamma (IFNγ) or to LPS are called classically-activated macrophages and are 'activated' in a way that promotes the presentation of antigens to T lymphocytes (e.g. upregulation of MHC class II proteins) or kills bacteria (production of reactive oxygen radicals). However, it has been found in more recent years that other signals can induce a range of other macrophage activation states that have been termed alternative activation [48]. The finding that *M. tuberculosis* Hsp60.2 protein stimulated the production of pro-inflammatory cytokines suggested that it was inducing a classically-activated state. However, while the exposure of human monocytes to *M. tuberculosis* Hsp60.2 induced the production of the same amount of cytokines released by cells exposed to IFNγ together with LPS, cells stimulated with Hsp60.2 did not show the increased expression of Fcγ-receptors, MHC classII proteins or the release of reactive oxygen intermediates. Thus *M. tuberculosis* Hsp60.2 is not inducing a classically activated state [49]. Further evidence that the Hsp60.2 protein from *M. tuberculosis* produces an activation state different from that of other cell activators, such as LPS, is the finding that this mycobacterial protein induces cultured human vascular endothelial cells to synthesise the classic leukocyte adhesion receptors (ICAM, VCAM, E-selectin) in a cytokine (IL-1, TNFα)-independent manner [50]. This contrasts with the literature, which suggests that the synthesis of these adhesion molecules requires the prior induction of IL-1 or TNFα [51].

Later in the 1990s, attention turned to the human Hsp60 protein with Hubert Kolb and co-workers being the first to show that this protein could stimulate human and mouse monocytes to synthesise pro-inflammatory cytokines (including TNFα and the Th1 cytokines IL-12 and IL-15) and mouse cells to produce nitric oxide. Human Hsp60 also synergised with IFNγ in inducing cytokine synthesis [52]. Activation of human monocytes and macrophages was then shown to depend, like LPS, on binding to CD14 with activation of p38 mitogen-activated protein (MAP) kinase [53] and Kolb's group reported that human Hsp60 failed to activate monocytes from the LPS-insensitive C3H/HeJ mouse which has a non-functional Toll-like Receptor (TLR)4 LPS-interacting protein. The inference was that human Hsp60 had to bind to, or somehow interact with, monocyte cell surface TLR4 to induce cell activation [54]. This has been the start of an unfinished journey into the 'receptorology' of molecular

chaperones, which is still a confusing aspect of these proteins and is a major problem with the biology of Hsp60 proteins. Kolb's work pointed to TLR4 as the key receptor for human Hsp60 and Vabulas and colleagues, using cell transfection methodology, reported that human fibroblasts transfected with TLR2 or TLR4 plus MD-2 (a co-receptor required for cell activation) gain responsiveness to Hsp60, while TLR2- or TLR4-defective cells displayed limited response. Moreover, these workers found that the Hsp60 had to be endocytosed to activate cells. Further analysis of human Hsp60 binding, using fluorescently-labelled recombinant Hsp60, revealed that it bound to cells and could be competed with unlabelled Hsp60 but not by unlabelled Hsp70, Hsp90 or gp96. The labelled Hsp60 bound to TLR4 negative cell lines (although it did not activate the cells), showing that the primary receptor is another cell surface protein. These findings clearly show that Hsp60 has properties distinct from the Hsp70 and Hsp90 family members [55]. Comparison of Hsp60 proteins from mammals and bacteria revealed that human, rat and mouse Hsp60 proteins all cross-compete for binding to murine macrophages. However, it was surprising to find that hamster Hsp60 could not compete the binding of human Hsp60. Neither did the Hsp60 proteins from *E. coli*, *Chlamydia pneumonia* or *Mycobacterium bovis* compete with the human Hsp60 protein for binding to mouse macrophages [56]. Using human Hsp60 truncation mutants and overlapping human Hsp60 synthetic peptides, the site in Hsp60 binding to macrophages was identified as the C-terminal region – residues 481-500 [57].

