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Review

Molecular chaperones and protein-folding catalysts as intercellular signaling regulators in immunity and inflammation

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ABSTRACT

This review critically examines the hypothesis that molecular chaperones and protein-folding catalysts from prokaryotes and eukaryotes can be secreted by cells and function as intercellular signals, principally but not exclusively, for leukocytes. A growing number of molecular chaperones have been reported to function as ligands for selected receptors and/or receptors for specific ligands. Molecular chaperones initially appeared to act primarily as stimulatory signals for leukocytes and thus, were seen as proinflammatory mediators. However, evidence is now emerging that molecular chaperones can have anti-inflammatory actions or, depending on the protein and concentration, anti- and proinflammatory functions. Recasting the original hypothesis, we propose that molecular chaperones and protein-folding catalysts are "moonlighting" proteins that function as homeostatic immune regulators but may also under certain circumstances, contribute to tissue pathology. One of the key issues in the field of molecular chaperone biology relates to the role of microbial contaminants in their signaling activity; this too will be evaluated critically. The most fascinating aspect of molecular chaperones probably relates to evidence for their therapeutic potential in human disease, and ongoing studies are evaluating this potential in a range of clinical settings. *J. Leukoc. Biol.* 88: 445–462; 2010.

Introduction

This review introduces the reader to the concept of protein folding and its relationship to classes of proteins known as molecular chaperones and protein-folding catalysts and also, to the hypothesis that many proteins can have more than one biological function—a phenomenon known as protein moonlighting. Armed with this background, the literature revealing the multifarious actions of molecular chaperones and protein-folding catalysts as signaling ligands will be reviewed.

Much of the early literature in this field suggested that molecular chaperones and protein-folding catalysts functioned as proinflammatory signals. In contrast, more recent research about these proteins is revealing that they can have profound anti-inflammatory effects. This has led to the evaluation of molecular chaperones and protein-folding catalysts as therapeutic agents in a variety of human inflammatory conditions/diseases, such as rheumatoid arthritis [1–3], psoriasis [4], diabetes [5], multiple sclerosis [6], and age-related macular degeneration [7]. These clinical trials are predicated on findings from a plethora of research laboratories, which support the hypothesis that molecular chaperones and protein-folding catalysts can be secreted from cells and that such secreted proteins can function as intercellular signaling molecules with a variety of cells [8, 9].

The original findings that molecular chaperones and protein-folding catalysts acted as proinflammatory signals raised the possibility that the biological actions of recombinant versions of these proteins were a result of contaminants from the *Escherichia coli*, in which these proteins were expressed [10]. This possibility has been reconsidered recently, and the evidence clearly shows that *E. coli* contaminants do not contribute to the activity of recombinant molecular chaperones [11]. Thus, the contamination issue will only be dealt with briefly herein.

AN INTRODUCTION TO MOLECULAR CHAPERONES AND PROTEIN-FOLDING CATALYSTS

The unknown research technician who carelessly increased the temperature of Dr. Ferrucio Ritossa's incubator one evening in

Abbreviations: ATL=adult T cell leukemia, Cpn=chaperonin, DC=dendritic cell(s), EAM=experimental autoimmune myocarditis, Hsp=heat shock protein(s), iDC=immature dendritic cell(s), MIF=macrophage inflammatory factor, Mip=macrophage infectivity promoter, Ni-NTA=nickel-nitrilotriacetic acid, PAMP=pathogen-associated molecular patterns, PDI=protein disulfide isomerase(s), PPD=purified protein derivative, PPI=peptidyl prolyl isomerase(s), PS=phosphatidylserine, TAMs=Trx80-activated monocytes, TNFRSF=TNFR superfamily, Trx=thioredoxin, Trx80=truncated thioredoxin

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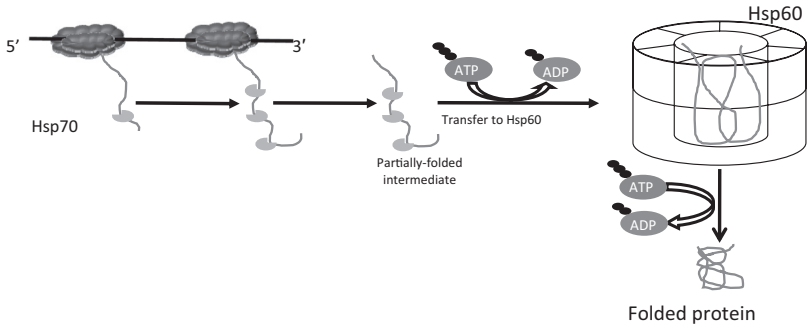

Figure 1. Functions of the major molecular chaperones in prokaryotes. Molecular chaperones of the Hsp70 family bind to and stabilize unfolded polypeptide chains during translation. The unfolded polypeptide is then transferred to chaperones of the Cpn (Hsp)60 family, within which protein folding takes place. ATP hydrolysis is required for release of the unfolded polypeptide from Hsp70 as well as for folding within Hsp60. In eukaryotes, Hp60 and one Hsp70 protein are found in the mitochondria.

the early 1960s could not have known the profound effect that this one moment of inattention would have on biological science. For in the incubator were *Drosophila* larvae, and the result of the increased incubation temperature was the appearance of new puffs in the polytene chromosomes, which were being studied by Ritossa. This was the first evidence that stress can influence gene transcription and induce the synthesis of new proteins [12]. For obvious reasons, when the products of these genes were identified initially by Alfred Tissières and colleagues [13] in the early 1970s, these proteins were termed "heat shock proteins", or Hsps. However, subsequent studies—demonstrating that a range of different stressors other than heat, such as viral infection, cytokines, oxidative stress, glucose deprivation, or exposure to toxins and certain metals, also induces the expression of such proteins—suggest that a more appropriate and descriptively correct term might be "cell stress" proteins [14]. It was some decades before the relationships among this heat-induced gene transcription, the role of protein folding in cell stress, and the roles of molecular chaperones in protein folding were consolidated [13, 15, 16]. Thus, by the late 1980s, it was recognized that a proportion of cellular proteins requires help with their folding and that this was facilitated via the actions of families of proteins termed "molecular chaperones" [16]. The accepted definition of the term molecular chaperone is "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" [17]. The role of the Hsp70 and Hsp60 proteins in folding nascent ribosomal proteins is shown in **Figure 1**. The approximate structures and interaction with client proteins of a number of the molecular chaperones described in this review are shown in **Figure 2**.

There are now a large number of families of molecular chaperones, many of which are involved in the ability of cells to cope with the various stresses that exist in the environment (**Table 1** and Fig. 2). If the transcription of the genes encoding molecular chaperones is increased by stress, then these proteins are molecular chaperones and cell stress proteins. It should be noted that the cellular levels of some molecular chaperones are not elevated by stress. Other proteins involved in modulating protein folding have enzymatic activity and are termed protein-folding catalysts. These include PDI and members of the Trx family, which are involved in controlling the formation of disulfide bonds (**Fig. 3**), and PPIs, which control the interconversion (*cis/trans*) of the peptide bond preceding proline residues [19]. In addition to being involved in protein folding, members of the Trx family play a role in the control of oxidative stress [20], which can result in protein misfolding [21]. The transcription of many of these proteins is also increased by stress.

Other cell stress proteins appear to have less of a role in protein folding. A good example is metallothionein, which was identified originally as a metal-binding, protective protein but has now been attributed to a range of potential functions including a role as an anti-inflammatory protein [22].

To avoid having to write molecular chaperones/protein-folding catalysts throughout this review, we will refer to these proteins under the joint term, cell stress proteins.

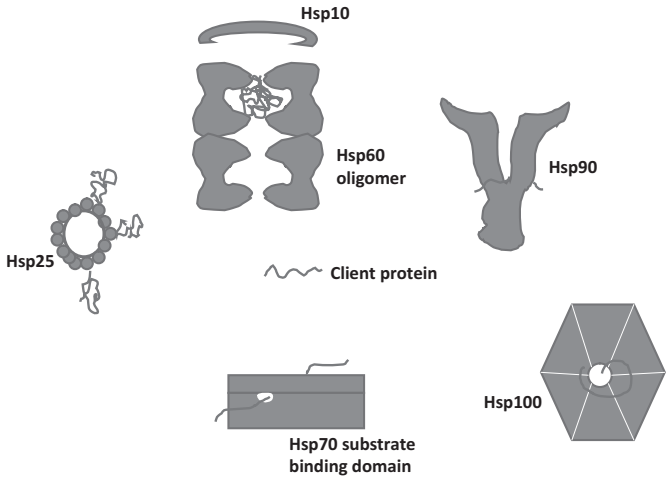

Figure 2. Schematic diagram showing the approximate structures of a number of the molecular chaperones discussed in this review and their interaction with the client proteins that they fold. Some of these molecular chaperones, such as Hsp60, Hsp70, Hsp90, and Hsp100, have ATP-ase activity and require the hydrolysis of ATP as part of the protein-folding mechanism. Others such as the small Hsps (Hsp25) and Hsp10 have no ATP-ase activity. The drawings approximate the molecular shapes of these molecular chaperones but are not to scale and have been redrawn from an original diagram from Professor Helen Saibil (Birbeck College, London, UK).

