Chaperonin 60: A Paradoxical, Evolutionarily-Conserved, Protein with Multiple Moonlighting Functions

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ABSTRACT

Chaperonin 60 is the prototypic molecular chaperone, an essential protein in eukaryotes and prokaryotes, whose sequence conservation provides an excellent basis for phylogenetic analysis. *Escherichia coli* chaperonin 60 (GroEL), the prototype, has an established oligomeric structure-based folding mechanism and a defined population of folding partners. However, there are a growing number of examples of chaperonin 60 proteins whose crystal structures and oligomeric composition are at variance with GroEL, suggesting that additional complexities in the protein folding function of this protein should be expected. In addition, many organisms have multiple chaperonin 60 proteins some of which have lost their protein folding abilities. This highlights the major paradox in the chaperonin 60 story. It is emerging that this highly conserved protein has evolved a bewildering variety of additional biological functions – known as moonlighting functions – both within the cell and in the extracellular milieu. Indeed, in some organisms, it is these moonlighting functions that have been left after the loss of the protein-folding activity. This article reviews the relationship between the folding and non-folding (moonlighting) activities of the chaperonin 60 family in an attempt to understand the emerging biology of this evolutionarily ancient protein.

**Keywords:** chaperonin 60, heat shock response, protein folding, protein moonlighting, paralogues, protein evolution

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I. INTRODUCTION

The chaperonin (Cpn)60 protein appears to be rewriting the rules of protein behaviour. The genes encoding Cpn60 are highly conserved, such that they are becoming an alternative to 16S RNA genes for phylogenetic analysis (e.g. (Sakamoto & Ohkuma, 2010)). In prokaryotes and eukaryotes the Cpn60 protein is an essential molecular chaperone whose loss is incompatible with survival of either bacteria (Fayet, Ziegelhoffer & Georgopoulos, 1989) or mammals (Christensen et al., 2010). The key role of this protein in cellular survival would be expected to constrain evolutionary change in Cpn60. However, many prokaryotes have multiple cpn60 genes (up to seven in the root nodulating alpha proteobacteria), which have arisen by gene duplication (Lund, 2009). It is estimated that these duplicated genes can undergo rapid evolutionary change (Goyal, Qamra & Mande, 2006; McNally & Fares, 2007). The major paradox exhibited by Cpn60 proteins is the evolution of multiple biological activities that have nothing to do with protein folding. Such additional biological activities exhibited by proteins are known as moonlighting activities and the proteins with such multiple actions are defined as moonlighting proteins (Jeffery, 1999). Currently, it is recognised that there may be more than 100 examples of proteins with moonlighting activity (Henderson & Martin, 2011a; Henderson & Martin, 2011b; Jeffery, 2009). However, none have the number, or the range, of moonlighting activities that are now attributable to the Cpn60 protein. These moonlighting actions are not just interesting artefacts, as at least two human diseases: early onset diabetes and juvenile rheumatoid arthritis, are likely to result from the cell signalling moonlighting actions of the human Cpn60 protein (in the human known as HSPD1) on cells of the human immune system (Ronaghy et al., 2011; Tuccinardi et al., 2011; Zonneveld-Huijssoon et al., 2011). This, in turn, reflects the enormous potency of the Cpn60 protein as a cell signalling molecule (Zanin-Zhorov et al., 2005a; Zanin-Zhorov et al., 2005b). In addition, the Cpn60 protein from organisms ranging from bacteria to humans have a diversity of functions including acting as cell surface receptors for various ligands and for bacteria, to inhibiting apoptosis, replicating the actions of soluble immune signals and acting as toxins. This review brings together the diverse literature on the moonlighting actions of Cpn60 proteins and attempts to provide insight into the mechanisms involved in such moonlighting by considering the potential relationships between the oligomeric and tertiary structure of Cpn60, its sequence, molecular evolution and folding function, and the development of additional biological (moonlighting) functions for this protein.
II. Chaperonin 60: An Evolutionarily Diversifying Molecular Chaperone

The term chaperonin (Cpn) is employed to describe two types of protein-folding oligomers. The type I chaperonins are found in the eukaryotic mitochondrion, chloroplast and hydrogenosome and also in the bacterial cytosol (Broadley et al., 2009). These proteins require a second oligomer, chaperonin 10, to function as a lid in the substrate encapsulation process in the central cavity. In contrast, the type II chaperonins contain an inbuilt 'lid' and are found in the eukaryotic cytosol and in the Archaea (Horwich et al., 2007). This review focuses only on the type I chaperonins. This section explores the findings that the Cpn60 protein may have greater structural variation than was previously assumed, and asks if this could contribute to the moonlighting actions of this protein?

The Cpn60 protein was discovered as a result of two separate areas of study. One was on the chloroplast Rubisco large subunit binding protein (Hemmingsen & Ellis, 1986) and the other was on an E. coli gene product, GroEL (Georgopoulos et al., 1972), essential for cell viability and for assembly of bacteriophages. It was recognised that both proteins were homologues and the term chaperonin (60) was employed to describe this novel molecular chaperone (Hemmingsen et al., 1988). Given that chaperonin (groEL/ES) genes of E. coli had been the subject of intensive study the E. coli protein came to be used as the prototype to analyse the structure and mechanism of the proteins that they encode. It was assumed that what was true for this commensal bacterium would be applicable to all other organisms – an assumption that is now undergoing some revision.

Electron microscopic studies and high resolution crystallography of GroEL reveals two rotationally symmetrical rings, each containing seven protomers, stacked back-to-back with dyad symmetry (Braig et al., 1994; Ranson et al., 2001; Xu, Horwich & Sigler, 1997) (Fig 1). The individual 57kDa subunits of GroEL are composed of three domains. The largest, termed the equatorial domain, is the foundation of the oligomeric structure at the centre of the GroEL machine and provides the contacts that hold the two rings together. This is then connected to the smaller intermediate domain that forms a connection with the third domain, the apical domain, which forms the ends of the GroEL cylinder (Braig et al., 1994) (Fig 1). Both EM and crystallographic studies showed that the GroEL oligomer has two central cavities, one formed by each ring, suggesting the obvious hypothesis that Cpn60 folds proteins within these cavities. Mutational analysis of GroEL identified a polypeptide binding site on the inside surface of the apical domain consisting of hydrophobic amino acids (Fenton
et al., 1994). This site also binds GroES in a process that results in the release of the bound client protein from the GroEL oligomer into the central cavity. A key residue, Asp87, which is within the nucleotide-binding pocket, was shown to be essential for ATP hydrolysis and release of bound proteins (Fenton et al., 1994) and the residues responsible for ATP binding have been identified (Xu & Sigler, 1998). In the intervening years research into the mechanism of GroEL protein folding within the cavity has suggested a model in which the cavity functions as a passive ‘Anfinsen folding cage’ (with a so-called ‘non-stick surface’) allowing sequestered monomeric proteins the space and time to fold into their most energetically favourable conformation (Horwich, Apetri & Fenton, 2009), although different models of the precise mechanism of protein folding are still under review (Jewett & Shea, 2010). Thus GroEL appears to function rather passively, mainly by sequestering aggregation-prone intermediates in the cavity, where they can fold without interacting with other proteins, though it may also provide an environment which is more favourable energetically for protein folding. More recent results from single-molecule spectroscopy on GroEL suggests that with any one client protein (in this study – rhodanese) no ‘universal chaperonin mechanism’ exists with folding being a competition between intra- and inter-molecular interactions (Hofmann et al., 2010).