There is still obviously much to learn about the ligand-receptor systems that drive Hsp60 stimulation of myeloid cells. More recent studies of human Hsp60 have revealed that this protein has unexpected effects on macrophages, dendritic cells and on lymphocytes. There are a number of reports that human Hsp60 can promote dendritic cell maturation and activate mature dendritic cells [58]. Addition of Hsp60 to murine bone marrow-derived dendritic cells (DC) potently stimulated the secretion of IL-1β, IL-12 and TNFα, but only a small amount of IL-10 suggesting a Th1 bias. The dendritic cells exposed to Hsp60 were able to induce proliferation of allogeneic T lymphocytes. These changes in DC behaviour were mirrored by changes in kinase signalling. Of interest was the finding that the MAP kinase activation induced by Hsp60 was also mediated by LPS but that the kinetics of the LPS activation was slower than that of Hsp60 [59]. Another study has suggested that unlike LPS, human Hsp60 stimulates both murine macrophages and dendritic cells to secrete IFNα and neutralisation of this cytokine blocked Hsp60-induced IFNγ production by DCs. This suggests that IFNα production contributes to Hsp60-specific immune stimulation [60].

The interaction of Hsp60 with T lymphocytes opens up a considerable debate, because it was recognised from about the time of the identification of Hsp60 as a molecular chaperone, that this protein was also a potent immunogen and T/B cell modulator. An early finding was that human T cells from healthy individuals, and from sites of disease, were able to recognise Hsp60 proteins, including the human protein and that such reactivity

contributed to the autoimmune-type phenomena often found in infections [61]. One of the most puzzling aspects of the Hsp60 molecule is its association with T cell modulation and immunity. An early observation was that T cell immunity to a specific epitope in the *M. tuberculosis* Hsp60.2 protein could be responsible both for inducing AND preventing adjuvant arthritis in the rat [62]. Adjuvant arthritis is a curious chronic inflammatory and massively destructive disease of the joints of rats which somewhat resembles human rheumatoid arthritis. Thirty years after this discovery the mechanisms of disease induction and cure by Hsp60 is still somewhat mysterious but is now becoming the basis for specific therapies [63]. In most of this work the Hsp60 has been regarded as an immunogen with limited specific biological activity in its own right. The finding that Hsp60 proteins can modify the actions of myeloid cells has prompted a reappraisal of the direct influence of Hsp60 proteins on lymphocyte functions.

Hill Gaston was among the first to show that human Hsp60 stimulated the proliferation of naive human T lymphocytes [64]. Broeler and co-workers [65] then showed that human Hsp60 promotes antigen-specific production of IFNγ in conditions when the antigenic stimulus is not sufficient to activate T lymphocytes. This same group then reported that human Hsp60 could act in the stimulation of CD8 lymphocytes [66]. Another study showed that when added to murine T cells (in the presence of peritoneal exudate cells as antigen-presenting cells (APCs)) human Hsp60 induced the specific secretion of large amounts of IFNγ. In contrast, dendritic cells (DCs), which are efficient APCs, released less IFNγ when exposed to Hsp60. The ability to induce IFNγ is dependent on the co-stimulatory pairing of CD80/86 with CD28. Similar results were seen with recombinant murine Hsp60, although one difference was the finding that the murine protein did not bind to CD14. This is in spite of the fact that the human and murine proteins share >98% sequence identity [67], emphasising the fact that it is not possible to treat the Hsp60 proteins as a single biological entity.

How does human Hsp60 cause the activation of T cells? Irun Cohen's group have examined the interaction of highly purified recombinant Hsp60 with T lymphocytes in the past five years. The first conclusion was that the direct action of human Hsp60 with T lymphocytes required the participation of TLR2 [68]. In this study T cell adhesion to fibronectin was stimulated to the same extent as established activators of this process and at concentrations as low as 0.1ng/ml (around 100pM). Surprisingly, Hsp60 inhibited T cell chemotaxis induced by the chemokines CXCL12 and CCL19 and with long-term exposure down-regulated the expression of the chemokine receptors CXCR4 and CCR7 [68]. This finding that human Hsp60 has inhibitory actions was expanded in later papers from this group. HSP60 is also reported to inhibit synthesis of the transcription factors: T-bet, NF-κB, and NFATp and to increase production of GATA-3, leading to decreased secretion of pro-inflammatory TNFα and IFN-γ but increased production of the anti-inflammatory cytokine, IL-10. Administration of Hsp60 to Balb/c mice with ConA-induced hepatitis inhibited clinical, serological and histological markers of