TABLE 1. Examples of the Molecular Chaperones/Protein-Folding Catalysts of Prokaryotes and Eukaryotes

Family	Eukaryotes	Prokaryotes
PapD	absent	present in some
NAC[a]	present	absent
Trx	present	present
Calnexin and calreticulin	present	absent
Trigger factor	absent	present
Prefoldin	GimC	absent
Small Hsps	Hsp25, Hsp27, crystallins	Acr1/2, ibpA, crystallins
PDI	PDI	DbsA
Hsp40	Ydj1, Sis1, Sec63, etc.	DnaJ, CbpA, RcsG
Cpns	Cpn10, Cpn60, CCT	Cpn10, Cpn60
Hsp70	many (see Table 4)	DnaK, Hsc66
Hsp90	Hsp82, Hsp83, Hsp90, Grp93	HtpG
Hsp100 (Clps)	Hsp104, Hsp110	ClpA/B/X
Hsp110 (SSE)	Hsp110, Grp170	absent

Note that there has been a recent attempt [18] to introduce a standard nomenclature for molecular chaperones. This has not been applied in this review, as this new nomenclature is still not standard and its introduction was felt to be too confusing for the readership. Note that only a small number of the above proteins have been studied for their cell–cell signalling activity, and only those proteins will be covered in this review.
[a]NAC, Nascent chain-associated complex.

PROTEIN MOONLIGHTING

This review proposes that cell stress proteins can exit cells (**Table 2**) and function as intercellular signaling proteins, which are of importance to the regulation of leukocyte functions. Many readers might feel that this is an unlikely statement. How can a molecular chaperone or a protein-folding catalyst also function as a signaling ligand for a leukocyte receptor? It is now 15 years since Campbell and Scanes [41] introduced the term "protein moonlighting" to describe the immunological functions of "endocrine peptides". Since then, a growing number of proteins from prokaryotes and eukaryotes have been shown to have moonlighting function(s) (reviewed in ref. [42]). The term moonlighting colloquially means to have one job during the day and a separate job at night. With proteins, the term moonlighting refers to the capacity of certain proteins to exhibit more than one biological function, as will be described. One of the most intriguing findings is the significant moonlighting actions of the highly conserved enzymes of the glycolytic pathway. Thus, phosphoglucose isomerase has been shown to have four actions in addition to its enzymatic one, including acting as a growth factor for neurons and as an implantation factor in the ferret [43]. GAPD takes the prize for the most moonlighting glycolytic enzyme with additional functions, including a uracil DNA glycosylase, an inhibitor of tubulin assembly, a transcriptional inhibitor, and an initiator of apoptosis [43, 44]. Its most recently identified moonlighting function is as a thiol-disulfide-exchange protein [45]. It has been established for some years that bacteria, especially the streptococci, possess many of the glycolytic pathway enzymes on their outer cell surfaces, and it has been proposed that these proteins are important in the virulence of *Streptococcus pyogenes* [45]. However, this hypothesis has been criticized on the basis that the enzymes could be present on the bacterial surface as the result of the death of other bacteria. *S. pyogenes* has only one gene coding for GAPD, and so, this gene cannot be inactivated. An alternative strategy that has been taken is to replace the chromosomal *gapD* gene with a modified gene coding for an enzymatically active GAPD mutant with a large C-terminal hydrophobic domain. It was thought that this extra domain would prevent enzyme release, and this turned out to be true. Significantly, bacteria expressing this mutant protein are significantly less virulent than wild-type bacteria. This has established that GAPD is a moonlighting, surface-located virulence factor in Group A streptococci [46]. Other glycolytic enzymes, such as enolase, are also found attached to the surface of many bacteria, where it can act as a binding factor for plasminogen, whose enzymic activity allows the bacterium to invade tissues [43]. There seems to be no limit to the discovery of moonlighting activity, and surprising results appear at regular intervals. For example, the cytokine-inducible transcription factor Stat3 has been discovered recently to be required for optimal function of the mitochondrial electron transport chain [47].

The above description suggests that, for example, all GAPD proteins exhibit moonlighting functions. However, a study of moonlighting proteins in yeast has revealed that this is not the case and that moonlighting functions are not conserved between different yeast species [48]. This is also the case with

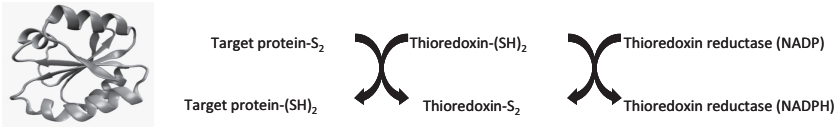

Figure 3. Ribbon structure of the Trx-fold and the mechanism catalyzed by this protein-folding catalyst.

TABLE 2. Nonintracellular Localization of Cell Stress Proteins

Protein	Cell surface	Secreted by cells	Present in body fluids	Reference
Hsp10			+	[23]
Trx		+	+	[24, 25]
Trx80		+	+	[26–28]
Cyclophilin A		+	+	[29, 30]
Hsp27	+	+	+	[31]
Hsp60			+	[32]
Hsp70	+	+	+	[33–37]
BiP		+	+	[3]
Hsp90	+	+		[38–40]

the moonlighting functions of cell stress proteins. This is probably one of the reasons why this area of research, on the additional biological actions of molecular chaperones, is so confusing for the neophyte.

CELL STRESS PROTEINS AND MOONLIGHTING

The literature about the moonlighting actions of cell stress proteins is growing apace, much of which is not directly relevant to this review. However, a few examples will be given to illustrate the range of actions which some of these proteins have evolved and to emphasize that in spite of marked sequence conservation, these moonlighting actions of cell stress proteins obey rules that have not yet been defined. Another complexity that faces the reader is that there are separate literatures about prokaryotic and eukaryotic cell stress proteins with only minimal points of contact.

Prokaryotic cell stress proteins have a growing number of moonlighting functions. Perhaps the most common of these is the ability to function as adhesins for a range of host components. This is particularly true for the bacterial Hsp60 and Hsp70 proteins (reviewed in ref. [49]). Bacteria also use PPI as virulence factors. For example, the well known virulence factor, Mip, of *Legionella pneumophila*, which enhances the survival of these bacteria within macrophages, is a PPI [50]. The most unusual activity of a bacterial cell stress protein is as an insect neurotoxin. The Hsp60 protein of *Enterobacter aerogenes*, a salivary commensal of the antlion larvae, functions as a potent insect neurotoxin [51].

With regards to eukaryotic cell stress proteins, a good example of a moonlighting function that does not cross the species barrier is the role of Hsp60 in sperm capacitation. This describes the processes that occur on the sperm surface membrane to allow fusion of the sperm with the ovum. The capacitation of murine sperm requires the presence of cell surface Hsp60 and the tyrosine phosphorylation of this protein [52]. Although it might be assumed that this incredibly important mechanism for survival is generic, this is not the case, as human sperm lack cell surface Hsp60. This protein is therefore not involved in human sperm capacitation [53].

Other functions of eukaryotic cell stress proteins, such as their interaction with leukocytes and vascular endothelial cells and the control of inflammatory and immune events, come under the focus of this review. One of the difficulties that critics of this literature cite is that there is no mechanism for cell stress proteins to exit cells. This is largely a result of a failure to understand that multiple pathways for protein secretion in bacteria [54] and eukaryotic cells [55] have now been identified, and new ones appear on a regular basis. Indeed, it has been known for decades that the mitochondrial Hsp10, Hsp60, and Hsp70 proteins exist in a variety of cell compartments, including the outer plasma membrane [56]. Furthermore, there is now strong evidence that a variety of cell stress proteins is secreted by cells in culture (e.g., PPIs, Hsp70, etc.), that Hsp10, Hsp27, Hsp60, Hsp70, immunoglobulin binding protein, and Trx are all found in the circulation, and that their circulating levels can alter during disease [57].