If Occam’s famous razor were to be applied to the Cpn60 paradigm, then it would suggest the hypothesis that all Cpn60 proteins have a tetradecameric structure with an active ATPase site, and that Cpn60 proteins are all chaperones that fold very similar populations of client proteins. However, this may not be the case. First, there are cases emerging of oligomeric and enzymatic variants of this protein. For example, the hydrogen-oxidizing bacterium Paracoccus denitrificans has a tetradecameric Cpn60 with 7-fold symmetry which differs from GroEL in terms of the subunit contact points (Fukami et al., 2001). The Gram-negative thermophilic bacterium, Thermus thermophilus, has a Cpn60 protein with significant differences in its cis-cavity, in which proteins are encapsulated, with the apical domain showing a large deviation from what is assumed to be the normal 7-fold symmetry of this oligomeric complex (Shimamura et al., 2004). The mycobacterial Cpn60 proteins exhibit the most unexpected structural features. These bacteria encode at least two Cpn60 proteins. It was reported many years ago that the Cpn60.2 protein of Mycobacterium bovis BCG eluted, on molecular sieving, as a tetramer of around 250kDa (De Bruyn et al., 2000), compared with the molecular mass of the GroEL tetradecamer of around 800kDa. More recently, it has been shown that both Cpn60
proteins of *Mycobacterium tuberculosis* fail to run as tetradecamers on native PAGE (Qamra, Srinivas & Mande, 2004; Tormay, Coates & Henderson, 2005). Further analysis of the *M. tuberculosis* Cpn60.2 protein by size exclusion chromatography, dynamic light scattering, analytical ultracentrifugation and electron microscopy (at concentrations ranging from 0.1-10.0mg/ml) only identified a dimer, with no evidence for the existence of a tetradecamer (Shahar et al., 2011). The ATPase activity of this mycobacterial protein was only 10% of that of *E. coli* GroEL under similar conditions (Shahar et al., 2011). In addition two independent crystal structures of *M. tuberculosis* Cpn60.2 at 3.2 and 2.8Å have been solved (Qamra & Mande, 2004; Shahar et al., 2011). Both structures are dimers, and it has been suggested that this may be due to the lack of the residues required to induce an oligomeric structure (Shahar et al., 2011). It has been argued that the *M. tuberculosis* Cpn60.2 structure and its low ATPase activity is a result of the slow growth and low metabolic demands of this organism. However, this protein can complement an *E. coli* groEL mutant revealing that it can function in a fast growing organism (Hu et al., 2008). It is unclear if oligomerisation occurs in *E. coli* or if the GroES can somehow accommodate itself to the dimeric constraints of the mycobacterial protein. This finding is not confined to the mycobacteria as the Cpn60 protein of the gastric pathogen, *Helicobacter pylori*, is also a dimer or a tetramer when analysed in solution (Lin et al., 2009). In addition, the cyanobacterium, *Synechoccus elongatus*, has two Cpn60 proteins with the Cpn60.1 protein forming an unstable tetradecamer while the Cpn60.2 forms a heptamer (Huq et al., 2010). It might be proposed that bacterial growth under unusual conditions might influence the oligomerisation state of Cpn60. However, the halophilic lactic acid bacterium, *Tetragenococcus halophila*, which can grow in 4 M sodium chloride, has a tetradecameric Cpn60 (Tosukhowong et al., 2005), so extreme conditions *per se* do not necessarily cause an evolutionary alteration of the oligomeric state of chaperonins.

The Cpn60 co-chaperone, chaperonin (Cpn)10, can also form oligomers of lower order than the heptamer seen with *E. coli* GroES. Again, this was first shown with the Cpn10 protein of *M. tuberculosis* which was found to form tetramers in solution and in bacterial lysates (Fossati et al., 1995). In addition, the Cpn20 protein (Cpn10 homologue) in the chloroplast appears to exist in multiple oligomeric permutations, including tetramers (Sharkia et al., 2003). Mutants of GroES that have a destabilised oligomeric structure are still active with GroEL (Seale et al., 1997). Thus, it is
unclear as to the extent that the structure, oligomeric composition and mechanism of protein folding of GroEL/GroES can be generalised to all chaperonins (Table 1). An obvious question is whether these variations in oligomeric structure could contribute to the moonlighting activity of Cpn60.

II (1). Evolution of the Client Binding Specificity of Chaperonin 60

Understanding how Cpn60 molecules bind to their client proteins may provide clues as to how they can perform so many different moonlighting functions, since many of these moonlighting activities involve interacting with specific proteins. Mutations in GroEL that interfere with the cavity or the ability of the oligomer to encapsulate protein substrates are lethal, demonstrating the essential role of the central cavity to protein folding and bacterial viability (Koike et al., 1995; Tang et al., 2008). What proteins in E. coli need GroEL/GroES to fold? Studies using a co-immunoprecipitation strategy to isolate clients bound to GroEL/GroES, and mass spectrometric peptide fingerprinting to identify the bound proteins, have reported that around 250-300 E. coli cytoplasmic proteins bind to the GroES/GroEL chaperonin complex (Houry et al., 1999; Kerner et al., 2005). These proteins have been divided into three classes (I – III). Class I proteins interact with GroEL but do not require it for folding at 37°C. Class II proteins require GroES/GroEL at 37°C but not at 25°C. The class III proteins, of which there are about 85 in E. coli, are obligate clients of GroEL. All are proteins of low-to-intermediate abundance. Among the class III proteins are 13 proteins that are essential for the viability of E. coli, which may explain the essentiality of the GroEL/GroES system for this bacterium (Kerner et al., 2005). These class III proteins tend to be relatively large with α/β and α+β domain topology and include proteins containing a (βα)_8 TIM barrel motif, though not all such proteins are GroEL clients. A more recent survey of GroEL/GroES substrates has suggested that only about 60% of the class III substrates identified by Kerner and colleagues (Kerner et al., 2005) are obligate GroEL-interacting proteins in vivo (Fujiwara et al., 2010). This study also defined a new subset of obligate GroEL binding proteins, the class IV proteins, which have a slight but statistically significant alanine plus glycine content bias. It is suggested that the short side-chains of these residues might confer more flexibility on such proteins, when denatured, leading to an increased propensity to aggregate. None of the GroEL specific-interacting proteins identified by Kerner and colleagues (Kerner et al., 2005) are homologous to the few proteins to which Cpn60 proteins are known to bind
when they are functioning as moonlighting proteins. This suggests that the moonlighting functions of Cpn60 proteins are not related to the protein binding specificities of this protein when acting as a molecular chaperone.

