Chaperonins are cell-signalling proteins: the unfolding biology of molecular chaperones

Julia C. Ranford, Anthony R.M. Coates and Brian Henderson

The chaperonins are a subgroup of oligomeric molecular chaperones; the best-studied examples are chaperonin 60 (GroEL) and chaperonin 10 (GroES), both from the bacterium *Escherichia coli*. At the end of the 20th century, the paradigm of chaperonins as protein folders had emerged, but it is likely that during the 21st century these proteins will come to be viewed as intercellular signals. Indeed, it is possible that the chaperonins were among the first intercellular signalling proteins to evolve. During the past few years, it has emerged that chaperonin 10 and chaperonin 60 can be found on the surface of various prokaryotic and eukaryotic cells, and can even be released from cells. Secreted chaperonins can interact with a variety of cell types, including leukocytes, vascular endothelial cells and epithelial cells, and activate key cellular activities such as the synthesis of cytokines and adhesion proteins. Much has been made of the high degree of sequence conservation among the chaperonins, particularly in terms of the immunogenicity of these proteins. However, different chaperonin 60 proteins can bind to different cell-surface receptors, including the Toll-like receptors, suggesting that this family of proteins cannot be treated as one biological entity and that several subfamilies may exist. Chaperonins have been implicated in human diseases on the basis of their immunogenicity. The finding that chaperonins can also induce tissue pathology suggests that they may play roles in infections and in idiopathic diseases such as atherosclerosis and arthritis.

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The last decades of the 20th century saw the discovery of the heat-shock or cell-stress response (changes in the expression of certain proteins), and the elucidation of the function of the proteins that mediate this essential cell-survival strategy. The stresses that can trigger this response vary widely, and include heat or cold, osmotic imbalance, toxins, heavy metals and pathophysiological signals such as cytokines and eicosanoids. The proteins that are synthesised in response to such environmental stresses have been variously called: heat-shock proteins (hsps), stress proteins or molecular chaperones. The term molecular chaperone, coined by Laskey and colleagues in 1978 (Ref. 1), has been used in this review article. The cell-stress response is an evolutionarily ancient, ubiquitous and essential mechanism for cell survival. This fact is reflected in the conservation of the peptide sequences of the molecular chaperones throughout living organisms (Refs 2, 3). During the past 20 years, it has been established that, within the cytoplasm of the cell, the molecular chaperones interact with other proteins to fold, refold or maintain the folding of the interacting proteins (Ref. 4). This mechanism protects cells from the damaging effects of environmental stresses, and the associated misfolding (denaturation) of intracellular proteins. Molecular chaperones are involved in many essential cellular functions, such as metabolism, growth, differentiation and programmed cell death, through protein assembly and transport. They also influence the activation of enzymes and receptors (Ref. 3).

One of the first molecular chaperones to be identified was a 60-kDa protein, which has been given the generic term chaperonin 60 (cpn60; Ref. 5). Fifteen different groups of proteins are now classified as molecular chaperones (Ref. 3; see Table 1, for a description of the major families of chaperones). Many researchers worldwide are attempting to determine the mechanism of action of these various molecular chaperones, many of which exist as families of related proteins. This research effort is driven largely by the need to understand the roles that molecular chaperones play in normal cell functioning. However, increasing attention is being concentrated on the potential roles of these proteins in human diseases, including infection and idiopathic conditions such as arthritis and atherosclerosis. One subgroup of molecular chaperones, the chaperonins, has received the most attention (Ref. 6). The chaperonins consist of two protein

Table 1. Diversity of the major molecular chaperone families (tab001jrl)

Family	Chaperone functions
Chaperonin 10 (cpn10, hsp10, co-chaperonin, early pregnancy factor, GroES)	'Co-chaperonin' to chaperonin 60; promotes folding of substrates that are bound to chaperonin 60
Small heat-shock proteins (hsps)	Diverse class of proteins; chaperone function is independent of adenosine triphosphate (ATP); bind non-native proteins
Hsp40 (DnaJ related)	Co-chaperones that regulate the activity of hsp70 proteins; some can bind non-native proteins themselves
Chaperonin 60 (cpn60, hsp60, hsp65, GroEL)	ATP-dependent folding and/or refolding of ~15–30% of total cellular proteins
Hsp70	Prevent the aggregation of unfolded polypeptides; disassemble multimeric protein complexes; involved in protein trafficking; regulate the heat-shock response
Hsp90	Specific functions in regulating signal transduction pathways, through their actions on certain kinases and steroid receptors; might also have 'general' chaperone activity
Hsp100 (Clp)	Disassemble protein oligomers and aggregates
Hsp110	High degree of homology with the hsp70 family; little known about functions

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families, the chaperonin 60 family and the chaperonin 10 family. Because of the attention that the chaperonins have received, and the discovery that they have the additional property of acting as cell-to-cell signalling molecules, the chaperonins have formed the focus of this review.

The chaperonins: structure and function

The chaperonins are a well-characterised, sequence-related subgroup of molecular chaperones, which includes the GroE and TCP-1 (T-complex polypeptide 1) subclasses. The proteins of the GroE subclass, namely chaperonin 60 and chaperonin 10, have formed the subject of this review. To date, GroEL, the *E. coli* chaperonin 60 protein, is the most extensively studied molecular chaperone (Refs 7, 8, 9). Together with GroES (the *E. coli* chaperonin 10 protein), GroEL is critical for the correct folding of many proteins in the cell, under both normal and stress conditions. The deletion of the *GroE* operon in *E. coli* is lethal; GroEL and GroES are essential for cell survival under any conditions (Ref. 10). In 1997, Ewalt and colleagues (Ref. 11) determined that under normal growth conditions, GroEL folds 10–15% of all cytoplasmic proteins; under heat stress, this figure increases to 30%. Houry and colleagues have recently identified the proteins that interact with GroEL in the cytoplasm of *E. coli* (Ref. 12).