EXTRACELLULAR CELL STRESS PROTEINS AS SIGNALS FOR LEUKOCYTES AND VASCULAR ENDOTHELIAL CELLS

Having described some of the functions of cell stress proteins and shown that these proteins have a variety of moonlighting actions, the remainder of this review will deal with the literature that supports the hypothesis that secreted cell stress proteins can act as signals for leukocytes and vascular endothelial cells and therefore, have a controlling action in immune and inflammatory mechanisms. Individual cell stress proteins ranging in mass from Hsp10 (10 kDa) to Hsp90 (90 kDa) will be described (Table 1, Fig. 2), and for each protein family member, the literature about bacterial homologues will be described first, followed by that of the eukaryotic homologues. As alluded to earlier, critics of this literature have suggested that much of the proinflammatory activity of cell stress proteins is a result of microbial contaminants, principally, PAMPs, such as LPS, which copurify with the recombinant proteins, the majority of which has tended to be generated using *E. coli* expression systems [10]. This is a valid point, and one that every experiment using recombinant proteins that have been expressed in *E. coli* needs to address. Unfortunately, this was rarely undertaken in the early studies. Although these criticisms have been addressed in a recent review [11], a concurrent discussion about the controls used to ensure that the specific actions of recombinant cell stress proteins are a result of recombinant protein and not some *E. coli*-derived contaminant will be provided briefly herein.

Much of the evidence to be provided about the cell–cell signaling actions of the cell stress proteins is based on the premise that these proteins must be secreted from cells and be found in the extracellular fluids of the body. To date, there have been no consistent studies about cell stress protein release, and what is known is presented in Table 2.

Hsp OR Cpn10

The Hsp10/Hsp60- or Cpn10/Cpn60-folding machine is really the prototypic molecular chaperone complex, which in the

guise of the *E. coli* GroES/GroEL proteins, exhibits an oligomeric complex with the Hsp60 monomers, forming a tetradecamer of two back-to-back, seven-membered rings of 60 kDa subunits (Fig. 2). To match this, the 10-kDa Hsp10 monomer forms a hepameric complex, which caps the Hsp60 oligomer and thereby, allows protein folding to occur (Fig. 2). As will be mentioned when discussing mycobacterial homologues, other Hsp10/Hsp60 proteins appear not to show this sevenfold symmetry.

Studies about the immunomodulatory actions of bacterial Hsp10 have been confined to the protein from *Mycobacterium tuberculosis*. Injection of rats with an aqueous solution of synthetic *M. tuberculosis* Hsp10 after immunization with *M. tuberculosis* Hsp65 to induce adjuvant arthritis (but before arthritis occurred) has been shown to delay the onset and ameliorate the severity of this disease. Synthetic Hsp10 peptides derived from the N-terminal region of the protein have a similar therapeutic effect. In contrast, neither the *E. coli* nor the rat homologues (both synthetic) has any therapeutic efficacy. The use of synthetic peptides clearly excludes a role for bacterial contaminants [58]. *M. tuberculosis* Hsp10 has also been shown to inhibit experimental allergic asthma in mice [59]. We clearly do not understand the interactions of Hsp10 with leukocytes, as *M. tuberculosis* Hsp10 has been shown to act as an osteoclast growth factor and to promote resorption of murine bone cultures. Again, this activity has been replicated using synthetic peptides [60].

When we turn to the human Hsp10 homologue, we encounter a long history of the study of this protein, which goes back to 1977, 11 years before the identification of Hsp60 as a molecular chaperone. At this time, Australian scientists discovered an immunosuppressive factor ("early pregnancy factor") in the serum of pregnant women in the first trimester [23, 61]. It was not until 1994 that this activity was identified as the cell stress protein Hsp10 [62]. Recombinant human Hsp10 was then shown to be immunosuppressive in a rat skin graft model [63] and in experimental autoimmune encephalomyelitis in rats [64, 65]. An Australian biopharmaceutical company has become interested in the therapeutic potential of recombinant human Hsp10. Early studies from the company revealed that human Hsp10 could inhibit LPS-induced activation of monocytes [66]. Since then, the company has manufactured recombinant human Hsp10 in *E. coli* to good manufacturing practice standards. As bacteria have limited post-translational protein modification ability, including acetylation, the therapeutic Hsp10 preparation has an added alanine residue to mimic the missing N-terminal acetyl group. This recombinant human protein, which has been tested extensively for bacterial contaminants, has been shown to have some therapeutic benefits in a variety of human inflammatory diseases [1, 2, 4, 6]. Clearly, the final product has minimal, if not nonexistent, contamination with PAMPs. The search for additional therapeutic applications is continuing.

Although identified initially as a factor in pregnant blood, more sensitive immunoassays have found Hsp10 in the blood of nonpregnant individuals. Levels of circulating Hsp10 are lower in patients with periodontal disease than in controls, and the treatment of this disease results in increases in circulating levels of this protein. This suggests that Hsp10 might be a natural anti-inflammatory molecule that is consumed during inflammatory events [67]. Thus, Hsp10 clearly appears to be a cell stress protein with an immunosuppressive activity that is of sufficient efficacy to attract support from the biopharmaceutical industry.

Trx

Members of the Trx superfamily are essential proteins with a common fold structure (the Trx-fold; see Fig. 3) containing the canonical CXXC motif, which is also found in the CXXC chemokine family. Although Trxs might show limited sequence conservation and different reactivities, the link between them is the fold [68]. Trx was discovered initially in 1964 as an electron donor to ribonucleotide reductase, an enzyme that is essential for DNA synthesis [68]. There is currently only one report of a bacterial Trx having immunomodulatory activity [69], and one of the authors refuted this hypothesis by showing that the recombinant version of this Trx, although enzymically active, had no influence on T cell function [70]. Human Trx (now classified as cytosolic Trx1 but termed Trx in this article) was identified initially as a cytokine (ATL-derived factor) from the supernatants of cultured ATL.2 cells. Cloning of the gene encoding this protein identified it as human Trx [24]. Inactivation of the gene encoding Trx1 in mice causes early embryonic lethality [71], thereby revealing the importance of Trx for organismal homeostasis.

It has become apparent that Trx has protein-folding, antioxidant, and signal transduction properties within cells. For example, Trx inhibits apoptosis signal-regulating kinase 1 [72]. Trx was identified as a T cell growth cytokine and is clearly released by cells. Indeed, oxidative stress increases its release from T lymphocytes, which shows that this is a redox-sensing cytokine system [73]. Over the past 10 years, Trx has been shown to have a range of actions, suggesting that it might be a natural regulator of inflammation. Early studies revealed that Trx is a unique chemoattractant for human leukocytes, which acts in a G-protein-independent manner. Mutation of the cysteines in the active site results in loss of activity [74]. The demonstration that elevating Trx levels in the circulation of mice have a marked effect on LPS- or chemokine-induced neutrophil migration into skin air pouches confirms that the chemotactic action of Trx occurs in vivo [75]. This ability of circulating Trx to inhibit leukocyte chemotaxis appears to be important for the pathogenesis of infection with the HIV. Thus, the survival of HIV-infected individuals with low numbers of $CD4^+$ T cells (<200 $CD4^+$ T cells/μl blood) and high plasma levels of Trx is impaired significantly compared with those with lower Trx levels. It is hypothesized that the Trx prevents the innate immune system functioning to destroy opportunistic pathogens by blocking leukocyte chemotaxis into tissues [25]. These results clearly suggested that Trx had anti-inflammatory effects and may have therapeutic benefits. That this was true was first shown in an EAM model in mice. Trx administration reduced the severity of EAM, as measured histologically. In contrast, administration of anti-Trx antiserum markedly enhanced the disease in a Trx mutant lacking enzymatic activity

[76]. It was also shown that Trx had the ability to block almost completely experimental acute pancreatitis in mice by inhibiting many aspects of the pathology including proinflammatory cytokine synthesis [77]. Administration or transgenic up-regulation of Trx has also been shown to block a range of other experimental diseases and conditions (**Table 3**).

The mechanism of action of extracellular Trx is speculated to be a result of: antichemotactic activity; an anti-MIF action [89]; inhibition of leukocyte-vascular endothelial cell interactions [90]; and interaction with complement factor H and inhibition of the alternative pathway C3 convertase [91]. Curiously, it is only in recent years that the search for a receptor for Trx has begun to yield some degree of success. One report has found that Trx binds to the TNFRSF8 (CD30) [92]. The exact functions of CD30 are still the subject of debate [93], and it is unclear if this is the only cell surface receptor for Trx.