One of the major problems in the study of chaperonin 60 client proteins is the lack of knowledge of other systems. Although a number of studies have been published showing that Cpn60 homologues can complement for loss of GroEL in *E. coli*, revealing that these proteins must at least be able to chaperone the folding of the essential GroEL clients, there have been few studies of Cpn60 client proteins in other bacteria. In one study, crystals of the Cpn60 protein from the Gram-negative thermophile, *Thermus thermophilus*, were found to contain 24 additional proteins which are potential clients, only three of which were homologues of known GroEL clients (Shimamura et al., 2004). In another study, a single ring mutant of the Cpn60 protein of the Gram-positive soil organism (and human pathogen), *Bacillus subtilis*, was used to trap potential client proteins. Approximately 110 proteins were detected in association with the *B. subtilis* Cpn60 and, of these, 28 were identified by MALDI-TOF MS peptide fingerprinting. The proteins ranged in mass from 20 to 100kDa, ten of which had been identified as Cpn60 clients in *E. coli*, though there was minimal overlap with the potential clients identified in *T. thermophilus* (Endo & Kurusu, 2007). Caution must be exercised in the interpretation of these studies, as potential artefacts can confound the interpretation of these experiments, unless appropriate controls are included. Cpn60 clients have also been studied in the unusual archaeon, *Methanosarcina mazei*, which possess a large number of bacterial genes, including the Cpn60/Cpn10 chaperonins, as well as the archaeal thermosome (Deppenmeier et al., 2002). Analysis of the proteins binding to Cpn60/Cpn10 and to the thermosome in *M. mazei* revealed that 13% of the soluble proteins in this organism bind to one or other of the two chaperonins, with the Cpn60 system binding around 180 proteins, and the thermosome approximately 250 proteins (Hirtreiter et al., 2009). While a proportion of the proteins in *M. mazei* are of bacterial origin, Cpn60 does not particularly favour these, and binds to many archaeal proteins (Hirtreiter et al., 2009). Clearly more examples of the profiles of cytosolic proteins bound by the Cpn60 protein would be needed to ascertain how much structural variety this chaperonin can cope with in terms of protein client binding. However, it is clear that extrapolations from studies on *E. coli* GroEL must be done cautiously.
The genomes of free-living bacteria range in size from around 500 genes to over 8,000 genes and thus the cytosolic proteome that requires interaction with the Cpn60/Cpn10 system could vary by at least a log order. In addition, the Cpn60/Cpn10 system presumably has to co-evolve with its clients and this may act to slow the rates of evolution of the chaperonin and its clients, as has been observed (Raineri et al., 2010). Duplication of chaperonin genes, followed by co-evolution of one of the genes and evolution of a specific subset of client proteins, may have occurred in some or all of the organisms that possess more than one chaperonin gene. Indeed, one study showed how directed evolution of a duplicated copy of *E. coli* GroEL resulted in GroEL variants with an enhanced ability to fold green fluorescent protein (GFP) (Wang et al., 2011). Alteration of only a handful of residues in GroEL/GroES resulted in a dramatic increase in rates of GFP folding, which was not due to changes in expression of GroEL or GFP. Strikingly, changes which increased the ability of GroEL to fold GFP resulted in a decreased ability of GroEL to function as a general inhibitor of protein aggregation and led to serious cellular growth defects, or inhibition of growth at elevated temperatures (Wang et al., 2011); thus, specialisation could only take place after gene duplication.

**II (2). A Proportion of Organisms Possess Multiple Chaperonin 60 Proteins**

An important factor in the evolution of moonlighting in the Cpn60 protein is the finding that many organisms encode more than one such chaperonin, each of which may have evolved distinct folding and moonlighting functions. *Escherichia coli* requires only one, indispensable, Cpn60 protein to be viable. However, analysis of 669 complete bacterial genomes has revealed that almost 30% of bacteria have more than one *cpn60* gene. This may be matched with only one *cpn10* gene or there may be more than one copy of the *cpn10* gene, with the individual *cpn10* and *cpn60* genes usually forming operons. The possession of multiple *cpn60* genes appears not to be a random process, as some bacterial groups (e.g. *Spirochaetes*) only possess one gene while in others (e.g. *Actinobacteria*) almost all possess multiple *cpn60* genes. Further groups such as Cyanobacteria and Chlamydiae all possess multiple *cpn60* genes (Lund, 2009). This non-random distribution of multiple *cpn60* genes suggests that there must be selective pressure for their maintenance. Several examples of mycoplasmas which lack Cpn60 homologues are known. Not all mycoplasmas have lost their Cpn60
proteins and in their review of Cpn60 function in the Mollicutes, Clark and Tillier suggest that those organisms with Cpn60 are not using this protein as a molecular chaperone but as an adhesin/invasin (proteins involved in binding to and invading host cells) (Clark & Tillier, 2010). This suggests that it is this moonlighting function of the Cpn60 protein of these organisms that is evolutionarily more important than the protein folding function.

Three explanations could account for the presence multiple cpn60 genes: (i) all gene products have similar function but are part of a complex regulatory system; (ii) the extra genes encode proteins evolved to only fold specific proteins and/or; (iii) the extra gene(s) encode proteins with non-folding, moonlighting, actions. What experimental evidence is there to support any of these hypotheses? The Alphaproteobacteria contain organisms that have the greatest number of cpn60 genes, with Bradyrhizobium japonicum USDA110 having seven such genes (Kaneko et al., 2002). This group contains organisms that form symbiotic relationships with plants in the form of nitrogen-fixing root nodules. It is not established that all these multiple Cpn60 proteins play a role in nitrogen fixation. However, the groEL1 gene of Sinorhizobium meliloti is required for expression of the nod gene proteins in this bacterium, and this gene function could not be replaced by E. coli GroEL (Ogawa & Long, 1995). This suggests some degree of folding specificity with this Cpn60 protein. In spite of this, S meliloti, which has four groESL operons and one groEL gene, can survive in the absence of the loss of individual cpn60 genes, although groESL1 or groESL2 are necessary for viability and growth (Bittner, Foltz & Oke, 2007). In Rhizobium leguminosarum, another nitrogen-fixing bacterium, there are three groESL operons with the cpn60 gene products being termed Cpn60.1, Cpn60.2 and Cpn60.3 (Rodriguez-Quinones et al., 2005). Analysis of this organism revealed that Cpn60.1 was present at higher levels than Cpn60.2. In contrast, the Cpn60.3 protein could not be detected under normal growth conditions, although the gene for this protein is transcribed under anaerobic conditions. Gene inactivation revealed that the Cpn60.1 protein is essential for viability and growth. However, both the cpn60.2 and cpn60.3 genes could be individually inactivated without loss of viability and a double mutant (lacking both cpn60.2 and cpn60.3) was also fully viable (Rodriguez-Quinones et al., 2005). Comparison of the protein-folding activities of the Rh. leguminosarum Cpn60 proteins has shown that Cpn60.1 and Cpn60.2 were equally able to fold lactate dehydrogenase and were similar in activity to E. coli GroEL. Unexpectedly, the Cpn60.3 protein could not fold this protein
(George et al., 2004). Surprisingly, the Cpn60.2 protein cannot be over-expressed in Rh. leguminosarum and so cannot replace the Cpn60.1 protein. In contrast, Cpn60.3 can be over-expressed and the transformant can grow in the absence of Cpn60.1, however, this strain has a temperature-sensitive phenotype (Gould, Burgar & Lund, 2007).