Members of the chaperonin 60 family have a characteristic double-ring structure comprising 14 subunits (Ref. 8; see Fig. 1); these form a large central cavity in which the unfolded protein substrate binds via hydrophobic interactions (Ref. 13). The crystal structure of GroEL has been solved (Ref. 14). Each subunit of GroEL has three domains: an apical domain, to which both the substrate and GroES bind; an equatorial domain, which contains a binding site for adenosine triphosphate (ATP) and the contacts for ring binding; and the intermediate domain, which connects these two domains. The intermediate domain acts as a hinge, effecting conformational changes when ATP is bound (Ref. 8), and causing the substrate-binding surface to alternate between hydrophobic and hydrophilic states. When the surface is in the hydrophobic state, a protein substrate can bind to GroEL, thus preventing the incorrect association of the substrate with other proteins, which might lead to misfolding. When ATP binds to GroEL, the hinge opens up, altering the substrate-binding surface such that it becomes hydrophilic, and the protein substrate is released. The folding function of GroEL has been covered in detail elsewhere (Refs 4, 7, 8, 9).

The chaperonin 60 oligomers associate with chaperonin 10 oligomers to effect their functions (Refs 15, 16). Chaperonin 10 forms single-ring heptamers that have a dome-like structure (Refs 17, 18). When ATP is bound to chaperonin 60, the chaperonin 10 forms a lid on top of the chaperonin 60 barrel (Refs 19, 20), and causes the central cavity to enlarge, thus aiding protein folding.

The biology of chaperonins

Immunobiology of chaperonins

Chaperonins are potent immunogens (i.e. they induce a strong, specific immune response) in humans and rodents (see Table 2). This finding was established before the functions of the chaperonins were known (Refs 21, 22), and has given rise to much immunological 'head scratching'. Infection is a stressful process, both for the pathogen and the host, and must, therefore, result in the increased production of molecular chaperones by both the pathogen and the host (Refs 23, 24). However, given the high degree of sequence similarity between bacterial and mammalian molecular chaperones, the immune reactivity to these bacterial proteins would be expected to be only minimal. The immune system is designed to ignore 'self', that is, host constituents; however, paradoxically, this is not the case with the chaperonins. It is still unclear why immunity to molecular chaperones is such a common and marked characteristic of infection (Refs 25, 26). For example, in mice that are infected with *Mycobacterium tuberculosis*, up to 20% of the reactive T cells respond just to *M. tuberculosis* chaperonin 60.2 protein, despite the fact that many other proteins are presented to the immune system during infection (Ref. 27). Anti-chaperonin antibodies are also found in a wide range of autoimmune diseases (Refs 28, 29, 30, 31). Other molecular chaperones, complexed to peptides from cancer cells or virus-infected cells, can elicit the production of antigen-specific cytotoxic T lymphocytes (CTLs; Refs 32, 33), a process that could be harnessed therapeutically to stimulate the immune response to kill cancer cells (Ref. 34; see Fig. 2).

The possibility that T-cell reactivity to bacterial chaperonins could lead to the autoimmune

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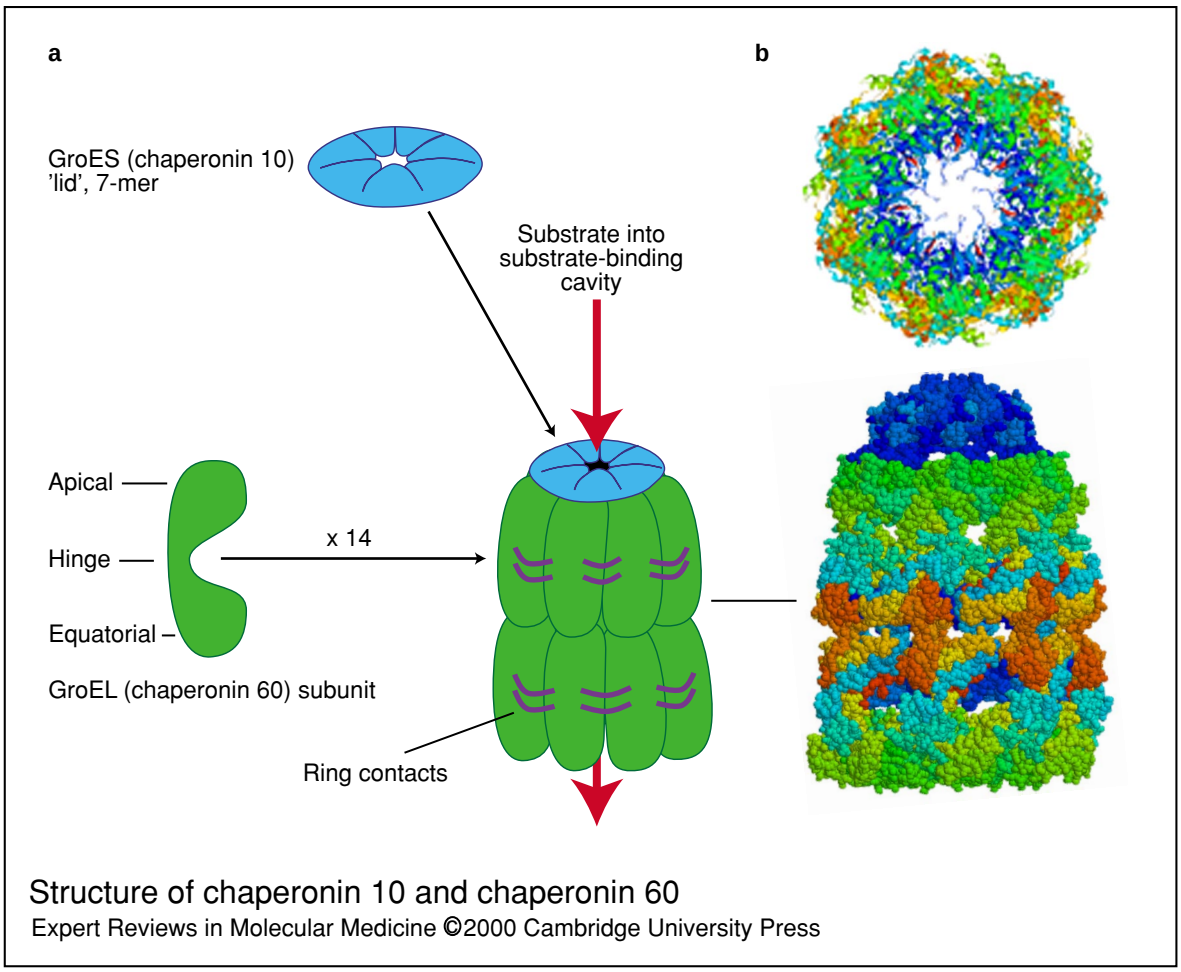