Trx80

In addition to Trx, with its many extracellular biological functions, there is a naturally occurring truncated form of this protein (Trx80), which comprises the 80 or 84 N-terminal residues of Trx. This protein was purified originally from plasma and was identified as an eosinophilic cytotoxicity-enhancing factor [26]. The intact and the truncated proteins are secreted by human monocytes following exposure to a variety of stimuli, including LPS [27]. Unlike Trx, Trx80 is a potent mitogen for resting human peripheral blood monocytes [94] and promotes the expression of cell surface CD14, CD40, CD54, and CD86 and the synthesis of IL-12 [28]. Trx80 differs from the parent protein in that it is a dimer and lacks the reductase activity [95, 96]. These actions of Trx80 appear similar to those induced by LPS. However, a more detailed analysis about the interaction of Trx80 with human peripheral blood $CD14^+$ monocytes has identified a unique activation state, termed TAMs, which to some extent, resemble iDCs. Like iDCs, the TAMs have increased surface expression of CD1a and mannose receptor. However, unlike iDCs they express a high proportion of CD14 but lower proportions of CD83 and HLA-DR. Trx80-stimulated cells have high pinocytic activity and produce large amounts of the anti-inflammatory cytokine IL-10. This is an activation profile that is not associated with LPS [97] and suggests Trx80 is an alternative macrophage activator.

Hsp27

This small ATP-independent molecular chaperone is a member of the small Hsp group and has several important intracellular functions [98]. Extracellular recombinant Hsp27 stimulates human monocytes to generate an activation state that is characterized by the overproduction of IL-10 relative to TNF-α, suggesting that this protein has anti-inflammatory properties [99]. This activity has been replicated recently using murine monocytes, and Hsp27 was able to inhibit acetylated low-density lipoprotein stimulation of monocyte IL-1β synthesis and to stimulate the formation of IL-10 directly. Moreover, when apolipoprotein $E^{-/-}$ mice were crossed with mice overexpressing Hsp27, the resultant offspring showed a remarkable inverse correlation between Hsp27 levels in blood and the size of atherosclerotic lesions [100]. These data strongly suggest that Hsp27 is antiatherogenic and have supported at least one clinical study [101]. It has also been suggested that circulating Hsp27 is linked to diabetic neuropathy [102].

The effects of Trx80 [97] and Hsp27 [99] on macrophages suggest that they are inducing some form of alternative activation state that is distinct to that which is induced by LPS or IFN-γ [103]. A more detailed examination of the action of extracellular recombinant Hsp27 has found that this molecular chaperone interferes with the normal pathways of monocyte differentiation into macrophages and DCs. For example, the addition of Hsp27 to monocytes being induced to differentiate into iDCs enhances the programmed cell death ligand 1 coinhibitor and decreases the CD86 costimulator expression levels in parallel to a decreased iDC-induced MLR [104]. Thus, the available evidence suggests that Hsp27 is an anti-inflammatory signal with properties that are clearly distinct from those of LPS. Clearly, Hsp27 is another potential therapeutic molecular chaperone.

PPIs

There are three distinct families of PPIs: parvulins, cyclophilins, and FK506-binding proteins. Most attention has focused

TABLE 3. Therapeutic Actions of Trx

Condition	Effect of Trx	Reference
Bleomycin-induced lung injury	inhibits	[78]
OVA-induced murine asthma	inhibits inflammation and hyper-responsiveness	[79]
LPS-induced bronchial inflammation	inhibits	[80]
Airway remodeling in chronic allergic asthma	inhibits	[81]
Cigarette smoke-induced lung inflammation and emphysema	inhibits	[82]
Goblet cell hyperplasia in asthma model	inhibits	[83]
Focal ischemic brain injury	inhibits	[84]
Bacteria induced gastritis	inhibits	[85]
Murine colitis	inhibits	[86]
Choroidal neovascularization	inhibits	[7, 87]
Contact hypersensitivity	inhibits	[88]

on the cyclophilins, the actions of which are blocked by the immunosuppressive agent cyclosporine. Cyclophilin was first identified as a secreted factor of LPS-stimulated murine macrophages, which had inflammatory activity in mice, and was a chemoattractant for neutrophils and monocytes. That this activity was a result of cyclophilin and not some contaminant was shown by the ability to block these effects with cyclosporin A but not the inactive structural analog cyclosporine H [29]. An early finding was the role played by cyclophilin in the uptake of HIV-1 into T lymphocytes and macrophages [105]. The signaling receptor for cyclophilin A and B is CD147, a member of the Ig superfamily with roles in lymphocyte development [106]. However, it appears that cyclophilin B is more important than cyclophilin A in T cell signaling, particularly with respect to the control of T cell adhesion [107]. The importance of cyclophilins to in vivo inflammation has been shown in an acute lung injury model, in which the inhibition of neutrophil accumulation by antibodies to CD147, which block cyclophilin interaction with this receptor, has been shown to be associated with a decrease in lung pathology [108]. Likewise, the blockade of cyclophilin-CD147 interactions is effective in inhibiting collagen-induced arthritis in mice [109]. There is also evidence for the participation of cyclophilins in cardiovascular disease [110] and in sepsis [111]. Clearly, more attention needs to be focused on the role of this family of molecular chaperones as secreted pathological factors.

The identification of the receptor for cyclophilins and the specificity of this binding, as defined by the suppression of cyclophilin signaling by selective anti-CD147 antibodies, demonstrate that the actions of the cyclophilins are not caused by bacterial contaminants such as LPS.

MEMBERS OF THE 60-KDA STRESS PROTEIN FAMILY

Hsp60, also known as Cpn60, is an essential cellular protein in prokaryotes and eukaryotes and was one of the first molecular chaperones to be identified as having cell signaling activity. This chaperone was identified initially as a protein that was able to stimulate human monocyte proinflammatory cytokine synthesis without inducing monocyte activation. This led to the identification of this protein as a proinflammatory signal that acts via the same receptors as LPS and has resulted in a number of workers dismissing the literature about the signaling actions of Hsp60 proteins [10]. This section will attempt to dissect the literature into a number of strands and thereby, demonstrate that Hsp60 has multiple, source-dependent functions with widely different cellular actions.

Bacterial Hsp60 proteins

The first report of the signaling actions of Hsp60 used the Hsp60.2 (Hsp65) protein of *M. tuberculosis* (this organism produces two Hsp60 proteins) and revealed that it could induce human monocytes to synthesize proinflammatory cytokines [112]. This suggested that Hsp60.2 had identical actions to LPS, which induces a so-called classical activation state in macrophages [103], and has led to significant criticisms of this work. This is largely a result of the fact that workers in this field have focused on the biology of mammalian Hsp60 proteins and ignored work on bacterial Hsp60 proteins.

The report that *M. tuberculosis* Hsp60.2 stimulated proinflammatory cytokine synthesis by human monocytes suggested that this protein generated a classic activation state associated with antigen presentation and T cell activation. However, there is a major difference between Hsp60.2 and the classical activators (IFN-γ and LPS). Although the exposure of human monocytes to *M. tuberculosis* Hsp60.2 induces the production of the same amount of cytokines as is released by cells exposed to IFN-γ plus LPS, unlike cells that have been stimulated with IFN-γ plus LPS, monocytes exposed to Hsp60.2 do not show the increased expression of FcγRs, MHC class II proteins, or the release of reactive oxygen intermediates [113]. This established that *M. tuberculosis* Hsp60.2 is not inducing a classically activated state in macrophages and clearly delineates the effect of this molecular chaperone from IFN-γ/LPS [113]. Additional evidence for the specific action of the Hsp60.2 protein from *M. tuberculosis* and its distinction from activators such as LPS is the report that it induces cultured human vascular endothelial cells to synthesize the leukocyte adhesion receptors (ICAM-1, VCAM, and E-selectin) in a cytokine (IL-1, TNF-α)-independent manner [114]. This contrasts with the literature, which suggests that the synthesis of these adhesion molecules requires the prior induction of the proinflammatory, early response cytokines, IL-1 and/or TNF-α [115]. Further evidence that the properties of bacterial Hsp60 proteins and PAMPs, such as LPS and bacterial lipoproteins, are distinctive arises from reports that these bacterial proteins can stimulate TLR4-negative mice [116] and that they can stimulate monocyte cytokine synthesis in a CD14 [117]-, TLR2-, TLR4-, or Myd88-independent manner [118]. Thus, these proteins from *Aggregatibacter actinomycetemcomitans*, *E. coli*, and *Helicobacter pylori*, respectively, have cell signaling actions that are independent of LPS and other PAMPs, which interact with TLR4 or TLR2.

The Hsp60 protein "family" is also a diverse moonlighting family of proteins. For example, the Hsp60.1 and Hsp60.3 proteins from *Rhizobium leguminosarum* share approximately 70% sequence identity. Although the recombinant proteins have the same level of LPS contamination, the Hsp60.1 protein, which is the major chaperone of this bacterium, cannot stimulate human monocyte proinflammatory cytokine synthesis, whereas the Hsp60.3 protein is a potent inducer of monocyte cytokine synthesis [119].