The Chlamydiae all have three cpn60 genes (Karunakaran et al., 2003; Lund, 2009) and, as will be described, some of these proteins play a role in tissue pathology. The Cpn60.2 and Cpn60.3 proteins are more related to each other than to the Cpn60.1 protein. Indeed, it is proposed that the Cpn60 protein in the Chlamydiae was duplicated at the initiation of this lineage to generate three distinct proteins which includes the original Cpn60.1 protein and the paralogous proteins, Cpn60.2 and Cpn60.3 (McNally & Fares, 2007). The most striking finding is that mutations have occurred during the evolution of the cpn60.2 and cpn60.3 genes in the normally highly conserved ATP-binding site (Fig 2). Thus the conserved sequence GDGTTT has mutated to GDGAKT in Cpn60.2 and to ADGVIS is Cpn60.3, presumably accounting for the failure of these two proteins to complement a groEL mutant (Karunakaran et al., 2003). So what role do these three proteins play? This question cannot yet be definitively answered, but there is evidence for differential regulation of the Cpn60 proteins under different conditions. In HeLa cells, Karunkaran et al (Karunakaran et al., 2003) reported that Cpn60.1 was the most highly expressed protein and the only one to be increased under heat shock. However, a separate report found that in Hep2 cells (a distinct cell line) the cpn60.3 gene was most highly expressed, whereas, when infecting monocytes, the cpn60.2 gene was the most expressed protein (Gérard et al., 2004). Chlamydia can cause arthritis in humans and in tissues from Chlamydia-infected joints the cpn60.1 and cpn60.2 genes were overexpressed with the latter always being more highly expressed than the former. In contrast, there was no expression of cpn60.3 (Gérard et al., 2004). This suggests a complex and distinct interaction between bacterium and host, dependent upon the cell type invaded.

The Cyanobacteria generally encode two Cpn60 proteins (Lund, 2009). As has been briefly described, a recent study has compared the two Cpn60 proteins of Synechococcus elongatus PCC 7942. Both Cpn60 proteins (referred to in this study as GroEL1 and 2) of this organism could prevent protein aggregation, but the folding activity was much lower than E. coli GroEL and the ATPase activity of GroEL1 was only 15% that of E. coli GroEL and the GroEL2 protein had insignificant
ATPase activity. This is similar to the ATPase activity of the two Cpn60 proteins of *M. tuberculosis* (Qamra & Mande, 2004; Shahar *et al*., 2011). The GroEL1 protein (native or recombinant) formed a tetradecamer, but the GroEL2 protein formed a heptamer or dimer. Moreover, both GroEL oligomers were very unstable (Huq *et al*., 2010). It is not known if the other cyanobacterial Cpn60 proteins have similar physicochemical characteristics. However, it has been shown that the *groEL1* gene from two other cyanobacterial species (*Synechocystis* sp. PCC 6803 and *Synechococcus vulcanus*) can complement *E. coli* GroEL, but that the *groEL2* gene either poorly complements or fails to complement (Kovács *et al*., 2001; Tanaka *et al*., 1997). This is different from the situation in the *Actinobacteria* where it is generally the *cpn60.2*, and not the *cpn60.1* gene, that can complement an *E. coli* groEL mutant (Lund, 2009).

A final example of the role of multiple Cpn60 proteins in the lifestyle of prokaryotes is the two Cpn60 proteins of the Gram-negative bacterium, *Myxococcus xanthus*, an example of a myxobacterium (Jiang *et al*., 2008). The myxobacteria have a complex social behaviour, forming gliding colonies called swarms that can feed on other microorganisms. They also form multicellular resting structures called fruiting bodies when nutrients are depleted (Shimkets, 1999). In *M. xanthus*, deletion of either *cpn60* gene did not affect cell viability, but inactivation of both genes was not possible. The *cpn60.2* gene appeared to be the key to survival at high temperatures (42°C). When the two different *cpn60* mutants were grown in medium containing hydrolysed proteins they grew as well as the wild type. However, when grown in medium containing casein or *E. coli* cells as nutrients the mutant lacking Cpn60.2, but not Cpn60.1, was deficient in bacterial predation activity and in utilisation of casein. In contrast, deletion of the *cpn60.1* gene, but not the *cpn60.2* gene, resulted in the virtual failure of this organism to develop fruiting bodies under low nutrient conditions (Li *et al*., 2010).

It is not only bacteria that exhibit multiple Cpn60 proteins. The chloroplast Cpn60 protein oligomer is actually generated from two different subunit types – Cpn60α and Cpn60β (Levy-Rimler *et al*., 2002). It is generally accepted, from reconstitution studies, that the Cpn60 complex in chloroplasts is α7β7 (Dickson *et al*., 2000). The model plant organism *Arabidopsis thaliana*, has two genes coding for Cpn60α proteins and four genes encoding Cpn60β subunits (Hill and Hemmingsen 2001). These proteins show different expression profiles with Cpn60α1, Cpn60β1 and Cpn60β2 being the dominant subunits with the others being expressed at low levels (Peltier *et al*., 2006; Weiss *et al*., 2009).
Inactivation of the \textit{cpn60a1} gene is lethal (Apuya \textit{et al.}, 2001) as is a \textit{cpn60β1β2} double mutant (Suzuki \textit{et al.}, 2009). It can be concluded that these gene products are involved in the generation of the housekeeping chaperonin function in the chloroplast. Studies of \textit{A. thaliana} mutants defective in NADH dehydrogenase-like complex (NDH) have revealed that the folding of a subunit of this complex, NdhH, requires the specific participation of the Cpn60β4 subunit (Peng \textit{et al.}, 2011). This is somewhat reminiscent of the result with directed evolution of GroEL to increase its binding of GFP described earlier (Wang \textit{et al.}, 2002) and is one of the few such reports of folding specialisation in the eukaryotic Cpn60 protein. The only metazoan that we have information about multiple chaperonins is \textit{Drosophila melanogaster} which encodes four Cpn60 proteins (Hsp60A to D) which have different functions and tissue distributions (Sarkar & Lakhotia, 2005).