Figure 1. Structure of chaperonin 10 and chaperonin 60. (a) The complex that is formed between GroEL (chaperonin 60, in green) and GroES (chaperonin 10, in blue). It comprises the two heptameric rings of GroEL, which have a characteristic 'double doughnut' structure, and the attached GroES heptameric 'lid'. (b) The central substrate-binding cavity can be seen on this diagram, constructed using RasMol from protein database file 1AON.pdb (from http://www.rcsb.org/pdb), by rotating the structure to the plan view shown **(fig001jrl)**.

recognition of host chaperonins has generated much interest (Ref. 35). Molecular chaperones have been implicated in autoimmune diseases such as systemic lupus erythematosus (SLE), Crohn's disease and rheumatoid arthritis, and they might provide a molecular link between bacterial infection and autoimmunity. Perhaps the strongest evidence for chaperonin 60 being involved in autoimmunity has been provided by studies of the common disease atherosclerosis (i.e. hardening or ulceration of the innermost portion of the arteries). This is an inflammatory disease, although it may not be immediately discerned as an autoimmune condition. Xu and Wick have built up a strong case for this disease being the result of the action of antibodies generated by a human host after exposure to bacterial chaperonin 60 proteins (Ref. 36; see Fig. 3). It has been hypothesised that these antibodies cross-react with the human chaperonin 60 protein, and that this protein is expressed on the surface of stressed human vascular endothelial cells. The binding of anti-chaperonin 60 antibodies to the surface of the vascular endothelial cells results in complement-mediated cytotoxicity, and the denuded areas of the vasculature then become sites of development of atherosclerosis (Refs 36, 37).

In addition to being the putative causes of certain human diseases, molecular chaperones can inhibit some animal models of autoimmune diseases such as adjuvant arthritis in rats (Ref. 38) and experimental insulin-dependent diabetes mellitus in mice (Ref. 39). As described

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Table 2. Evidence for the involvement of chaperonins in disease (tab002jrl)

Chaperonin	Disease and evidence	Refs
Immunogenic aspects		
Mycobacterium tuberculosis chaperonin 60.2	Tuberculosis – 20% of reactive T cells recognise *M. tuberculosis* chaperonin 60.2	27
Human chaperonin 60	Juvenile arthritis – anti-chaperonin 60 antibodies	28
Human chaperonin 60	Atherosclerosis – presence of human chaperonin 60 in atherosclerotic lesions	29, 36
Escherichia coli chaperonin 60; *M. tuberculosis* chaperonin 60.1 and chaperonin 60.2	Directly induces intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM) and E-selectin expression by endothelial cells	65, 66
Mycobacterial and human chaperonin 60	Kawasaki disease – anti-chaperonin 60 antibodies	30
Mycobacterial chaperonin 60	Psoriasis – anti-chaperonin 60 antibodies	31
Human hsp70 or gp96 from cancer cells	Cancers – can induce T-cell response	32, 33
Chaperonin 60 from various bacteria	Inflammation – can activate leukocytes, fibroblasts and epithelial cells to produce cytokines	44, 45, 46, 53, 54, 55, 56, 57, 58, 59, 60
M. tuberculosis chaperonin 10	Synthesis of cytokines in monocytes	P. Tabona[a]
E. coli chaperonin 60; *Actinobacillus actinomycetemcomitans* chaperonin 60; *M. tuberculosis* chaperonin 10	Induces bone resorption in mouse model; *M. tuberculosis* chaperonin 10 shown to be a growth factor of osteoclasts	50, 62
M. tuberculosis chaperonin 10	Induces the proliferation of the mouse P19 teratocarcinoma cell line	81
Human chaperonin 60	Stimulates cytokine synthesis by monocytes	61
Immunosuppresive aspects		
Rat chaperonin 60	Adjuvant arthritis model – chaperonin 60 inhibits disease	38
Mouse chaperonin 60	Experimentally induced diabetes – inhibited by chaperonin 60	39
Human chaperonin 10	'Early pregnancy factor' is immunosuppressive in pregnancy	74, 75, 76

[a] P. Tabona and colleagues, Eastman Dental Institute, London, UK, unpublished

later, mammalian chaperonin 10, which is also known as early pregnancy factor (EPF), is immunosuppressive during early pregnancy. Thus, molecular chaperones appear to suppress the immune system in some instances, probably by modulating T-cell function.

A very interesting (but highly controversial) hypothesis in this context is Matzinger's 'danger model', which attempts to explain immune responsiveness in terms of the recognition of host components that signal that the body is under attack. Molecular chaperones are one of the

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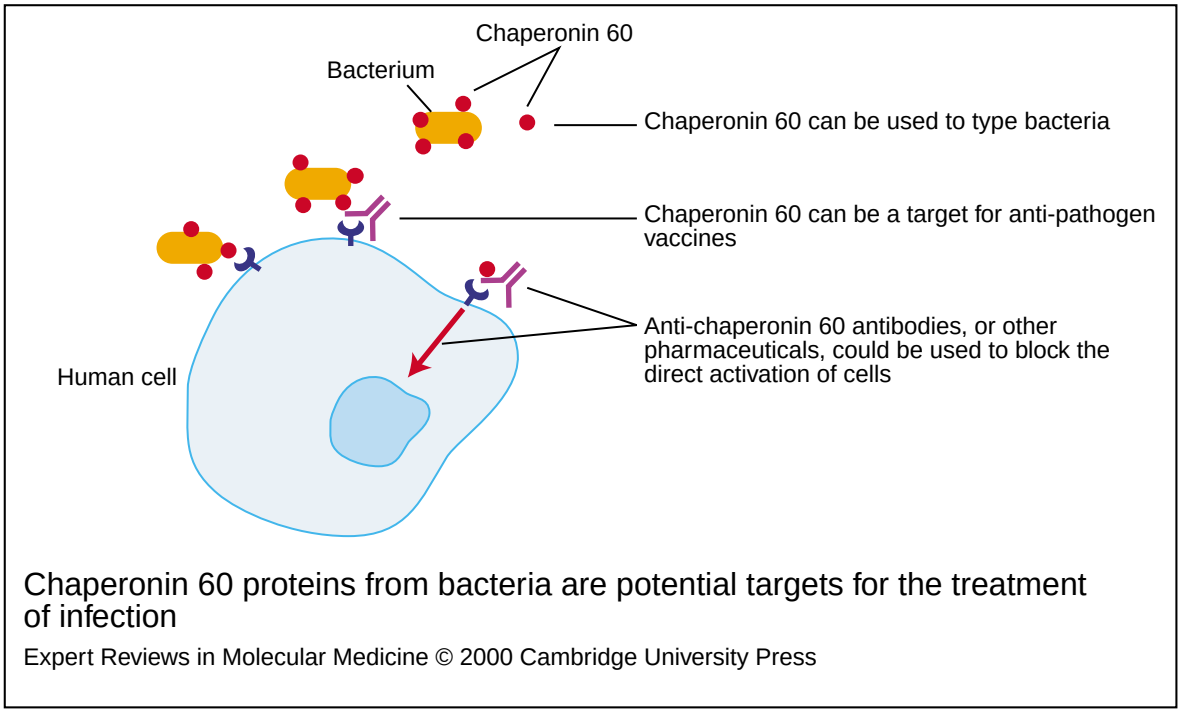