disease and increased levels of the transcription factors SOCS3 and GATA-3 while downregulating Tbet expression in T lymphocytes [69]. It was further shown that Hsp60 stimulates SOCS3 expression in T lymphocytes via the TLR2 receptor causing signalling via the JAK/STAT pathway which modulates T cell chemotaxis. A surprising finding was the concentration dependency of these effects. Hsp60 was active at concentrations of 0.1 to 1.0ng/ml (approximately 0.1-1.0pM) but not active in the range 10-100ng/ml but again active at concentrations of 1µg/ml. One possible explanation is the involvement of different receptors with different affinities or of some form of receptor activation complex such as is postulated for LPS [70]. A fascinating suggestion is that Hsp60 acts as a co-stimulator of human T-regulatory cells (Tregs). Cohen's group has presented data that Hsp60 co-stimulation of Tregs leads to activation of various intracellular kinases and the suppression of target T cells both by cell-cell contact and by the secretion of TGFβ and IL-10 [71]. Addition of Hsp60 (but not *E. coli* GroEL or *M. tuberculosis* Cpn60.2) to naive murine B cells stimulated cell proliferation, the release of IL-6 and IL-10 and increased surface expression of CD40, CD69 and CD86. This cell modulation was dependent not on TLR2 binding but on the TLR4/MyD88 signalling system. Thus both mammalian T and B cells respond to Hsp60 in unexpected ways [72].

Two other aspects of the Hsp60 protein will be briefly discussed. The first concerns the insect known as the Doodlebug. The larval form of this insect lives in sandy soil and hunts prey. It captures its prey by biting them and injecting a paralysing neurotoxin. It turns out that the neurotoxin is actually produced by a symbiotic salivary bacterium – *Aerobacter aerogenes.* When this toxin was isolated and sequenced it turned out to be the Hsp60 protein of the bacterium and a protein that was virtually identical to *E. coli* GroEL. Indeed, single amino acid mutations can turn GroEL, which has no neurotoxic properties, into a potent neurotoxin [73]. So like a molecular Meccano© set one can turn GroEL into a new type of molecule with minimal tinkering. The second use to which Hsp60 has been put has been briefly touched on with regard to Hsp10 and sperm capacitation. It was reported in 2004 that mouse sperm capacitation required the presence of Hsp60 on the sperm head and that this protein had to undergo tyrosine phosphorylation to trigger conformational changes facilitating the formation of a functional zona pellucida receptor complex on sperm surface [74]. Surprisingly, the same role for Hsp60 cannot be accorded to human sperm capacitation [75] revealing major differences in the role played by molecular chaperones in different species.

Hsp70 Family

Humans encode at least 12 Hsp70 proteins [76]. This brings with it some degree of uncertainty in the literature as it is often not exactly clear what Hsp70 protein individual authors are describing. For readers who want to know more about these and other molecular chaperones see [77] which describes new guidelines for the nomenclature for molecular chaperones (also Table 1). This has not been used in this review as most of the

literature that readers would refer to use the older nomenclature. However, it is likely that this will change to the new nomenclature in the coming years.

Alexzander Asea and Stuart Calderwood were the first to describe that commercially available recombinant Hsp70 (the exact protein was not specified, but was probably Hsp70-1a (HSPA1A) or Hsc70 (HSPA8)) stimulated human monocytes to produce pro-inflammatory cytokines by a mechanism involving rapid intracellular calcium flux [78]. They termed Hsp70 a chaperokine, a term that does not seem to have caught the scientific imagination. Importantly, this calcium flux was not seen in cells stimulated with LPS [78], which is always a potential contaminant in *E. coli*-derived recombinant proteins. Other groups repeated and extended this finding with murine monocytes [79] and showed that purified murine Hsp70 (a mixture of Hsp70 proteins) stimulated nitric oxide production [80]. Initial studies employing selective inhibitors and appropriately transfected cell lines came to the conclusion that 'Hsp70' activation of myeloid cells was through a receptor complex involving CD14, TLR2 and TLR4 [79,81]. Thus the original hypothesis was that 'Hsp70' was a pro-inflammatory signal interacting with the classic innate immune signalling receptors.