Returning to the three hypothesis previously ascribed to account for the presence of multiple \textit{cpn60} genes, the evidence, such as it is, might be stretched to support a role for these chaperonins in both folding specific proteins and in protein moonlighting. There does not appear to be evidence for organisms with multiple \textit{cpn60} genes using them in some form of regulatory system.

\textbf{III. A Brief Introduction to Protein Moonlighting}

Having described in detail the structure, the function, the folding substrates of Cpn60 and briefly mentioned the evolution of multiple Cpn60 proteins, the discussion will now turn to the unexpected multiple moonlighting activities of the Cpn60 molecule. To aid the reader who may have no understanding of protein moonlighting, this section will provide a brief introduction to this new area of protein function.

It has been assumed since the studies of protein (mainly enzyme) behaviour began that each protein has a single ‘active site’ and therefore a single function. It was Joram Piatigorsky, working at the National Eye Institute, in Bethesda, USA, who first reported that a protein could have more than one function. He reported that the lens crystallin protein in the duck was the metabolic enzyme, argininosuccinate lyase (Piatigorsky \textit{et al.}, 1988). He termed this phenomenon ‘gene sharing’ (Piatigorsky, 1998; Piatigorsky, 2007). Further work from his laboratory revealed that a whole range of metabolic enzymes, and other proteins, have evolved to function as transparent lens structures in a variety of animal species (Piatigorsky, 1998). Gene sharing has transmogrified into protein
moonlighting, a term that has been publicised by the structural biologist, Connie Jeffery, who has also been responsible for codifying this phenomenon (Jeffery, 2009). The original term, moonlighting, meant to have a second job, at night, in addition to the daytime occupation. Jeffery (Jeffery, 2009) attempted to define and categorise the various functional facets of moonlighting proteins. Proteins generated by gene fusions, homologous but non-identical proteins, splice variants, protein decoration variants, protein fragments and proteins operating in different locations or utilising different substrates are not defined as moonlighting proteins (Jeffery, 2009). Enzymes which have two metabolic functions or utilise two different substrates are categorised as bifunctional enzymes (Moore, 2004). The term ‘catalytic promiscuity’ has also been applied to the situation of an enzyme which has an active site able to catalyse two different reactions (Copley, 2003). Thus the term protein moonlighting refers to proteins that have one, or more, independent biological activities, in addition to the initial activity by which the protein was first known.

Moonlighting proteins are generally described when someone studying a known protein finds that it does something unexpected, or when individuals discover a new biological activity and then find that it is caused by a known protein. Thus moonlighting is normally discovered by accident. At the time of writing, there may be between one and two hundred moonlighting proteins described. An important point is that homologues of moonlighting proteins may not share the same moonlighting activity. Another unexpected finding, and the reason for writing this manuscript, is that certain moonlighting proteins have multiple moonlighting functions. At the present time there are only a few of these multiple moonlighting proteins and they all tend to be evolutionarily ancient proteins. Among such proteins are the glycolytic enzymes, phosphoglucoisomerase (PGI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase and, of course, Cpn60 (Henderson & Martin, 2011a; Henderson & Martin, 2011b)

Phosphoglucoisomerase is an excellent example of the discovery of different biological activities which are then ascribed to the same protein. Thus at the present time PGI has five defined moonlighting actions, in addition to its role as a glycolytic enzyme and these are: (i) a neurotrophic activity termed neuroleukin (Chaput et al., 1988); (ii) a factor which promotes cell motility, and is involved in tumour malignancy, called autocrine motility factor (AMF – (Watanabe et al., 1996)); (iii) differentiation and maturation mediator, which promotes myeloid cell differentiation and may play
some role in leukaemia (Xu et al., 1996); (iv) an implantation factor activity in the ferret (Schulz & Bahr, 2003) and (v) a protein with the ability to regulate endoplasmic reticulum (ER) stress by a novel mechanism involving controlling ER calcium levels (Fu et al., 2011). For all but the last of these moonlighting functions, the PGI must be secreted from cells and bind to a receptor. Most studies of the moonlighting actions of PGI have focused on its role as AMF, and it has been established that this protein is important in breast cancer progression and is associated with a poor prognosis. This is due to secreted PGI/AMF promoting epithelial-to-mesenchymal transition (EMT), which is a precursor to tumour metastasis (Ahmad et al., 2011). An unexpected aspect of the activity of AMF, and one that may be telling us something about the commonality of protein moonlighting, is that the receptor for AMF is itself a moonlighting protein. The receptor is a protein called gp78, an ER membrane-anchored ubiquitin ligase involved in ER-associated protein degradation (Fairbank, St-Pierre & Nabi, 2009). Glyceraldehyde-3-phosphate dehydrogenase has at least 12 different biological actions (Piatigorsky, 2007; Sirover, 2011).

Thus a small number of proteins have evolved to express a range of different biological actions. These actions, as have been described with PGI, and as will be described with Cpn60, have both homeostatic function (PGI and implantation) as well a pathological activity (AMF in breast cancer). The evolutionary pressure behind the development of multiple moonlighting activities is not understood.

IV. An Introduction to the Moonlighting Actions of the Chaperonin 60 Proteins

In this and the following sections, we will discuss the surprisingly large, and initially potentially confusing, range of different biological functions that have been ascribed to Cpn60 proteins. While large in number, these moonlighting actions have to be set in the context of the astronomical number of peptide sequences that can be generated when evolution is allowed combinatorial free-play with 20 different amino acids. Thus it is theoretically possible to synthesise 3.2 million amino acid pentamers and an enormous $1.02 \times 10^{13}$ decamers. This mind-boggling number of potential proteins able to be generated has been termed protein hyperspace (Smith & Morowitz, 1982). Thus within a protein, like Cpn60, with over 500 residues, there could be an enormous number of biologically active pentamers, decamers and so on, providing this protein with an potentially unlimited capacity to
promote moonlighting actions. Of course, the same is true of any protein, resulting in the obvious question – is the growing number of moonlighting actions ascribed to Cpn60 proteins unique to this protein – and - if so – why?

It is now 20 years since the first intimation that the Cpn60 protein may exhibit additional biological activities. This was the report that a mucus binding protein of the enteric pathogen, *Salmonella typhimurium*, was the Cpn60 protein of this organism (Ensgraber & Loos, 1992). Clearly, to act in this manner, the Cpn60 protein had to be secreted by the organism and bind to the bacterial cell wall.