Figure 2. Chaperonin 60 proteins from bacteria are potential targets for the treatment of infection. Chaperonin 60 proteins could be used in several different ways to treat bacterial infections. Bacterial isolates could be identified (or typed) by sequencing their chaperonin 60 proteins, and this might lead to better targeted treatments. Vaccines could potentially be made from bacterial chaperonin 60 proteins, and used to immunise the body against the pathogenic effects of the bacteria they are derived from. Alternatively, because chaperonin 60 proteins directly activate cells that lead to the inflammatory response, specific anti-chaperonin 60 antibodies could be used to block this response **(fig002jrl)**.

proposed groups of 'danger molecules' (Ref. 40; see also Ref. 41).

The literature abounds with controversy about the roles of molecular chaperones in immunity. This confusion might in part be due to the increasing evidence that molecular chaperones are not simply inert immunogens but can participate in lymphocyte activation. It has been suggested that molecular chaperones (particularly the chaperonins) should be classified as 'multiplex antigens' because of their ability to interact with, and activate, different cells. Many of these cells, including monocytes, dendritic cells and endothelial cells, can present antigens (Refs 26, 42). The ability of intact chaperonins and, as described later, chaperonin-derived peptides to activate antigen-presenting cells (APCs) may enhance antigen presentation, resulting in a greater lymphocyte response than would be induced by antigens that are inert and fail to activate the interacting leukocytes.

During the past 5 years, evidence has accumulated to support the hypothesis that molecular chaperones can activate a variety of cellular functions that might be important in tissue pathology, and that might also explain the immunoreactivity of these proteins. The chaperonins have received the most attention, and the literature on the biological activity of these proteins has been reviewed in the next section.

Chaperonins as intercellular signalling proteins

One should always expect the unexpected in life and this is particularly true in science. The 1990s were a period of enormous advancement in our understanding of the molecular, structural and cellular biology of the molecular chaperones. By the mid-1990s, understanding of the structure and function of the chaperonins was relatively clear. It was known that the two families of intracellular oligomeric proteins, whose synthesis could be dramatically increased under stressful conditions, could fold and refold other proteins intracellularly. However, while these studies of the structure–activity relationship of the protein-

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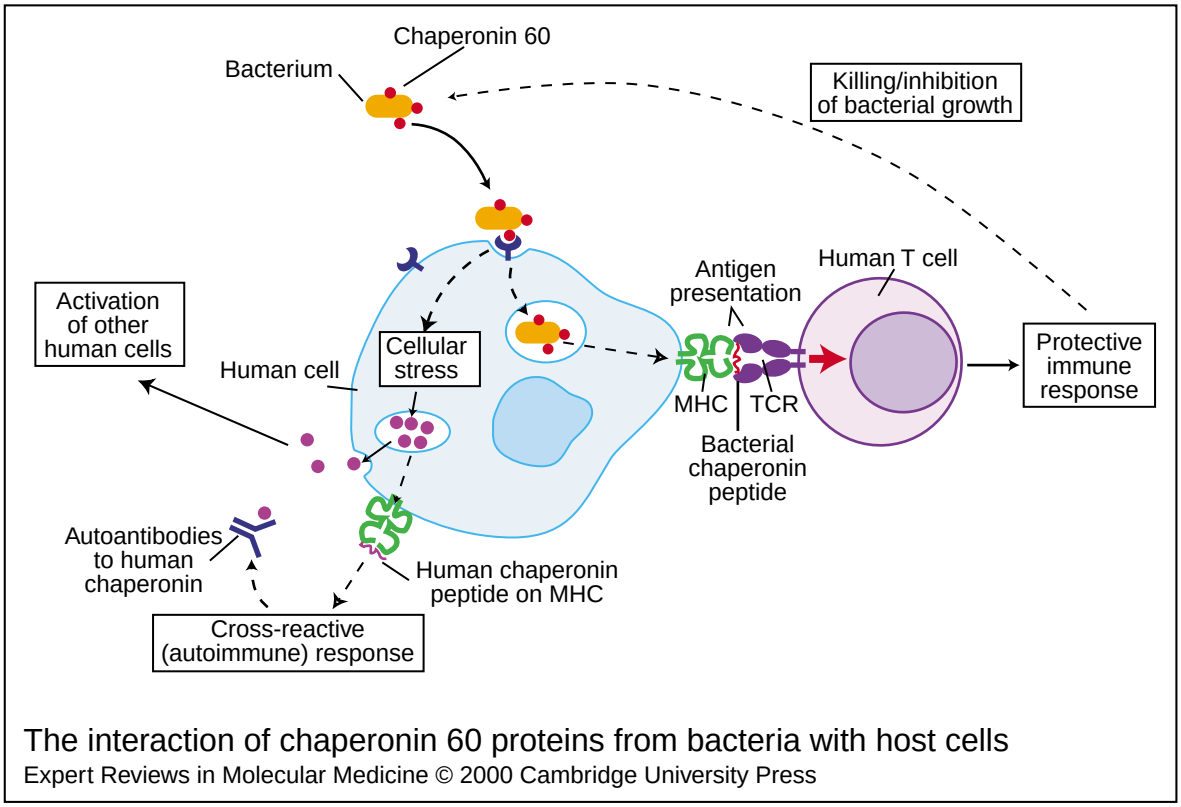