As stated above, most of the mycobacteria have at least two Hsp60 proteins. A comparison of the monocyte-activating ability of the Hsp60.1 and Hsp60.2 proteins of *M. tuberculosis* has revealed that the Hsp60.1 protein is more potent and efficacious than the Hsp60.2 protein [120], despite the fact that both of these proteins had the same (low) level of LPS contamination [120]. In spite of this, neither of the two *M. tuberculosis* Hsp60 proteins was able to stimulate the breakdown of the murine calvarial bone in vitro [60, 116]. In contrast, bone breakdown could be induced by the *E. coli* Hsp60 protein (GroEL) [121] and the human Hsp60 protein [122] and is a result of the chaperone acting as an osteoclast growth and differentiation factor. It should be noted that LPS is an incredi-

bly potent inducer of murine calvarial bone breakdown [116, 120, 121], and this lack of bone-resorbing activity shows that LPS contamination in these recombinant mycobacterial proteins must be minimal. Osteoclasts are multinucleate cells derived from monocytes, and the process of osteoclast formation can be thought of as a unique form of macrophage activation.

A more detailed analysis of the effect of *M. tuberculosis* Hsp60 proteins on bone remodeling has revealed that the Hsp60.2 protein can neither stimulate nor inhibit bone resorption or the generation of osteoclasts. In contrast, Hsp60.1 is a potent inhibitor of bone resorption and of the receptor activator for NF-κB ligand-stimulated osteoclast production in vitro. The *M. tuberculosis* Hsp60.1 protein is also able to completely suppress the massive osteoclastic bone damage which occurs in rats with adjuvant arthritis without, at the same time, inhibiting the joint inflammation [123]. The mechanism of action of *M. tuberculosis* Hsp60.1 appears to be the inhibition of the transcription of the key osteoclast transcription factor NFATc1, without blocking the activity of NF-κB or MAPKs [123]. This appears to be a potentially novel mechanism for blocking osteoclast formation and has therapeutic potential.

It has also been shown that the Hsp60.1 protein of *M. tuberculosis* inhibits the activation of monocytes, which is induced by the proinflammatory mixture of mycobacterial components known as PPD [124]. *M. tuberculosis* Hsp60.1 protein can also inhibit experimental allergic asthma in mice, whereas the Hsp60.2 protein is inactive [59]. Of interest, another group has found that the Hsp60.2 protein of *Mycobacterium leprae* is a potent inhibitor of this allergic asthma model in mice, whereas the *M. tuberculosis* Hsp60.2 protein and a range of other bacterial Hsp60 proteins are inactive [125]. A particularly interesting feature of this study was the fact that these two mycobacterial Hsp60.2 proteins share 95% sequence identity. Thus, only tiny sequence changes in the Hsp60.2 protein can confer novel biological actions. This has also been the finding of Yoshida and co-workers [51], who found that the Hsp60 protein from *E. aerogenes* was an insect neurotoxin, whereas the highly homologous *E. coli* Hsp60 protein, GroEL, was inactive. However, single residue mutations in GroEL could produce potent neurotoxic activity [51].

The activity of the Hsp60/Hsp10 proteins of *M. tuberculosis* suggested that they could have some role in the pathogenesis of tuberculosis. To determine such roles, attempts were made to inactivate the *Hsp10*, *Hsp60.1*, and *Hsp60.2* genes in the virulent *M. tuberculosis* strain H37Rv. Only the *Hsp60.1* gene could be inactivated. Further, it was shown by complementation studies in an *E. coli* Hsp10/Hsp60 conditional mutant that the Hsp60.1 protein does not function as a molecular chaperone [126]. This is a surprising finding. Even more surprising was the finding that the infection of mice or guinea pigs with the *Hsp60.1* isogenic mutant resulted in the failure of the bacterium to induce a granulomatous response [126]. The wild-type, isogenic mutant and complemented mutant all grew at the same rate, and so, the differences were not a result of bacterial numbers. Granuloma formation is the hallmark of the pathology of tuberculosis, and it would appear from these studies that the Hsp60.1 protein of *M. tuberculosis* is a, or the, key cell signal for the generation of granulomas. Again, this suggests that Hsp60.1 is a signaling protein for inducing a novel form of macrophage activation, the granuloma macrophages (epithelioid cells and Langhans giant cells).

A key question was whether the activity of the *M. tuberculosis* Hsp60 protein required the whole oligomeric complex of Hsp60 or the individual 60 kDa protein or some individual domain of the protein. The crystal structure of the Hsp60 of *E. coli* reveals a tetradecameric structure. This has been assumed to be the oligomeric state in which all Hsp60 proteins would exist. However, it now emerges that the Hsp60 proteins from *M. tuberculosis* behave physicochemically as monomers-dimers, and the crystal structure of the Hsp60.2 protein is a dimer [127, 128]. This is a completely unexpected finding and shows that even the prototypic Hsp60 still has many surprises to offer. It is still not clear why these proteins are so different from GroEL and what these differences mean in terms of protein folding and extracellular signaling. However, it has been found by generation of the three individual recombinant domains of *M. tuberculosis* Hsp60.1 that the monocyte-stimulating activity resides in the equatorial domain [129]. Thus, the oligomeric state is not required for cell signaling activity, which resides within a specific part of the Hsp60 monomer.

The Hsp60.1 proteins of the mycobacteria are beginning to exhibit a variety of moonlighting actions. Thus, the *Mycobacterium smegmatis* [130] but not the *M. tuberculosis* [126] Hsp60.1 protein is vital for biofilm formation. More recently, the Hsp60.1 protein of *M. tuberculosis* has been shown to be able to bind to and condense DNA and acts as a DNA-protective molecule [131]. Another recent, fascinating finding is that the Hsp60.2 protein of *M. tuberculosis* is secreted and functions as an adhesin, allowing this pathogenic bacterium to bind to macrophages as part of the macrophage-invasion process, which lies at the heart of the pathology of tuberculosis [132].

Thus, it is now emerging that the mycobacterial Hsp60 proteins have pro- and anti-inflammatory actions (**Fig. 4**) and that such actions appear to be concentration-dependent. At micromolar concentrations, these proteins are proinflammatory, whereas at lower concentrations, they can be anti-inflammatory. The major conclusion from these studies is that each bacterial Hsp60 protein must be treated as a separate case and its function considered in the context of its concentration in the organismal microenvironment. As will be discussed in a later section, the Hsp70 (DnaK) protein of *M. tuberculosis* also functions as a virulence factor, suggesting that for the mycobacteria, the cell stress proteins should be looked at more closely for immunomodulatory activity.

Eukaryotic Hsp60 proteins

The previous section has provided evidence that the interaction of bacterial Hsp60 proteins with leukocytes or vascular endothelial cells results in activation or inhibition of the cells via mechanisms that do not involve contaminating PAMPs. However, studies of the human Hsp60 protein have been the subject of controversy over the role of such PAMPs. To some extent, the fault has lain with the level of LPS contamination, which has been present in the principal commercial recombinant Hsp60 preparations and the difficulty in identifying the

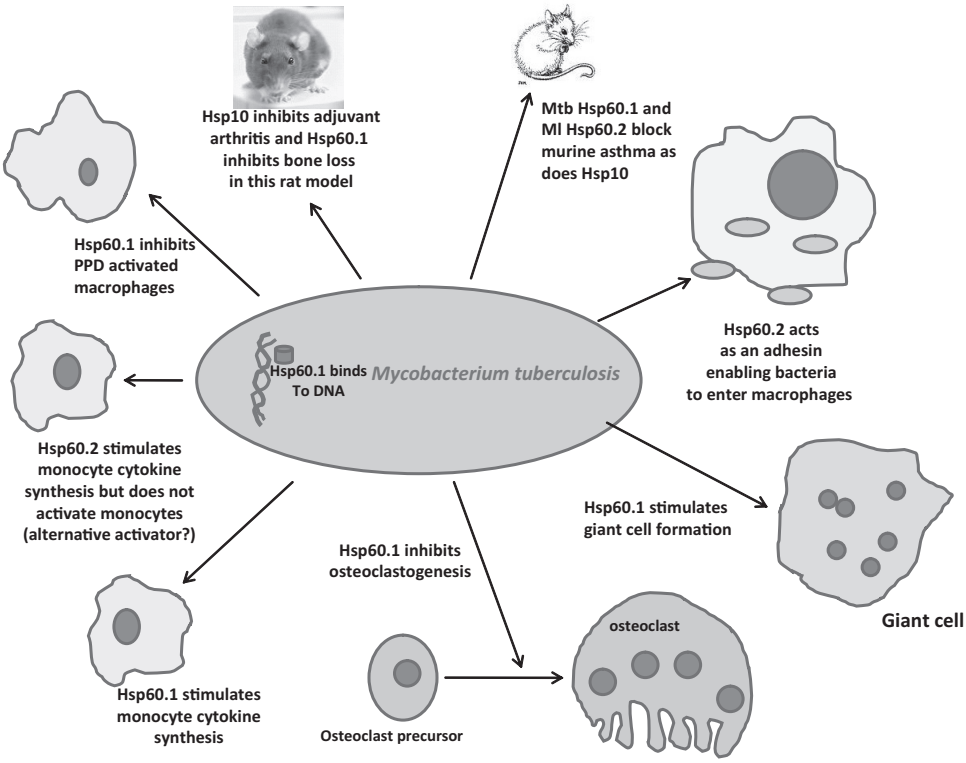

Figure 4. A summary of the moonlighting actions of the mycobacterial Hsp60 proteins (Ml, *M. leprae*; Mtb, *M. tuberculosis*).

precise origin and nature of the preparations being used in the reported studies. Although more commonly considered as an inflammatory molecule and the archetypal "danger" signal, a number of investigators have reported the human 60-kDa stress protein to have a number of anti-inflammatory properties; the literature dealing with this has been considered in-depth elsewhere [133–137] and will therefore be summarized only briefly here. These findings confirm a moonlighting function for eukaryotic Hsp60 in the context of its immunological properties.