This encapsulates two of the key findings with Cpn60. The first is an activity which is presumably nothing to do with protein folding – in this case binding to mucus. The second is that this activity requires that the supposedly intracellular Cpn60 protein can get out of the cell. In this case, the secreted Cpn60 then associates with the bacterial cell surface. It turns out that the Cpn60 protein is reported to be found on the cell surface of 22 different bacteria, where it is assumed to function as an adhesin, binding to components of the host (Table 2). It would be expected that the ligands for these cell surface bacterial Cpn60 proteins would be identical or, at least, limited to one type of molecule. However, the identified binding ligands for the bacterial cell surface Cpn60 proteins range from glycosphingolipids to mucus to invertase and all the way to known proteins such as DC-SIGN and CD43. In addition, it has been reported that the Cpn60.2 protein of *M. bovis* BCG and *E. coli* GroEL bind non-covalently to fatty acids and methylglycosides (De Bruyn et al., 2000). This ability of bacterial Cpn60 proteins to bind to constituents of the cell surface of human cells may go some way towards explaining why these proteins are also able to act as intercellular signalling molecules.

The second reported moonlighting activity of Cpn60 was that the *M. tuberculosis* Cpn60.2 protein (also known as Hsp65) stimulated human monocytes to synthesise pro-inflammatory cytokines (Friedland et al., 1993). This was the first intimation that the Cpn60 protein could act as an intercellular signalling molecule. Twenty years have now elapsed since these primary reports and it is now established that the Cpn60 protein from bacteria and metazoans has an extremely wide range of moonlighting biological activities (described fully in Table 3). These biological actions can be roughly divided into: (i) intracellular functions not related to protein folding with, in eukaryotes, the Cpn60 protein often being found outside of the mitochondrion; (ii) cell surface localisation associated with ligand binding with the Cpn60 acting as a receptor; (iii) ligand binding, non-receptor, interactions
inside or outside of cells and; (iv) secreted Cpn60 functioning as an intercellular signalling molecule with a wide variety of cells. Only a small number of Cpn60 proteins have been examined and, surprisingly, not all exhibit the same moonlighting activities. It is important to define which Cpn60 proteins produce a specific biological activity and which do not. So in Table 3, where a Cpn60 protein has been found not to produce the activity of another homologue/paralogue, this fact is noted by highlighting the species name. To aid the reader, Figure 3 provides a diagrammatic overview of the multiple moonlighting activities of Cpn60 proteins.

As can be seen in Table 3 there is, at the time of writing, a very large number of distinct moonlighting functions ascribed to the Cpn60 protein. Some of this work has been reviewed in recent articles (Henderson, Lund & Coates, 2010; Henderson & Martin, 2011a; Henderson & Martin, 2011b; Henderson & Pockley, 2010) and only the highlights of Cpn60 moonlighting activity will be described here. These will be divided between moonlighting functions in bacteria and in mammals. Note that in eukaryotes, the type I chaperonins are found in the mitochondria and chloroplast, both of which are of bacterial origin (α-proteobacteria and cyanobacteria) respectively. Thus these proteins are evolutionarily linked although the gene(s) encoding eukaryotic type I Cpn60 proteins are now found in the cell nucleus.

IV(1). Selected Moonlighting Actions of Bacterial Cpn60 Proteins

Tables 2 and 3 reveal the growing number of bacteria which utilise the Cpn60 protein, largely for interactions with the host species. Most attention has focused on the causative agent of tuberculosis, *Mycobacterium tuberculosis*, an organism with two Cpn60 proteins (Cpn60.1 and 60.2). Tuberculosis can be thought of as a disease of the macrophage (Pieters, 2008) with the infection of these cells by *M. tuberculosis* resulting in the generation of a selective inflammatory pathological state in which the bacteria are walled off in structures called granulomas. These are characterised by the presence of unusual subpopulations of macrophages, and in particular the multinucleate giant cell (Russell, 2007). As stated, the initial report that Cpn60 could signal to macrophages used the Cpn60.2 (Hsp65) protein of *M. tuberculosis*. The finding that this protein could stimulate macrophage pro-inflammatory cytokine synthesis, and later studies showing that the human protein acted via TLR4 (which is also the lipopolysaccharide (LPS) receptor), has resulted in some controversy about the
immune cell stimulating actions of Cpn60 proteins. The main claim is that such activity is due to contamination of the Cpn60 with LPS from the *E. coli* strain used to express these proteins (Tsan & Gao, 2009). However, the next paper to report on the influence of *M. tuberculosis* Cpn60.2 on human monocytes revealed that, while this protein did stimulate these cells to produce pro-inflammatory cytokines, it did not induce other signs of macrophage activation (such as would be induced by LPS). Macrophage activation, or as it is now termed, classical macrophage activation, is an essential process defending vertebrates against microbial attack. It is induced by microbial pathogen-associated molecular patterns (PAMPs), bacterial components such as LPS and peptidoglycan, and by the host cytokine, gamma-interferon (γ-IFN). These produce changes in the macrophage such as enhanced expression of MHC class II proteins and activation markers such as CD80 (required for antigen presentation) and Fc receptors, plus increased production of free radicals for killing microbes (Martinez, Helming & Gordon, 2009). Exposure of human monocytes to LPS and/or γ-IFN, induces these cellular changes, but exposure to *M. tuberculosis* Cpn60.2 only caused cytokine synthesis (Peetermans *et al.*, 1994). Another paper from this same group showed that *M. tuberculosis* Cpn60.2 stimulated human vascular endothelial cells to produce the leukocyte adhesion proteins, ICAM and E-selectin, by a novel mechanism distinct from that of LPS and pro-inflammatory cytokines (Verdegaal, Zegveld & van Furth, 1996). Thus, this mycobacterial protein is a novel, so-called, alternative macrophage activator, inducing a particular profile of gene transcription which seems only able to cause the production of cytokines, without the other changes which would make macrophages pro-inflammatory and potentially dangerous.

The *cpn60.2* gene in *M. tuberculosis* is essential, so it is not possible to directly ascertain the importance of this protein for this bacterium. However, indirect evidence suggests the importance of this protein. This is based on the discovery that a mycobacterial predicted protease, Rv2224c, is responsible for the release of the Cpn60.2 protein. However, when the gene encoding this protease is inactivated, the bacterium has significantly lower survival rates both in macrophage cultures and within infected animals, where disease pathology is much reduced (Rengarajan *et al.*, 2008). This is not due to changes in intracellular Cpn60.2 levels. More recently it has been shown that the Cpn60.2 released by the bacterium binds to the bacterial cell surface where it functions as an adhesin allowing the organism to bind to macrophages, via the cell surface receptor, CD43. This results in the
internalisation of the bacterium and may be a key step in induction of tuberculosis (Hickey et al., 2009; Hickey et al., 2010).