A consensus appeared to have been reached by early 2000 concerning the receptors and intracellular signalling pathways utilized by mammalian 'Hsp70'. These appeared to be the same as were being interacted with by the majority of the Hsp60 proteins. However, Tom Lehner in London, who has concentrated on the Hsp70 (DnaK) protein from *M. tuberculosis*, reported that this recombinant protein, but not human 'Hsp70', stimulated human monocyte chemokine and cytokine synthesis through binding to CD40, a member of the TNF receptor family [82,83]. Biological activity resided in the C-terminal peptide-binding part of the molecule [83]. Further analysis using synthetic peptides has identified activating and inhibiting epitopes within this region [84]. In contrast, Ulrich Hartl and colleagues have reported that human 'Hsp70' binds to CD40 via the amino-terminal nucleotide binding domain when in the presence of ADP (but not when ATP was present) and activates NF-κB [85]. Hsp70 proteins interact with co-chaperones such as DnaJ (Hsp40), Hip and Bag-1. Hartl has shown that Hip competes with 'Hsp70' for binding to CD40 [85]. The potential binding specificity of 'Hsp70' is further confused as a range of other receptors have been claimed to bind to 'Hsp70'. Lehner has recently reported that *M. tuberculosis* Hsp70 binds to the chemokine receptor/HIV co-receptor, CCR5 [86]. This raises the obvious question of whether members of the human Hsp70 family bind to this receptor – which could have interesting implications for the control of HIV infection. Human 'Hsp70' has been reported to bind to a range of other human cellular receptors including: CD91 [87], the scavenger receptors (SRs) such as LOX-1 [88,89] and C-type lectin receptors [90]. To further confuse the situation with the 'Hsp70' receptor, Stuart Calderwood, who has done much of the work on identifying receptors for Hsp70 (including identifying CD14/TLR2/TLR4 as receptors [78]), now reports that human 'Hsp70' only binds to members of the scavenger receptor family ([90] and personal

communication). One member of the human Hsp70 family (Hsp70-8 also called Hsc70, Hsp73 or in the new proposed nomenclature HSPA8) has been reported to bind to EWI-2, - an early activation marker of dendritic cells [91]. Binding of HSPA8 to EWI-2 resulted in enhanced chemokine-dependent migration of activated mature dendritic cells but their antigen-specific stimulatory actions were attenuated [91]. Hartl has recently shown how complex are the interactions of Hsp70, and its two separate domains, with the surface of leukocytes [92].

As will be described, 'Hsp70' is present in human blood and a number of studies report that circulating concentrations are negatively correlated with symptoms of cardiovascular disease suggesting that 'Hsp70' is 'cardioprotective' [93-95]. This possibly flies in the face of the evidence that 'Hsp70' is a pro-inflammatory factor. This was the situation with Hsp60 until more recent studies, described above, revealed that the human and *M. tuberculosis* Hsp60.1 proteins can inhibit leukocyte activation [see 29,69]. Thus it is possible that Hsp70 proteins may have anti-inflammatory properties at low concentrations.

In fact, another Hsp70 family member, BiP, has been shown to be a potent anti-inflammatory protein. Valerie Corrigall's group identified BiP as an autoantigen in rheumatoid arthritis [26 41]. Assuming BiP would induce arthritis, the recombinant protein was administered to mice and rats, but without effect [26 41]. However, administration to rodents with experimental arthritis inhibited the initiation and/or severity of disease [26 41]. The explanation appears to be that BiP (in contrast to'Hsp70') stimulates leukocytes to produce an anti-inflammatory cytokine network involving the predominant synthesis of IL-10 and IL-4 [27,96]. To gain more information about the effects of BiP on monocyte activation, Affymetrix microarrays were used to measure global transcriptional responses of these cells to BiP. Two normal subjects were used, and a single time point, 24h, was selected for study. The results were remarkable in showing changes in around 900 genes, with 75% of these genes being downregulated. Of note, was the 3 fold downregulation of HSP70-1a mRNA levels following BiP stimulation. This suggests the hypothesis that Hsp70 family members regulate each other's synthesis. To date, the receptor for BiP has not been identified [97] and it is not any of those that binds 'Hsp70' [97]. Corrigall has shown that BiP is present in synovial fluid and serum of rheumatoid patients [97]. Henderson has recently shown that patients with periodontitis, have significantly lower circulating levels of BiP than comparable normal controls [43].

The most distant members of the Hsp70 family, the Hsp110/SSE and Grp170 proteins, share similarity to Hsp70 but have additional domains. These proteins act as regulatory factors for Hsp70 [98,99] and, importantly, they have also been implicated as potent extracellular signalling proteins [100,101].