With regards to proinflammatory effects, human Hsp60 has been reported to induce the secretion of IL-6 from macrophages [138] and the rapid release of TNF-α, NO, IL-12, and IL-15 from human macrophages [139]. It has also been shown to up-regulate the expression of costimulatory molecules (CD86) and CD40 in a murine macrophage cell line, enhance the maturation of DCs, and increase the antigen-presenting capacity of APCs [140]. The reports that Hsp60 (and Hsp70) binds to the same receptors as those of bacterial endotoxins, such as *E. coli* LPS also binds (CD14, TLRs), were the driving force behind the suggestion that the proinflammatory activities of these proteins result from the effects of LPS or other molecules associated with the bacterial surface [141, 142]. However, there is a wealth of data suggesting that eukaryotic Hsp60 can have potent anti-inflammatory effects [133], and animal studies demonstrate its ability to prevent/attenuate experimental autoimmune disease [143–147]. An inverse association between the severity of disease and the production of the regulatory cytokines IL-4 and IL-10 by T cells stimulated with human Hsp60 in patients with rheumatoid arthritis has also been reported [143]. Furthermore, in patients with juvenile chronic arthritis, in whom the disease follows a relapsing-remitting course, circulating T cells responsive to human Hsp60 are of the regulatory Th2 phenotype, and their presence is beneficial [148]. In addition, the spontaneous remission of juvenile idiopathic arthritis is associated with the presence of human Hsp60-reactive $CD30^+$ T cells producing IL-10 [149].

These anti-inflammatory properties appear to involve the induction and/or maintenance of Th2-type $CD4^+$ T cell populations secreting regulatory cytokines such as IL-4 and IL-10 [149]. Hsp60 can activate naturally occurring $CD4^+CD25^+$ regulatory T cells via interactions with cell surface-expressed TLR2 [150]. Such interactions enhance the ability of these cells to regulate $CD8^+$ T cell populations via cell–cell contact and IL-10/TGF-β-mediated mechanisms [150]. Of importance, when considering the potential involvement of endotoxin contamination in the immunological properties of Hsp60, studies show that Hsp60-derived peptides (which are endotoxin-free) can have potent, anti-inflammatory properties and attenuate human autoimmune diabetes [5]. Studies have also reported a clear differentiation between the immunological properties of human and bacterial Hsp60-derived epitopes, and the former induces an anti-inflammatory phenotype in T cells from patients with rheumatoid arthritis and the latter, a proinflammatory phenotype [151]. Taken together, these and other studies suggest that rather than being a universal proinflammatory molecule, eukaryotic Hsp60 can function as an anti-inflammatory protein with the potential to attenuate rheumatoid arthritis [152].

It is clear that the biological/immunological properties of Hsp60 cannot be attributed exclusively to the presence of endotoxin contamination. The most convincing evidence relates

to the findings that synthetic, eukaryotic, Hsp60-derived peptides, which are not contaminated with or carry endotoxin, can induce anti-inflammatory phenotypes in responding leukocyte populations [153] and attenuate arthritis [154, 155], autoimmune disease [5, 150, 156], and atherosclerosis [157]. It should be noted that naturally occurring $CD4^+CD25^+$ immunoregulatory T cells that express TLRs can also be activated by LPS [158]. Although this suggests that endotoxin contamination could, at least in part, explain some of the anti-inflammatory properties of Hsp60 and other stress proteins that are proposed to use TLRs as their cell surface ligand, human T cell adhesion responses via TLR2 can be 1000-fold more sensitive to mammalian Hsp60 than they are to LPS [159]. It is therefore unlikely that endotoxin accounts for the anti-inflammatory properties of these cell stress proteins.

MEMBERS OF THE 70-KDA STRESS PROTEIN FAMILY

The literature about the biological actions of Hsp70 is somewhat similar to that of Hsp60, and bacterial and human proteins are studied separately and provide different results. To complicate matters further, there are at least 14 human Hsp70 proteins and only a few of these are examined for leukocyte-modulating activity (**Table 4**). Such study has identified a clear-cut divergence in immunomodulatory activity between, for example, Hsp701a (HSPA1A) and BiP (grp78 or HSPA5).

Bacterial Hsp70 (DnaK)

The majority of studies of bacterial Hsp70 proteins has concentrated on the protein from *M. tuberculosis*. Thomas Lehner et al. [160] were the first to discover that recombinant *M. tuberculosis* Hsp70 dose-dependently stimulated the $CD8^+$-enriched T lymphocytes of naïve, nonhuman primates to produce the β-chemokines or CC chemokines: CCL3 (Mip1α), CCL4 (Mip1β), and CCL5 (RANTES). As will be described later, at around the same time, other workers were reporting that human Hsp70 stimulated human monocytes to generate proinflammatory cytokines by binding to the receptors for PAMPs such as CD14 and TLR2/4. However, Lehner and co-workers [161] found that *M. tuberculosis* Hsp70, but not human Hsp70, induced human mononuclear cells to synthesize chemokines by binding to CD40, another member (like CD30) of the TNFRSF. To determine the active site in the *M. tuberculosis* Hsp70, the N-terminal domain containing the ATP-ase active site (aa 1–358) and the C-terminal peptide-binding domain (aa 359–610) were cloned and expressed independently. The parent recombinant protein was isolated by ATP affinity chromatography, whereas the His-tagged recombinant protein domains were purified by metal affinity chromatography on Ni-NTA matrices. This approach clearly revealed that the C-terminal domain was responsible for stimulating chemokine synthesis. The C-terminal domain stimulated THP1 cells to generate IL-12, TNF-α, and NO. Curiously, the native recombinant Hsp70 was incapable of inducing IL-12 synthesis and was less active than the C-terminal domain in stimulating TNF-α and NO synthesis [162]. This enhanced activity of the C-terminal domain was shown not to be a result of the ATP-ase-binding domain having inhibitory activity and remains unexplained. In addition to these effects, *M. tuberculosis* Hsp70 induces the maturation of DCs [162]. Further analysis of the C-terminal domain revealed that the cytokine-inducing and DC-differentiating activity resided in a fragment comprising residues 359–494. Further refinement of the active site was undertaken using overlapping peptides. This revealed that peptide 407–426 was the major stimulating peptide in *M. tuberculosis* Hsp70 protein. Alanine-scanning mutagenesis revealed that the residues Q407, P408, S409, and V410 were critical for the activity of this Hsp70 peptide. In addition to this myeloid cell stimulatory site, *M. tuberculosis* Hsp70 contains an inhibitory peptide site, residues 457–496 [163]. The action of both peptides seems to be via activation or inhibition of p38 MAPK [163]. The importance of CD40 in mycobacterial infection has been shown (by a separate group) by the greatly enhanced susceptibility of CD40 knockout ($CD40^{-/-}$ but not CD40 $ligand^{-/-}$) mice to infection with *M. tuberculosis* [164]. This study showed that

TABLE 4. Members of the Hsp70 (HSPA) Family of Molecular Chaperones

Gene	Alternative name	Human gene ID	Gene locus	Number of amino acids	Mol mass (Kd)
HSPA1A	HSP70-1, HSP72, HSPA1	3303	6p21.3	641	70.0
HSPA1B	HSP70-2	3304	6p21.3	641	70.0
HSPA1L	hum70t, hum70t	3305	6p21.3	641	70.4
HSPA2	HSP2 70 kD	3306	14q24.1	639	70.0
HSPA5	BIP, GRP78, MIF2	3309	9q33-q34.1	654	71.0
HSPA6	HSP6 70 kD (HSP70B′)	3310	1q23	643	71.0
HSPA7[a]		3311	1q23.3	?	?
HSPA8	HSC70, HSC71, HSP71, HSP73	3312	11q24.1	646/493	70.9/53.5
HSPA9	GRP75, HSPA9B, MOT, MOT2, PBP74, mot-2	3313	5q31.1	679	73.7
HSPA12A	FLJ13874, KIAA0417	259217	10q26.12	1296	141.0
HSPA12B	RP23-32L15.1, 2700081N06Rik	116835	20p13	686	75.7
HSPA13	Stch	6782	21q11	471	51.9
HSPA14	HSP70-4, HSP70L1, MGC131990	51182	10p14	509	54.8

[a]Possibly a pseudogene.