Unexpectedly, the Cpn60.2 protein, which is 61% identical at the sequence level to the Cpn60.1 protein of this organism, shows significant differences from this Cpn60.1 paralogue in its moonlighting activity, being: (i) less potent as an activator of monocyte cytokine synthesis (Lewthwaite et al., 2001); (ii) failing to inhibit murine experimental asthma when administered to animals (Rha et al., 2002; Riffo-Vasquez et al., 2004) and; (iii) having no positive or negative effect on bone matrix breakdown (Meghji et al., 1997) or osteoclast formation (Winrow et al., 2008). This is likely to be explained by the fact that the M. tuberculosis Cpn60.2 and Cpn60.1 proteins fail to compete for binding to human monocytes, suggesting they bind to different receptors (Cehovin et al., 2010). However, the nature of the receptors for these two proteins still has to be defined.

The Cpn60.1 protein of M. tuberculosis, while it stimulates human monocyte cytokine synthesis, is a potent inhibitor of the process of bone resorption and of osteoclast formation both in vitro and in animals with adjuvant arthritis – a model in which massive osteoclast-induced bone remodelling occurs (Winrow et al., 2008). Osteoclasts are multinucleate cells which are involved in the normal process of skeletal remodelling by causing breakdown of the bone matrix (Edwards & Mundy, 2011). This is a curious finding as the Cpn60 proteins of Aggregatibacter actinomycetemcomitans, E. coli (Kirby et al., 1995; Reddi et al., 1998) and Homo sapiens (Meghji et al., 2003) are potent stimulators of bone matrix breakdown and osteoclast growth. The osteoclast-inhibitory action of M. tuberculosis Cpn60.1 is, at least in part, due to its ability to inhibit the transcription of the key osteoclast transcription factor - NFATc1 (Winrow et al., 2008). What would be the evolutionary pressure to enable the M. tuberculosis Cpn60.1 protein to evolve into an inhibitor of osteoclast formation? The answer probably lies in the response of the virulent strain of M. tuberculosis (H37Rv) to the inactivation of the chaperonin genes. Attempts were made to inactivate both cpn60 genes and the cpn10 gene. Only the cpn60.1 gene could be inactivated, suggesting that the other two gene products are essential for bacterial survival. The cpn60.1 isogenic mutant behaved normally in culture and when grown within macrophages (Hu et al., 2008). It was only when this mutant was used to infect animals that a phenotype emerged. While growing at the same rate as the wild type
and complemented strains, the $\Delta cpn60.1$ mutant failed to induce a local granulomatous response, suggesting that this chaperonin was driving the process of granuloma formation (Hu et al., 2008). This was confirmed when it was shown that the $\Delta cpn60.1$ mutant failed to induce the formation of multinucleate giant cells in a human whole blood granuloma assay (Cehovin et al., 2010). Moreover, when used to complement an $E. coli$ groESL mutant, the $cpn60.1$ gene failed to complement while the $cpn10$ and $cpn60.2$ genes could replace the $E. coli$ homologues (Hu et al., 2008). Thus it can be concluded that the $cpn60.1$ gene product in virulent $M. tuberculosis$ is an important virulence factor causing granulomatous inflammation, but that it has probably evolved away from being a molecular chaperone (Henderson et al., 2010). It is assumed that this promotion of pathological giant cell formation is somehow linked to the ability of this Cpn60.1 protein to inhibit the formation of the normal multinucleate myeloid cell population of bone – the osteoclast (Winrow et al., 2008).

In addition to inhibiting bone breakdown in rats, administration of the $M. tuberculosis$ Cpn60.1 protein can block experimental allergic asthma in mice. In contrast, the $M. tuberculosis$ Cpn60.2 protein is inactive in this model (Riffo-Vasquez et al., 2004). Surprisingly, the $M. leprae$ Cpn60.2 protein is a potent inhibitor of this experimental asthma lesion (Rha et al., 2002). Now these two mycobacterial Cpn60.2 proteins have >95% sequence identity, revealing that relatively small evolutionary changes in the Cpn60 protein can introduce novel biological actions.

Other moonlighting actions of the mycobacterial Cpn60 proteins include: (i) protease activity of $M. leprae$ Cpn60.2 (Portaro et al., 2002); (ii) the binding of $M. tuberculosis$ Cpn60.1 to single stranded DNA (Basu et al., 2009) and (iii) the ability of $M. smegmatis$ Cpn60.1 to promote biofilm formation (Ojha et al., 2005). However, the Cpn60.1 protein of $M. tuberculosis$ is not required for biofilm formation (Hu et al., 2008).

As has been described, the Chlamydiae have three Cpn60 proteins. Of these, only Cpn60.1 has been studied in any detail and been shown to have a growing number of functions (see Table 3 for references). These include the ability to promote monocytes and macrophages to produce cytokines and metalloproteinases and to induce the maturation of dendritic cells. In addition, this protein also has effects on vascular endothelial cells including stimulation of proliferation of these cells, inhibition of their ability to produce nitric oxide (NO) and the induction of Lox-1. This is one of the few Cpn60 proteins to have been administered to normal animals and it generally appears to induce a pro-
inflammatory response. In addition to these effects on leukocytes and endothelial cells, the C. pneumoniae Cpn60.1 protein also functions as a cell adhesin and is capable of promoting the oxidation of low density lipoprotein (LDL). Note that the Cpn60.2 and Cpn60.3 proteins (which do not appear to function as a molecular chaperone) do not exhibit adhesive activity (Karunakaran et al., 2003).

The Helicobacter pylori Cpn60 protein also shows the ability to promote epithelial cells and myeloid cells to generate pro-inflammatory cytokines (Table 3) including IL-6 and IL-8 which are believed to be important in the process of H. pylori-induced gastritis (Sugimoto, Yamaoka & Furuta, 2010). In addition to acting as a stimulating agonist for myeloid cells, the H. pylori Cpn60 protein is the first to be shown to potentially regulate the bacterium. This is inferred from the ability of an antibody to the H. pylori Cpn60 protein being able to inhibit the growth of the bacterium (Yamaguchi et al., 1997). It is often suggested that Cpn60 could be a useful vaccine candidate for bacterial infection. However, antibodies raised in patients and animals to H. pylori Cpn60 actually accentuate the pro-inflammatory activity of this protein (Liao et al., 2011).

One of the most curious biological actions reported for a bacterial Cpn60 protein is the ability of the Cpn60 protein of Legionella pneumophila (a cell-invasive bacterium), when attached to beads taken up by epithelial cells or macrophages, to attract mitochondria and to modulate the cell's actin cytoskeleton. This activity was suggested to be specific for this one Cpn60 protein as E. coli GroEL could not replicate the effect (Chong et al., 2009), however as no other Cpn60 protein was tested this conclusion is still speculation. This Legionella Cpn60 protein is present on the bacterial surface and is also required for the invasion of the bacterium into non-phagocytic cells (Garduño et al., 1998).