The interaction of chaperonin 60 proteins from bacteria with host cells

Figure 3. The interaction of chaperonin 60 proteins from bacteria with host cells. During bacterial infection, host cells are activated by bacterial chaperonin 60 proteins that are secreted or expressed on the outer surface of bacteria. This induces host cells to produce cytokines or other intercellular signals. It may also cause them to release their own chaperonins, which in turn activate other host cells. Bacterial or host chaperonins are identified by the host via presentation of a part of the chaperonin on the major histocompatibility complex (MHC). This activates T cells via the T-cell receptor (TCR). Additionally, B cells are activated by chaperonins to produce antibodies. Errors in this response may lead to some forms of autoimmune disease, owing to the recognition of self-chaperonin molecules **(fig003jrl)**.

folding activity of the chaperonins were under way, other researchers were beginning to discover that the chaperonins had another, distinct set of functions. Perhaps the earliest indication that the chaperonins were likely to have actions other than protein folding was the report that chaperonin 60 could produce pores in membranes (Ref. 43).

Non-chaperone activities of chaperonin 60

The first piece of evidence that molecular chaperones can activate cells if they are delivered onto their external surface was the report that *M. tuberculosis* chaperonin 60.2 protein (which is usually referred to as heat-shock protein 65 or hsp65) could stimulate the release of pro-inflammatory cytokines from human monocytic cells (Ref. 44). This finding was confirmed by other researchers using either chaperonin 60 (Refs 45, 46) or hsp70 (Ref. 47). A major problem associated with the interpretation of these experiments is the possible contamination of the molecular chaperone preparations with other bacterial components that can induce cytokine synthesis independently of the chaperonins. Host cells have evolved to recognise bacteria and synthesise cytokines in response to this. Thus, it was possible that these findings were not due to the molecular chaperones themselves, but to contaminating bacterial components. Such components include the ubiquitous bacterial cell activator lipopolysaccharide (LPS), other components that similar are to LPS, such as peptidoglycan and lipoarabinomannan (LAM), and protein activators, such as the bacterial exotoxins (Refs 48, 49). The antibiotic polymyxin B binds to LPS and inactivates it, and has therefore been used by most researchers to control for LPS contamination in experiments

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(which is a major problem with recombinant preparations of molecular chaperones produced in *E. coli*). However, to date, only one study has totally excluded the possibility that the cell-activating properties of chaperonin 60 are due to LPS contamination (Ref. 50). These investigators were studying the in vitro induction of cytokine-driven bone resorption by GroEL. The LPS-insensitive C3H/HeJ mouse strain [which lacks a functional Toll-like receptor 4 (TLR4) – the co-receptor for LPS] was used to show that murine calvarial bone tissue did not respond to LPS (as expected) but was sensitive to the administration of GroEL (Ref. 50). This study demonstrated the direct activity of GroEL on this bone tissue. Chaperonin 60 preparations, even after purification by high-performance liquid chromatography (HPLC), can contain substantial amounts of other contaminating proteins (Ref. 51); thus, the reported bioactivity of the chaperonin 60 proteins could actually be due to such contaminating proteins. However, it has recently been shown that the removal of these contaminating proteins from a GroEL preparation had no effect on the cytokine-inducing activity of GroEL, and that the contaminants, in the absence of GroEL, did not induce cytokine activity (Ref. 52). Thus, these two experimental studies established that purified chaperonin 60 proteins can independently activate human and murine cells.

Other researchers have shown that chaperonin 60 preparations from various bacteria can also stimulate leukocytes (Refs 53, 54, 55, 56, 57, 58), fibroblasts (Ref. 59) and epithelial cells (Ref. 60) to secrete pro-inflammatory cytokines. It has also been claimed that human chaperonin 60 can stimulate cytokine synthesis by monocytes (Refs 58, 61). In our experience, human chaperonin 60 protein is only a very weak stimulant of cytokine synthesis in human monocytes (J.S.H. Gaston and colleagues, Department of Medicine, Cambridge University, UK, unpublished).

A very potent activity that has been ascribed to chaperonin 60 is bone resorption. Bone loss is a key factor in diseases such as Pott's disease (spinal tuberculosis) and periodontal disease, both of which are caused by bacterial infections, and also in osteoporosis. The chaperonin 60 protein from the bacterium *Actinobacillus actinomycetemcomitans* (which normally colonises the oral cavity) and that from *E. coli* (which normally colonises the gut) have been shown to be extremely active stimulators of the breakdown of murine calvarial bone in vitro (Ref. 50). Surprisingly, the homologous chaperonin 60.2 (hsp65) proteins from *M. tuberculosis* and *Mycobacterium leprae* were very weak agonists of bone resorption in this model (Ref. 62). Thus, there are some differences in the biological action of these supposedly homologous proteins; these differences have been discussed in more detail in the next section. The analysis of the mechanism of action of GroEL on bone revealed that this protein acts as a growth factor for murine osteoclasts, which are the cells that break down the extracellular matrix of bone (Ref. 63). Other molecular chaperones (including hsp70 and hsp90) have also been found to stimulate bone resorption in in vitro models (Ref. 64).

In addition to the actions of chaperonin 60 proteins on cytokine synthesis, several other activities have been ascribed to molecular chaperones that seem either to be independent of cytokine synthesis or to have an indirect effect on cytokine synthesis. Perhaps the most interesting report is that cultured human vascular endothelial cells respond to chaperonin 60 by upregulating the synthesis of the adhesion molecules that are involved in controlling leukocyte trafficking in inflammation. The chaperonin 60 proteins from *M. tuberculosis* (chaperonin 60.2) and *E. coli* induce the expression of intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM) and E-selectin by human vascular endothelial cells (Refs 65, 66). The induction of the transcription of the genes encoding these three adhesion proteins is controlled by pro-inflammatory cytokines including interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-α), and therefore the actions of chaperonin 60 proteins on these three proteins would be expected to involve the synthesis of these two cytokines. However, the *M. tuberculosis* and *E. coli* chaperonin 60 proteins induce human vascular endothelial cells to transcribe the genes for these adhesion proteins independently of the induction of the synthesis of either IL-1 or TNF-α, via another signalling pathway. As already mentioned, there is a great deal of interest in the role of chaperonin 60 in the pathology of atherosclerosis; therefore, the finding that chaperonin 60 can directly activate endothelial cells, which are central to the pathogenesis of this disease, is important, and of therapeutic and/or prophylactic interest.