M. tuberculosis Hsp70 stimulates antimycobacterial defenses [164], thereby suggesting the intriguing hypothesis that this molecular chaperone is a key factor in enabling the host to combat tuberculosis.

Having shown that *M. tuberculosis* Hsp70 acts by binding to CD40 and that it induces the synthesis of three chemokines that bind to the receptor CCR5, the question arose as to whether *M. tuberculosis* Hsp70 also bound to this chemokine receptor. The results of two separate studies have shown that *M. tuberculosis* Hsp70 does indeed bind to CCR5 and that interactions between CCR5 and CD40 occur [165, 166]. The importance of this finding is that CCR5 is a coreceptor for HIV, and so, *M. tuberculosis* Hsp70 could interfere with the uptake of HIV into cells. Indeed, it has been shown that *M. tuberculosis* Hsp70 and the stimulating epitope 407–426 do block the uptake of HIV, thereby suggesting that this bacterial protein might have therapeutic potential [167].

These studies of the recombinant *M. tuberculosis* Hsp70 protein have been criticized as being a result of LPS contamination [10] or contamination with nucleotides that have been used to purify the Hsp70 [168]. Lehner and co-workers [161] have comprehensively shown the first hypothesis to be false, as Hsp70 induces an intracellular Ca^{2+} flux, which LPS does not, and furthermore, the actions of Hsp70, but not LPS, can be blocked by the Ca^{2+} chelator BAPTA-AM; antibodies to CD40 block the effects of Hsp70, but not LPS, and antibodies to CD14 block the effect of LPS, but not Hsp70 [161]; proteinase K treatment of Hsp70 blocks activity but has no effect on LPS activity [161]; Hsp70 synthetic peptides, which are free of bacterial contaminants, replicate the activity of the parent protein [163]; the concentration of contaminating LPS in recombinant Hsp70 preparations is too low to account for any of the effects recorded; and up-regulation of endogenous Hsp70 produces responses similar to that produced by exogenous Hsp70 [11]. The hypothesis that the action of *M. tuberculosis* Hsp70 is a result of nucleotides remaining from the isolation of the Hsp70 on ATP columns is also false, as synthetic peptides are active, and the biologically active recombinant fragments of *M. tuberculosis* Hsp70 were isolated on Ni-NTA affinity columns, and no nucleotides were used to purify them [161–163].

Eukaryotic Hsp70

Members of the eukaryotic Hsp70 family of stress proteins also exhibit immunological moonlighting properties, as they too have been reported to have pro- and anti-inflammatory effects on a variety of different leukocyte populations (reviewed in refs. [137, 169]). However, the views that these proteins are potent inflammatory molecules and/or that their immunological properties result from contamination or recombinant proteins with endotoxin continue to take center stage.

There now appear to be 14 human Hsp70 proteins [170], and a new nomenclature for these and for the other classic molecular chaperones has been proposed recently [18] (Table 4). This plethora of Hsp70 proteins has created some confusion in the literature, as many papers fail to explain precisely which Hsp70 protein is being used. Furthermore, some groups have used purified preparations of Hsp70, which contain an admixture of gene products. As will be seen, there can be major differences in the cellular actions of Hsp70 proteins, and it is therefore essential to know which protein is being used to allow a comparison of like-with-like. It is with the Hsp70 proteins that most of the problems with regard to contamination with PAMPs have arisen. This is because the early studies of the human Hsp70 protein, presumably Hsp70-1 or Hsc70 (new nomenclature HSPAIA and HSPA8, respectively), proposed that Hsp70 uses a number of cell surface receptors on human monocytes, most notably, CD14 and TLRs (reviewed in ref. [171]). However, it should be noted that purified Hsp70 does not bind to null cells that have been stably induced to express CD14, CD40, TLR2, and TLR4 on their surface [172]. Although a number of factors could account for these discrepant findings, it is clear that further studies definitively identifying the receptors used by recombinant and purified preparations of Hsp70 are required. This issue has been explored at length elsewhere [137].

In the early studies of Hsp70 (a generic term to cover any of the proteins described in the literature), this protein was described as being able to stimulate human and murine monocyte proinflammatory cytokine synthesis or NO synthesis [173–176]. Early studies also reported that immunization of animals with Hsp70, which had been purified from tumor cells, elicits anti-tumor immunity and protects from subsequent challenge with the same, but not different, tumor cells [177–179]. With regards to the latter observation, the commonly held view is that immunization induces tumor-specific cytotoxic $CD8^+$ T cells (CTLs) that are specific for peptides associated with the Hsp rather than by the Hsp itself [180, 181]. Contrasting with the documented proinflammatory properties of Hsp70 are studies reporting that the induction of T cell reactivity to self-Hsp70 down-regulates disease in a number of experimental arthritis models by a mechanism that involves the development of Th2-type $CD4^+$ T cells producing the regulatory cytokines IL-4 and IL-10 [143–146]. Furthermore, DNA vaccines encoding for Hsp70 also inhibit experimental adjuvant arthritis and the development of diabetes in nonobese diabetic mice [147, 182]. The findings that biological and immunological effects can be elicited by the administration of Hsp70-derived peptides, Hsp70 protein, and DNA vaccines encoding for Hsp70 argue against the "active ingredient" being contaminating endotoxin. This is confirmed further by the finding that T cells responding to conserved, synthetic, peptide-defined sequences of Hsp70 are responsible for the arthritis-protective effects of this stress protein [146].

Although much attention has been focused on its immunological properties, eukaryotic Hsp70 has intercellular signaling functions other than those relating to immunoregulation. Work from the De Maio laboratory [183] has shown that the interaction of *E. coli*, expressed recombinant human Hsp70 with PS on the surface of PC12 cells (derived from a transplantable rat pheochromocytoma), decreases their viability. Furthermore, the Multhoff laboratory [184] has shown that the interaction of recombinant human Hsp70, which had been expressed using an insect (baculovirus) expression system with surface PS, reduces the clonogenic survival of normoxic and hypoxic tumor cells significantly, whereas proliferation was

only affected in hypoxic cells. It is also interesting to note that radiation-induced tumor cell kill was enhanced significantly by the addition of the Hsp70 [184]. These are important findings, as it is known that certain tumor cells can release Hsp70, the presence of which in the tumor microenvironment, might have profound implications for the sensitivity of the tumor to therapeutic strategies. In addition to binding to PS expressed on the surface of tumor cells, it has been shown that baculovirus-expressed recombinant human Hsp70 can bind to membrane lipid components, specifically, globotriaoslyceramide (Gb3) [185].

In addition to the above, it is apparent that extracellular Hsp70 can preferentially bind to and be internalized by endothelial cell populations, and it might be that this accounts for the apparent atheroprotective action of this protein (reviewed in ref. [186]) and its reported capacity to protect heat-stressed cynomolgus macaque aortic cells [187] and serum-deprived rabbit arterial smooth muscle cells [188]. It is therefore clear that Hsp70 has a number of extracellular functions, some of which involve immunoregulation and others that are more focused on cellular homeostasis and protection.

Arguably, one of the most important Hsp70 family members is BiP (HSPA5), which is a key endoplasmic reticulum-lumenal molecular chaperone intimately involved in the unfolded protein response. In recent years, BiP has been proposed to play a role in a range of human infections and syndromes [189]. The signaling actions of human BiP were found as the result of a search for the autoantigen responsible for rheumatoid arthritis. This search identified BiP as a key autoantigen [190]. Administration of BiP to animals failed, as expected, to induce arthritis. Indeed, further experimentation revealed that BiP actually blocked the induction of various forms of experimental arthritis in rodents [191]. It was shown further that BiP blocked antigen presentation and induced leukocytes to produce an anti-inflammatory profile of cytokines, including IL-10, the natural IL-1 antagonist (IL-1ra), and soluble TNFRII [191]. Thus, BiP somewhat resembles the actions of Hsp27 described earlier and is clearly able to induce some form of alternative macrophage activation state. Further studies of animal arthritis models revealed that BiP was able to inhibit established collagen-induced arthritis by inducing regulatory leukocytes that produce the inhibitory cytokine IL-4 [192]. More recent studies about the effect of BiP on DC and immunoregulatory T cell development have revealed that BiP is a major immunomodulatory protein with anti-inflammatory actions that are related to its ability to induce tolerogenic DCs and the related production of immunoregulatory T cells [193]. As has been described [3], BiP is now in clinical trial for the treatment of rheumatoid arthritis in the United Kingdom.