In conclusion, the evidence provided in this review and other studies support the hypothesis that 'Hsp70' proteins are potent intercellular signalling molecules with either pro- or anti-inflammatory actions, working through a bewildering variety of cell surface receptors to provide homeostatic or pathological network signaling

important in immune and other body functions [102]. The confusing nature of the literature is almost certainly the result of different workers using different proteins (either purified single proteins, 'purified' mixtures of proteins or recombinant proteins). Post-translational modifications (which will not be present in recombinant proteins prepared in *E. coli*) could influence activity. A more serious issue hindering the study of the Hsp70 system is a small number of papers which claim that the activity of 'Hsp70' protein is due to either contaminating *E. coli* components such as LPS [103] or flagellin [104], or to nucleotides left over as part of the isolation procedure [105]. The flagellin paper [104] focused on Hsp70 stimulation of T cells. However, Henderson has shown that recombinant *E. coli* flagellin is a very weak activator of human monocytes [33]. and Figueiredo and coworkers have recently reported that 'Hsp70' expressed in eukaryotic cells is still active as a T lymphocyte stimulator[106], refuting the work of Gao and Ye [103,104]. In addition, the work of Lehner and co-workers has comprehensively ruled out LPS as a contributor to the cellular effects produced by *M. tuberculosis* Hsp70 [84].

MOLECULAR CHAPERONES ARE FOUND IN THE EXTRACELLULAR FLUID AND ON THE EXTERNAL PLASMA MEMBRANE

Interesting as these studies are, they could be irrelevant if the molecular chaperones involved are not secreted. There is now increasing evidence for the passage of molecular chaperones both to the cell surface and to the extracellular fluids of the body. It is also emerging that proteins such as Hsp70 family members and Hsp60 can act as receptors for a variety of ligands.

The Hsp10, Hsp60 and HSPA9 (also known as Hsp70-9, Grp75, mtHsp75, Mortalin) proteins are nuclear-encoded proteins that target to the mitochondrion where they are considered to have their only functions [107]. The remaining Hsp70 family members are found in the cytosol, nucleus, lysosomes and ER [107]. However, it has been known for some years now, thanks to the work of Radhey Gupta, that these mitochondrial chaperones can be found in other parts of the cell including the outer surface of the plasma membrane [108]. Thus Hsp60 has been directly observed on the outer plasma membrane by using surface-labelling techniques linked to electron microscopy [109]. This Hsp60 protein is also found in other non-mitochondrial locations in cells. For example, in pancreatic acinar cells and pituitary cells (both secretory cells), Hsp60 is found in zymogen granules and growth hormone granules respectively [110]. Indeed, Hsp10 has also been immunolocalised in these sites [111]. The mitochondrial form of Hsp70 (HSPA9) has also been localised in the plasma membrane and in different cellular granules [reviewed in 108]. Intriguingly, many bacteria utilise cell surface Hsp60 and Hsp70 as bacterial adhesins which bind with a wide variety of host ligands [35].

Other members of the Hsp70 family have also been found in the plasma membrane. Possibly the most intriguing finding of Hsp70 in plasma membranes is the hypothesis forwarded by Triantafilou, based on biophysical measurements, that the receptor for the Gram-negative inflammogen, LPS, contains a number of

proteins including Hsp70. The other members of this so-called LPS activation complex are Hsp90, CXCR4 and growth differentiation factor (GDF)5 [112,113]. Other workers have reported that Hsp70 members in plasma membranes act as 'receptors' for viruses. This was reported for human T-cell lymphotropic virus type 1 [114]. Other viruses proposed to bind to cell surface Hsp70 (type normally unknown) include Dengue [115] and Japanese encephalitis virus [116].

There is still some degree of reluctance to accept the findings that molecular chaperones can escape from cells. This is a prejudice largely based upon ignorance of the number of mechanisms that eukaryotic cells (and bacteria) have for secreting proteins. It is now clear that a number of mechanisms in addition to the ER-Golgi-signal peptide mechanism exist for the secretion of proteins from eukaryotic cells [117]. The first secretion mechanism identified for Hsp70 release was through the formation of exosomes. These are small membrane vesicles that are secreted by numerous cell types. It was shown that Hsp70 (non-mitochondrial) was released by human peripheral blood mononuclear cells, in both unstressed and heat shocked states in the form of exosomes [118,119]. Exosomal release has also been proposed for the Hsp60 protein [120] although as a mitochondrial protein the secretion pathway is likely to be more complex than that of Hsp70. One of the most fascinating stories about protein secretion centres around IL-1. This key early response pro-inflammatory cytokine does not have a leader sequence and it is only in very recent years that the mechanism of secretion has begun to yield to investigation. There is evidence that IL-1 secretion is linked to a complex mechanism involving secretory lysosomes, phospholipase A_2 and the purinoceptor P2X7 [121,122]. The mechanism of release of Hsp70 has been described as being similar to that of IL-1, at least in respect to the involvement of the secretory lysosomal compartment [123]. Currently, there is no known mechanism to explain the secretion of Hsp10.