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Activity-dependent neurotrophic factor (ADNF) is a human 14-kDa protein that is homologous to chaperonin 60. It inhibits neuronal apoptosis (programmed cell death) and may provide protection against neurological diseases that are associated with neuronal death, such as Alzheimer's disease (Ref. 67). Interestingly, a 14-residue peptide derived from ADNF is a more powerful inhibitor of apoptosis than the parent ADNF molecule (Ref. 68), and protects experimental animals from experimentally induced memory loss (Ref. 69). Furthermore, ADNF actually increased the expression of the chaperonin 60 gene in cortical cells (Ref. 70). Brenneman's group has also reported that brain cells can release chaperonin 60, and has suggested that this release is part of a neuroprotective mechanism (Ref. 71). In addition to its neuroprotective effect, ADNF has also been reported to stimulate embryonic growth (Ref. 72). This activity might be similar to that reported for human chaperonin 10 (see next section). Another molecular chaperone, hsp90, has been shown to have neurite-promoting activity (Ref. 73).

Biological activities of chaperonin 10

The biology of chaperonin 10, a small heptameric protein, has received less attention than that of chaperonin 60. However, the role of human chaperonin 10 in the biology of EPF is still being actively debated. EPF is a secreted protein that has growth regulatory and immunomodulatory actions, both of which are required for the successful establishment of pregnancy, at least in a rodent model (Refs 74, 75). The purification and sequencing of EPF from rats have led to its identification as mammalian chaperonin 10 (Ref. 76); moreover, it has been suggested that there is a family of EPF genes in humans (Ref. 77). Other researchers have questioned the identification of EPF as chaperonin 10 on the basis of sequence homology (Ref. 78), and have suggested instead that it might be thioredoxin (Ref. 79) or an Fc-binding protein (Ref. 80). Support for the hypothesis that chaperonin 10 has growth-modulating actions was provided in 1996 by Galli and colleagues (Ref. 81), who reported that chaperonin 10 from *M. tuberculosis*, but not that from *E. coli* (i.e. GroES), increased the proliferation of the mouse P19 teratocarcinoma cell line. Surprisingly, in the absence of serum, *M. tuberculosis* chaperonin 10 increased the rate of apoptosis in this cell line.

The ability of certain bacterial chaperonin 60 proteins to stimulate bone resorption has been described above. Bone infection normally results in the rapid breakdown of the matrix of the affected areas of the skeleton. A few bacteria, including *Staphylococcus aureus* and *M. tuberculosis*, are the major causative agents of bone infections. Tuberculosis of the spine (which is caused by *M. tuberculosis* and is also known as Pott's disease) is one of the most serious bone infections because it causes severe deformation of the spine. It has recently been established that the major, if not the only, *M. tuberculosis* component that stimulates bone resorption, at least in in vitro bone resorption assays, is chaperonin 10 (Ref. 62). In these studies, *M. tuberculosis* chaperonin 10 was found to be a potent growth factor of murine osteoclasts (Ref. 62). The structure–activity relationship of *M. tuberculosis* chaperonin 10 has been examined using a series of synthetic peptides that were N- and C-terminal truncations of chaperonin 10. The active sites of *M. tuberculosis* chaperonin 10 were shown to lie in the two loop structures that make contact with chaperonin 60 (Ref. 62). Additionally, because there were no contaminating exogenous peptides or bacteria-derived LPS in these experiments, they strongly support the concept that chaperonins contain short peptide segments that can activate cells independently of other bacterial or host proteins. GroES has also been shown to be a potent inducer of the in vitro break down of mouse bone (Ref. 64).

In addition to stimulating the breakdown of bone in in vitro assays and cell cultures, *M. tuberculosis* chaperonin 10 can also induce human monocytes in vitro to synthesise and secrete pro-inflammatory cytokines. Structure–activity studies, using synthetic peptides, have revealed that the active site of *M. tuberculosis* chaperonin 10 is in the C-terminal helical domain. This domain is distinct from those that induce bone resorption by osteoclasts, suggesting that this small protein has at least two distinct sites for the activation of myeloid cells (P. Tabona and colleagues, Eastman Dental Institute, London, UK, unpublished).

Chaperonins: the unanswered questions

Release of chaperonins from cells

Only 7 years have passed since it was first reported that chaperonins might stimulate cells. There is now substantial supportive evidence

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for the hypothesis that chaperonins, when added to the external environment of cells in vitro, can act as intercellular signals. However, the key question 'Do chaperonins act as intercellular signals in vivo?' still needs to be answered. Many researchers studying molecular chaperones have the fixed idea that these proteins are intracellular, and therefore that any extracellular effects that are reported are artefactual. The reason for this is that, at present, there is no known mechanism to explain how cells can secrete molecular chaperones. At first sight, this knowledge gap seems to represent a significant problem for those studying the cell-to-cell signalling properties of molecular chaperones. However, to date, there is no known mechanism to explain the secretion of the potent pro-inflammatory cytokine IL-1 or the well-known redox chaperone protein thioredoxin (Ref. 82). Yet no one would discount the evidence that both of these proteins are potent and important secreted cell-to-cell signalling molecules.