Eukaryotic Hsp90

Hsp90 is a much-studied molecular chaperone with a wide variety of intracellular functions and is now regarded as being a major therapeutic target for the treatment of cancer [194]. This protein has also been identified to be on the surface of tumor cells and has been proposed to have a role in tumor cell invasion and possibly also in metastasis [38]. This involves interaction with other cell surface molecules, including matrix metalloproteinase-2 [38] and the extracellular domain of human epidermal growth factor receptor-2 [195]. Early studies reporting that immunization of animals with Hsp70, which had been purified from tumor cells, elicits anti-tumor immunity and protects from subsequent challenge with the same, but not different, tumor cells [177–179] also reported Hsp90 to have similar properties, albeit at a tenfold-less potency [178]. Hsp90α can also be secreted by cells [39, 196–199], and rather surprisingly, the topical application of Hsp90α in mice has been shown to improve wound healing [39]. It is interesting to note that such properties are not restricted to Hsp90α, as Hsp70 and gp96 (grp94, HSPC4) can also increase wound healing by a mechanism that appears to involve macrophage-mediated phagocytosis of wound debris [200]. Does this suggest yet another therapeutic function for cell stress proteins?

MOLECULAR CHAPERONES AS MEMBRANE RECEPTORS

In addition to functioning as ligands for cell surface/intracellular receptors, there is evidence that molecular chaperones can exist on the outer plasma membranes of prokarotic and eukaryotic cells and act as receptors for a variety of ligands. There is significant evidence that Hsp60 and Hsp70 proteins are present on the surface of a variety of bacteria, where they function as adhesins binding to a variety of cell surface constituents (reviewed in ref. [49]). The Multhoff laboratory [201–203] was the first to demonstrate that Hsp70 is expressed frequently on the membranes of a number of cancers, including mammary, and metastases derived there from but not on their nonmalignant counterparts. Membrane Hsp70 also acts as a tumor-specific recognition structure for $CD94^+$ NK cells, although it cannot induce these cells to kill membrane Hsp70-positive tumor cells in the absence of cytokine (IL-2/IL-15) coactivation [202, 204–206]. Furthermore, Hsp70 expressed on the membrane of tumor cells facilitates the internalization of granzyme B, a serine protease that is associated with the cytotoxic activities of NK and cytotoxic T cells and thereby, promotes perforin-independent apoptosis [207].

In the mammal, the strongest evidence for molecular chaperones acting as receptors has derived from the study of the receptor for the major PAMP, LPS. Binding of LPS to human cells has clearly been shown to involve the participation of Hsp70 and Hsp90 [208]. Molecular chaperones on the surface of host cells can also function as receptors for microbial pathogens. The most-fascinating example of this, at the moment, is the binding of the human pathogenic bacterium, *Listeria monocytogenes,* to enteric epithelial cells. This involves the bacterial adhesin termed *Listeria* adhesion protein, which actually turns out to be an enzyme, alcohol acetaldehyde dehydrogenase. The receptor for this bacterial adhesin on human epithelial cells is Hsp60. Thus, we have one moonlighting protein binding to another moonlighting protein. It should be noted that the Ka of binding between these two proteins was 5×10^8 $M^{-1}s^{-1}$, which is not greatly different from that of the binding affinity of a commercial high-affinity anti-Hsp60 antibody [209]. Viruses also bind to cells via cell surface molecular

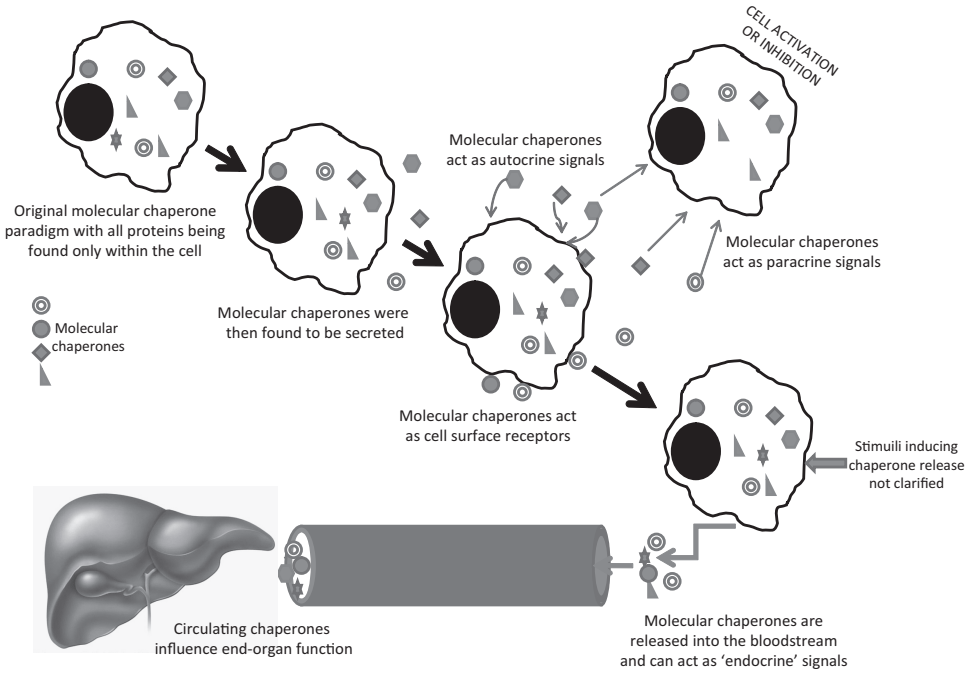

Figure 5. Schematic diagram showing the various roles played by secreted molecular chaperones emphasizing that they appear to have similar actions to cytokines in that they can cause autocrine, paracrine, and endocrine cell signaling.

chaperones [49]. The latest example of this is the role of cell surface BiP in the binding of Borna disease virus [210].

SUMMARY

It is clear that the concept that cell stress proteins are exclusively intracellular molecules, the sole function of which is to act as intracellular molecular chaperones, is now untenable. A wealth of data now demonstrate that certain stress proteins are constitutively released from cells or that their release can be induced by endogenous and exogenous factors. When released, cell stress proteins can act as one, or all, of the following: cell surface receptors; secreted autocrine signals; secreted paracrine signals; and secreted "endocrine" signals (**Fig. 5**). In these respects, the cell stress proteins are reminiscent of cytokines. Although in a few instances, LPS contamination might be able to account for the observed immunological/biological properties of these proteins, the overwhelming evidence argues against the intercellular signaling properties being a result of such contamination. This has arisen from studies, in which functionality has been demonstrated using endotoxin-free preparations, synthetic peptides, and appropriately controlled experiments, plus those in which immunoregulatory capacity has been indicated on the basis of stress protein-derived peptide specificity. The increasing number of examples of molecular chaperones acting as inhibitors of leukocytes, rather than as activators, also rules out LPS or other PAMP contamination. Understanding the systems biology of extracellular stress proteins in health and disease is likely to reveal new insights into fundamental homeostatic mechanisms and is undoubtedly the next challenge for stress protein biologists. This challenge will be markedly aided by the fact that there is increasing interest in the use of molecular chaperones as anti-inflammatory and immunomodulatory therapeutic agents, and a growing list of these proteins are tested clinically (**Table 5**). The most recent example is the oral administration of a peptide (dnaJP1), derived from the *E. coli* Hsp40 (DnaJ) protein, to patients with rheumatoid arthritis. This has revealed relevant changes in leukocyte function and a diminution of clinical indices of disease activity [211].

TABLE 5. Therapeutic Use of Recombinant Cell Stress Proteins and Peptides

Cell stress protein/peptide	Human condition
Hsp10	Rheumatoid arthritis, psoriasis, multiple sclerosis
Trx	Macular degeneration
Hsp60 peptide 437–460 (DiaPep 277)	Type I diabetes
BiP	Rheumatoid arthritis
DnaJP1	Rheumatoid arthritis

AUTHORSHIP

Brian Henderson drafted the first version of the manuscript, and this was subsequently expanded on and developed by Brian Henderson and Graham Pockley.

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KEY WORDS:
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