Thus there are appearing mechanisms to account for the presence of molecular chaperones in body fluids. This review will end with a brief review of the literature on the presence of Hsp10, Hsp60 and Hsp70 in body fluids.

Hsp10

As described, this was the first molecular chaperone to be identified in the blood as early pregnancy factor. Little more is known of this protein in body fluids. In a recent study Henderson & colleagues analysed blood from patients with periodontitis, a chronic inflammatory disease of the gums and compared them with age and gender matched controls. Suprisingly, Hsp10 was found in the majority of the bloods from the controls. In contrast, the levels of Hsp10 in patients with periodontitis were significantly lower. The patients were then divided into two groups. One had a very rigorous form of treatment which, in essence, cured the disease. The others were given a lesser form of treatment which meant that disease returned. In the first group, levels of circulating Hsp10 returned to normal whereas the second group remained lower – similar to pretreatment levels.

These findings suggest Hsp10 is a normal component in the plasma and that levels of Hsp10 are able to be influenced by degrees of inflammation. This seems to be at variance with the concept of Hsp10 only appearing in the blood during early pregnancy. This may depend on the sensitivity of the assays being used. The other interesting finding is that levels are controllable. However, while Hsp10 levels showed marked changes in circulating concentrations there was no changes in Hsp60 levels in these periodontal patients[43]. This finding is particularly intriguing as the Hsp60 (HSPD1) and Hsp10 (HSPE1) genes are linked head to head with 17 kb of DNA making up both genes which consist of 12 and 4 exons, respectively. The first exon of the human HSP60 gene is non-coding and the first exon of the human HSP10 gene ends with the start codon. The region in between is a bidirectional promoter with the transcriptional activity of the promoter fragment in the HSP60 direction being approximately twice that in the HSP10 direction under normal growth conditions [124]. Given this arrangement it is not clear how the levels of both proteins in blood could differ so markedly with one being responsive to environmental conditions and the other not [43]. Much more work is required to determine how levels of these two proteins are controlled in the circulation.

Hsp60

Graham Pockley was the first to determine the presence of Hsp60 in the blood of normal individuals (in transfusion samples) [125]. Given the work of Georg Wick, who had proposed a role for immunity to Hsp60 in the pathogenesis of atherosclerosis and the development of coronary artery disease [126], it seemed sensible to see if there was any correlation between Hsp60 levels and heart disease. Wick and co-workers looked at levels of circulating Hsp60 in participants in the Bruneck Study, an Italian study of a general population to identify disease risk factors. This revealed that circulating levels of Hsp60 were significantly elevated in subjects who showed signs of atherosclerosis as determined by measuring the common carotid artery intima/media thickness. Multiple logistic regression analysis determined that these associations were independent of age, sex, and other risk factors [127]. Follow up studies provided data confirming an association between high levels of blood Hsp60 and early carotid atherosclerosis [128]. Pockley studied a cohort of patients with borderline hypertension, compared to a comparable normotensive group, and reported that those with borderline hypertension had elevated levels of circulating Hsp60 which was associated with intima-media thicknesses [129]. However, there was no difference between Hsp60 levels in individuals with established hypertension and normotensives [130].

The Whitehall Study, a large prospective study of 10,000 British civil servants established that psychological stress is an important risk factor for cardiovascular disease [131]. Measurement of circulating Hsp60 in a small subset of these subjects revealed a correlation between blood Hsp60 levels and psychological distress in females [132]. In a larger cohort there was a significant correlation between circulating Hsp60 levels and