Fortunately, evidence has begun to accumulate in recent years to support the hypothesis that host-derived molecular chaperones can be released from cells as part of normal homeostatic control. Indeed, certain bacteria release large amounts of these proteins. For example, an early publication reported that chaperonin 10 made up 20% of the total protein content of culture filtrates of logarithmically growing *M. tuberculosis* (Ref. 83). An increasing number of studies have reported that chaperonin 60 is either found on the surface of bacteria or actively secreted by them. For example, at least some of the chaperonin 60 protein in *A. actinomycetemcomitans* is associated with its cell surface (Ref. 50). Since this publication, several groups have reported that *Helicobacter pylori* (Ref. 84), *Haemophilus ducreyi* (Ref. 85) and *Legionella pneumophila* (Ref. 86) express chaperonin 60 on their outer surfaces. Also, the surface location of chaperonin 60 on *A. actinomycetemcomitans* is known (Ref. 87). In this species of bacterium, the surface-expressed chaperonin 60 is believed to act as an adhesin (a protein that is responsible for the adhesion of the bacterium to host tissues). Initially, controversy existed as to whether the surface expression of this chaperonin was due to it being either actively secreted by the bacteria or simply the result of bacterial lysis. However, it has recently been conclusively demonstrated that the expression of chaperonin 60 on the outer surface of *H. pylori*, at least, is not due to cell lysis (Ref. 88). These findings have suggested that the chaperonins might be another class of bacterial virulence determinants, that is, bacterial components that are able to produce tissue pathology (Ref. 89).

What is the significance of the extracellular presence of chaperonins on eukaryotic cells? There is increasing evidence to suggest that various molecular chaperones are associated with the plasma membrane of eukaryotic cells. For example, chaperonin 60 has been detected on the surface of Chinese hamster ovary cells and on the human leukaemic $CD4^+$ T-cell line CEM-SS (Ref. 90). Daudi human lymphoma cells have also been found to express chaperonin 60 on their cell surfaces (Ref. 91). In mice infected with the intracellular bacterium *Listeria monocytogenes*, spleen and liver cells expressed murine chaperonin 60 on their plasma membranes (Ref. 92). Several transformed mammalian cell lines have also been reported to express hsp70 on their cell surfaces (Refs 93, 94, 95, 96). Leukocytes from some patients suffering from SLE expressed human hsp90 on their cell surfaces (Ref. 97), as did various tumour cells (Refs 94, 98) and even normal cells (Refs 99, 100). This cell-surface expression of hsp90 is presumably related to the reports that it can stimulate leukocytes to synthesise cytokines (Refs 101, 102), in that it implies cell–cell signalling by extracellular hsp90. Interestingly, it has also been reported that human interleukin 10 (IL-10) can activate human cells to express the human hsp90β gene (Ref. 103).

Moreover, it has been reported that eukaryotic cells secrete chaperonins. For example, human neuronal cells have been reported to secrete both a chaperonin-like protein, ADNF (Ref. 68), and a chaperonin 60 protein (Ref. 71). The existence of EPF (chaperonin 10) in the serum of pregnant women has already been discussed. Indeed, it has recently been reported that the serum of healthy human subjects contains both human chaperonin 60 and antibodies to chaperonin 60 (Ref. 104). Intriguingly, the serum concentrations of chaperonin 60 and antibodies to this protein are higher in women than in men, regardless of whether the women have been pregnant.

Therefore, there is mounting evidence that molecular chaperones can associate with the plasma membranes of eukaryotic cells, and can be secreted into the extracellular fluid.

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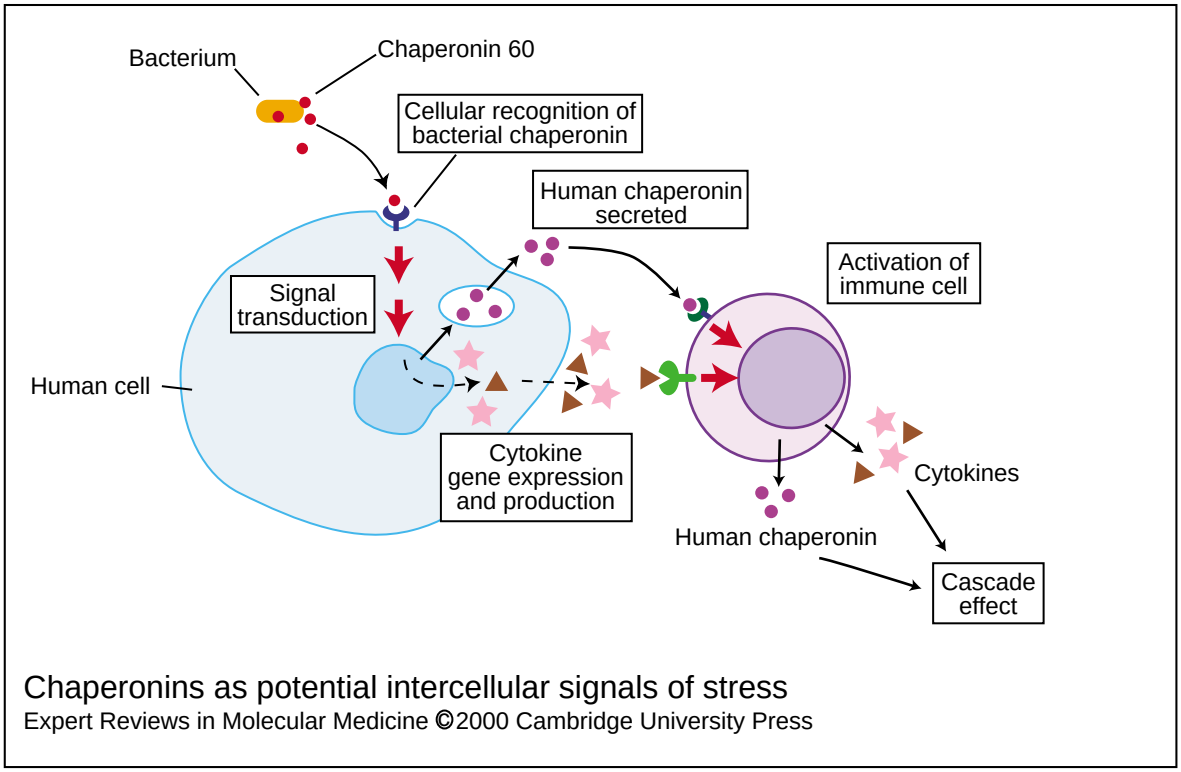

Figure 4. Chaperonins as potential intercellular signals of stress. A damaged or infected cell might secrete chaperonins to signal a stress to neighbouring cells. These neighbouring cells can then mount an inflammatory response. Chaperonins can therefore be seen as broad-range intercellular stress signals **(fig004jrl)**.