psychological distress in both males and females [133] and analysis of vascular damage by flow-mediated vasodilation measurements showed that Hsp60 levels correlated with such damage [134]. Measurement of Hsp60 levels in a cohort of healthy teenagers revealed that only 25% had circulating levels of Hsp60 but that these were the individuals who showed vascular damage as determined by flow mediated vasodilatation measurements [135]. This suggested that Hsp60 in the blood may be a predisposing factor for the development of atherosclerosis. Given the fact that Hsp60 has profound effects on leukocytes this may not be so far-fetched. One of the most fascinating findings about Hsp60 in the circulation is that only 50-60% of the population manifest this protein in their blood and that this seems to be a genetically defined event. Measurement of Hsp60 levels in individuals over a period of 30 days revealed complete stability in circulating levels. More strikingly, in blood samples taken 3 years apart, the same individual had virtually the same levels of Hsp60 in their blood. The other very striking finding is the range of concentrations of Hsp60 found in blood. 40-45% of the human race have no measurable Hsp60. These individuals have no circulating factors which interfere with the immunoassays used to measure Hsp60. The remaining 55-60% of the human race can have nanograms/ml, micrograms/ml or, in a small proportion of the population milligrams/ml. This is a million-fold range. No other protein has such a range of circulating levels [133]. We have used peptide fingerprinting and MALDI-TOF MS to identify the circulating protein, which is the intact Hsp60 monomer which has been into the mitochondrion and lacks the mitochondrial import peptide. How this protein exits from the mitochondrion and then from the cell is a key question, as is the source of the Hsp60 and its cellular effects.

Hsp70

Again Graham Pockley was first to report that Hsp70 was present in the blood of normal individuals [136]. The first evidence for changes of Hsp70 levels in disease was a study of patients with peripheral and renal vascular disease [137]. However, measurement of Hsp70 levels in a cohort of individuals who have been assessed for signs of coronary artery disease has revealed a negative correlation between Hsp70 concentrations in the blood and risk of coronary artery disease [138]. In a separate study progression of intimal-medial thickness of the carotids (a standard measurement of atherosclerosis development) was less pronounced in individuals with high levels of circulating Hsp70 [139]. Thus the hypothesis would have to be modified to one which classes Hsp70 as a protective factor for heart disease. A number of other reports have revealed the presence or changes in Hsp70 levels in various conditions or under various treatment regimes. This literature is rather diverse and to maintain the focus of this article the interested reader is advised to do the appropriate literature searches.

CONCLUSIONS

At the time of writing, 22 years have elapsed between John Ellis's seminal paper in Nature [140] on the function of the Hsp60 protein as a protein-folding chaperone and our emerging view that molecular chaperones are

serious moonlighting proteins with a growing array of diverse functions which are unrelated to protein folding. These functions are generated by molecular chaperones present on the cell surface or in the extracellular fluids. Some of the functions of a selected trio of human and mouse proteins have been described in this article and the role of bacterial homologues briefly touched upon. While the functions of molecular chaperones inside the cell are now generally clear and sensible, the growing functions for cell surface and secreted molecular chaperones tend to create a feeling that 'this can't quite be right'. For example, the finding that around 60% of the population have Hsp60 in their circulation and that levels of this protein can range from a few nanograms per ml to mgs/ml raises a plethora of questions, few of which can yet be answered. These results reveal that we have still a long way to go to understand the extracellular biology of chaperone proteins.

The most likely explanation for the evolution of moonlighting activity in the molecular chaperones is the enormous importance of the cell stress response to the survival of the individual cell and to the organism which these cells generate. It is a sensible hypothesis to suggest that stress in any particular part of the organism needs to be integrated into the homeostatic regulation of the whole organism. This is what is believed to be the evolutionary rationale for the signalling actions of molecular chaperones. They are the archetypal 'danger signals' as stress is the ubiquitous danger for all organisms. Thus it is likely that in the next decade the real biology of molecular chaperones will be identified as being a continuum between the protein folding actions within the cell to selective cell signalling actions on the cell surface and in the wider body milieu. In a previous publication we have termed this phenomenon – STRESS BROADCASTING [141]. A very simple diagrammatic view of this hypothesis is presented in Figure 2.

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Table 1. Molecular Chaperones, Alternative Names and Cellular Dispositions

Molecular Chaperone	Alternative Names	Cellular Disposition	Secreted
Heat shock protein (Hsp) 10	chaperonin (Cpn)10, GroES in *E. coli*	mitochondrion	yes
Cyclophilins	peptidylprolylisomerases	cytoplasm	yes
Hsp27	Hsp25, Hsp28	cytoplasm	yes
Hsp60	chaperonin (Cpn)60 GroEL in *E. coli*	mitochondrion	yes
Hsp70 (12 human genes)	DnaK in bacteria HSPA 1 onwards (new nomenclature)	all cell sites	some
BiP (Hsp70 family member)	Grp78, HSPA5	endoplasmic reticulum	yes
Hsp70-9	HSPA9 Grp75, mtHsp75, mortalin	mitochondrion	not established
Hsp90		cytoplasm	unclear