However, it is still not clear how this is achieved. The recent finding that chaperonin 60 has a lipid-binding domain, which allows it to interact with membranes (Ref. 105), might help to explain these findings (see Fig. 4).

Chaperonin biology: structure–activity relationships and receptors

The chaperonin 60 family of proteins is highly conserved, even between such unrelated species as bacteria and humans. The degree of sequence conservation between individual bacterial species is in the order of 50–60%. Thus, it has been thought convenient to regard these 60-kDa chaperonin proteins as identical biological molecules. This is certainly the working hypothesis of those who are interested in the folding properties of chaperonin 60 proteins. This was also our viewpoint until our group discovered (Ref. 50) that the biological activity of chaperonin 60 proteins derived from *E. coli* and *A. actinomycetemcomitans* differed significantly to that of chaperonin 60 proteins derived from *M. tuberculosis* and *M. leprae*. The *E. coli* and *A. actinomycetemcomitans* chaperonin 60 proteins were potent inducers of bone resorption whereas the *M. tuberculosis* and *M. leprae* chaperonin 60 proteins had only minimal activity (Refs 50, 62). What then is the structural basis for this difference in biological activity? To date, the only information we have about the relationship between the structure of chaperonin 60 and its biological activity is the finding that this oligomeric protein can be proteolysed with minimal loss of biological activity (Ref. 52). Our group has isolated (by HPLC) and identified (by N-terminal sequencing and mass spectrometry) the active chaperonin 60 peptides from *E. coli* but failed, as yet, to show that the corresponding synthetic peptides are biologically active. This might be owing to the failure to detect active contaminants in preparations of the purified protein. However, these studies do suggest that different linear peptide segments of chaperonin 60 proteins might mediate the different biological activities of these proteins, and this might explain why different chaperonin proteins within the same family can have different activities.

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An obvious question that has to be addressed is 'How do molecular chaperones activate cells?' The simplest hypothesis is that they bind to a cell-surface receptor, and activate cells via one or more intracellular signalling pathways. In a recent report, Kol and colleagues (Ref. 58) presented evidence that murine monocytes respond to both human and chlamydial chaperonin 60 proteins via the LPS CD14 receptor. CD14 is a glycosylphosphatidyl-inositol-anchored non-signalling receptor, which interacts with a second class of receptor, known as Toll-like receptors (TLRs), to induce cell activation (Ref. 106). The TLR that recognises LPS is now known to be TLR4. This work relied, in part, on the finding that anti-CD14 monoclonal antibodies blocked the activity of the chaperonin 60 proteins used in these studies. However, our group reported previously that anti-CD14 monoclonal antibodies did not block the activation of human monocytes by GroEL (Ref. 52); we have also shown that the activity of the *M. tuberculosis* chaperonin 60.1 and chaperonin 60.2 proteins is unaffected by anti-CD14 monoclonals (J. Lewthwaite and colleagues, Eastman Dental Institute, London, UK, unpublished). Further evidence that GroEL does not function via the complex formed between the CD14 receptor and the TLR comes from earlier published work, in which we used the C3H/HeJ LPS-unresponsive mouse strain. Bone derived from these mice was, as expected, unresponsive to LPS but underwent osteolysis in the presence of GroEL. Because the basic defect in these mice resides in the TLR4, which is nonfunctional owing to the mutation of a single base in its DNA, the CD14–TLR4 complex is ruled out as the receptor for either GroEL or the *M. tuberculosis* and *M. leprae* chaperonin 60 proteins. Further evidence to support this hypothesis comes from the use of signal-transduction inhibitors. Our group has shown that inhibitors of p38 mitogen-activated protein kinase block both LPS- and GroEL-activated human monocytes, but that only GroEL-activated monocytes are blocked by inhibitors of the src family of kinases (P. Tabona and colleagues, unpublished).

The preliminary conclusion that can be reached from all these studies is that human cells have more than one receptor for chaperonin 60 proteins. Thus, different chaperonin 60 proteins may be able to bind to different receptors on human cells and produce different patterns of cell activation.

Chaperonin biology: the future

Chaperonins are released from cells and/or are expressed on external cell membranes (of both eukaryotic and prokaryotic cells), and thereby become part of the population of proteins that act as cell-to-cell signals. Is signalling one of the evolved properties of chaperonins, or is it simply an artefact of the inappropriate release of these proteins? We believe the ability to signal to cells and activate them is a key attribute of a stress protein. Studies of molecular chaperones and their induction in response to stress have largely been performed in in vitro culture systems; in such systems, all cells are exposed to the same stress signals and do not, therefore, need to signal to other cells. But what happens in vivo? If cells in one part of a tissue (or in one tissue) are exposed to stress, it would be sensible to broadcast this 'news' to the rest of the tissue or organism. The simplest method of doing this would be to use the molecular chaperones, whose synthesis has been increased by the organism, to act as the messengers to signal to other cells that stressful events are occurring within their immediate environment. Indeed, the finding that human ADNF, a neuronal stress protein, can upregulate the expression of human chaperonin 60 in neuronal cells is the first example of molecular chaperones upregulating the synthesis of additional molecular chaperones. We would predict that this is likely to be a common mechanism by which molecular chaperones act to spread the 'bad news' about stress, so that the body can react appropriately to minimise its effects.

If the hypothesis that molecular chaperones are cell-to-cell signalling proteins is correct, then these proteins could play pathological roles in human diseases, and therefore be targets for therapeutic intervention. It is likely that the direct roles of molecular chaperones in human diseases will be defined in the near future.

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Further reading, resources and contacts

Cell Stress Society International is a US organisation that should be useful to anyone who has an interest in stress-response research.
http://www.sp.uconn.edu/~hightowe/cssi/

The Molecular Chaperone Club provides an informal discussion forum within the UK for all those interested in molecular chaperones. All aspects are covered, including synthesis, structure and function, as well as medical and biotechnological implications.
http://www.ocms.ox.ac.uk/ocms/molchap.html

Helen Saibil's webpage includes colourful images and movies of chaperonins, as well as some links to other relevant sites. Helen is a member of the Chaperone Group, in the Crystallography Department at Birkbeck College, London, UK.
http://www.cryst.bbk.ac.uk/~ubcg16z/chaperone.html

Features associated with this article

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