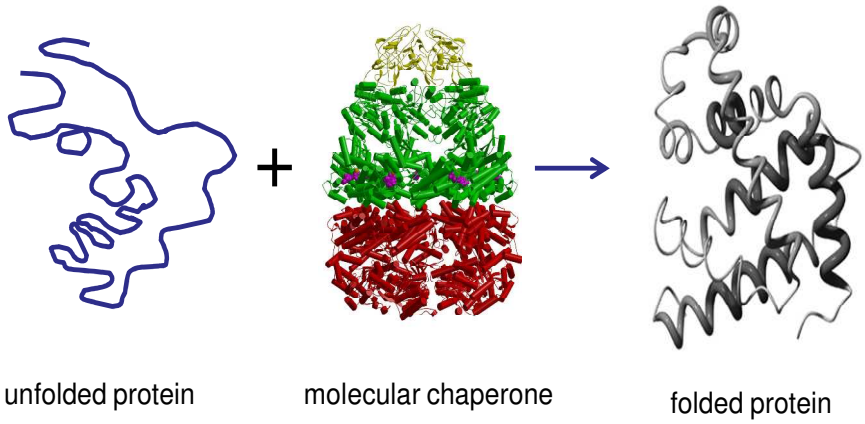

Figure 1. This schematic diagram encapsulates the basic biology of intracellular molecular chaperones (in this case Hsp60 or chaperonin 60). The function of molecular chaperones is to prevent unfolded proteins going to one of the potentially huge number of folding states (configurations) that they are capable of attaining. The molecular chaperone assists the unfolded protein to achieve its single correct three-dimensional configuration (by whatever mechanism it has evolved to generate this folded state) without becoming a constituent of the final folded protein. Molecular chaperones have evolved, it is believed, to aid protein folding inside the cell because in this milieu the protein concentration is enormously high leading to proteins inappropriately interacting with each other and failing to fold properly.

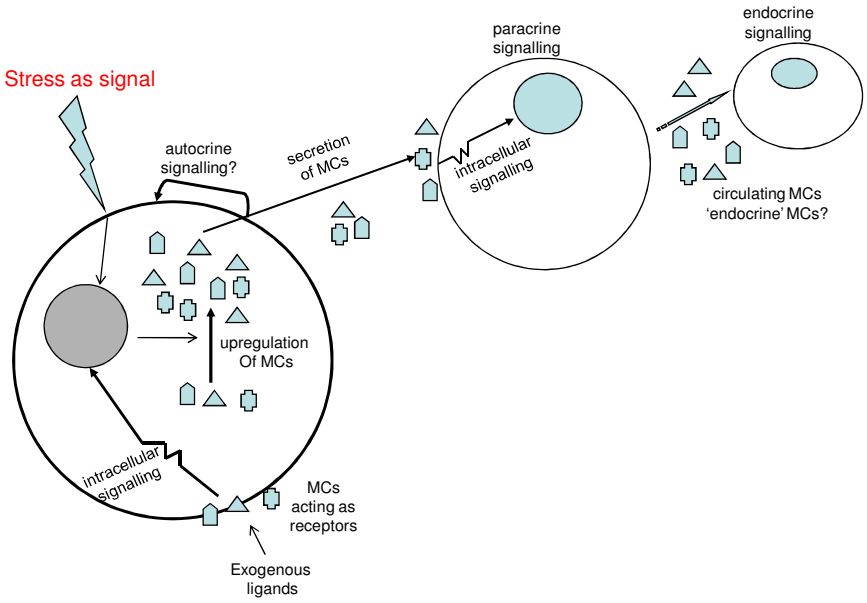

Figure 2. A simplified schematic diagram suggesting how stress received by a cell could lead to the upregulation of levels of intracellular molecular chaperones (MCs) which could lead either to these proteins exiting the cell to exist on the cell membrane (where they could act as receptors for host or microbial ligands) or to exit the cell and diffuse into the extracellular milieu to act as signals for other cells. If this process is amplified and sufficient cells are activated it could result in sufficient MC entering the blood for it to be measured. Thus it is envisaged that. Like cytokines, the MCs could act as autocrine, paracrine or endocrine signals. It is not clear if the very high levels of Hsp60 in some individuals could be explained in this way and possible alternative pathways may exist for the release of Hsp60.