The most unexpected action of a bacterial Cpn60 protein is its ability to function as an insect toxin. Surprisingly then, two different bacteria have evolved to use this protein in this manner. The antlion or doodlebug is a larval insect form that feeds on other insects and paralyses them with a neurotoxin. The neurotoxin is produced by a symbiotic bacterium, Enterobacter aerogenes, which lives within the saliva of this larva and is, in fact, the Cpn60 protein of this bacterium. The sequence of the A. aerogenes Cpn60 is virtually identical to that of E. coli GroEL. Unexpectedly, it was found that mutations of single residues in GroEL, which itself had no neurotoxic activity, could turn it into a potent insect neurotoxin. Among the mutants made in this study was mutation of a surface exposed
residue Thr347 which did not confer neurotoxic activity on GroEL (see next section) (Yoshida et al., 2001). Another member of the enterobacteriaceae is Xenorhabdus nematophila, a virulent insect pathogen. This bacterium also secretes a Cpn60 protein with insecticidal activity. Structure:function studies suggest that all three domains of the protein are needed for insecticidal activity and that this can be blocked by N-acetylglucosamine and chito-oligosaccharides. Generation of protein mutants identified the surface-exposed residues Thr347 (which, as stated above, was not involved in the neurotoxicity of GroEL) and Ser356 as essential for binding to the target insect gut epithelium and for insecticidal activity (Joshi et al., 2008). Clearly, these two Cpn60 proteins, turned insect toxins, are dramatically different in their structure:function relationships. However, nothing is yet known of the mechanism through which Cpn60 exerts its insect neuro-toxic and toxic effects. Yet again, this exemplifies the enormous variation that can occur in moonlighting proteins that have evolved to have similar moonlighting functions.

Another proposed role for bacterial chaperonins in insects is the binding of the Cpn60 proteins of insect bacterial endosymbionts with plant viruses to transmit these viruses to plants. Only one example of Cpn60/viral interaction will be described. Aphids are involved in the transmission of the potato leafroll virus (PLRV) which mainly replicates in the plant phloem (Sylvester, 1980). This virus is maintained in a persistent and circulative manner in the insect haemolymph and the virions are transported through epithelial cells lining the gut and salivary gland (Gildow, 1987). In the haemolymph, which acts as a viral reservoir, the virus is found in combination with the Cpn60 protein of an endosymbiotic Buchnera bacterial species – such interaction being required for the persistence of the virus in the aphid (van den Heuvel et al., 1997). The interaction between this virus and the bacterial Cpn60 involves the N-terminal segment of the so-called readthrough domain of the minor capsid protein of the virus which binds to the equatorial domain of the Cpn60 protein (Gildow, 1987; Hogenhout et al., 1998).

All the above examples have involved bacterial Cpn60 proteins interacting with host cells or tissues. There is one example of bacteria-bacteria interactions via Cpn60. This is the ability of the Cpn60 protein of Lactobacillus johnsonii to aggregate Helicobacter pylori, but, surprisingly, not other intestinal bacteria (Bergonzelli et al., 2006). This activity could have therapeutic potential.
IV (2). Selected Moonlighting Actions of Eukaryotic Cpn60 Proteins

This section deals with eukaryotes, and some workers use the terms chaperonin 60 to describe the eukaryotic/archaeal type II chaperonins. It must be emphasised that our use of the term Cpn60 refers only to the type I chaperonin 60 protein. It is also common to use the term Hsp60 (or more recently HSPD1 (Kampinga et al., 2009)) to refer to the human Cpn60 protein. However, to avoid confusion for readers not familiar with this field, the term Cpn60 will be used throughout.

Again, the details of the moonlighting actions of the Cpn60 proteins of the few eukaryotic species that have been studied are detailed in Table 3. Only a brief description will be provided of the key elements of the moonlighting actions of selected proteins. The fungus, *Histoplasma capsulatum* which causes histoplasmosis, an inflammatory disease generally affecting the lungs in immunocompromised individuals, has a Cpn60 protein which functions as a molecular chaperone (Guimarães et al., 2011b). It is also a fungal cell surface protein which recognises the integrin receptor CR3 (CD11b/CD18) on macrophages and uses this interaction to be internalised (Long et al., 2003). This protein is also an immunodominant antigen and antibodies raised to the fungal Cpn60 protein are protective in animals (Long et al., 2003) and these antibodies agglutinate the fungus and also modify macrophage functionality (Guimarães et al., 2011a). This raises the possibility that the *H. capsulatum* Cpn60 may be a vaccine candidate.

The remainder of this section will deal with multicellular organisms (metazoans). Previously, the moonlighting actions of the Cpn60 protein, with few exceptions, required that the chaperonin be on the bacterial surface or secreted from the bacterium. With multicellular organisms, there are significant reports of moonlighting actions of the intracellular protein. However, the Cpn60 protein may not necessarily be in its normal site – the mitochondrion. In the plants, the *Chlamydomonas reinhardtii* Cpn60 protein has been shown to also function as a Group II intron-specific RNA-binding protein (Balczun et al., 2006) and in *Arabidopsis thaliana*, both the Cpn60 α and β proteins have been reported to be necessary for plastid division (Suzuki et al., 2009). Mutants lacking the genes coding for these proteins had fewer and larger chloroplasts and it is not clear if this defect is due to interference with protein folding or is true moonlighting function of the chloroplast Cpn60 protein.

The most diverse multicellular organisms are the insects and two insects have already been described using bacterial Cpn60 proteins for their survival. Indeed, many insects have vertically
transmitted endosymbiotic bacteria which play major roles in reproduction, metabolism and defence (Gibson & Hunter, 2010) and it is suggested that the evolution of the \textit{cpn60} gene plays a role in such endosymbiosis (Fares, Moya & Barrio, 2005). Of note, a recent report suggests that interaction of the weevil antibacterial peptide coleoptericin A with its endosymbiotic bacterium (to regulate bacterial growth) requires the interaction with the bacterium’s Cpn60. However, this peptide did not bind to the host Cpn60 (Login \textit{et al.}, 2011). However, of the million or more species of insects on our planet only one has been examined in any detail for the role of the Cpn60 protein. This is the well studied organism, \textit{Drosophila melanogaster}. The finding that 30\% of bacteria can encode more than one Cpn60 protein has been addressed (Lund, 2009), suggesting that a similar number of eukaryotes may also require multiple \textit{cpn60} genes. \textit{Drosophila melanogaster} has four \textit{cpn60} genes which encode homologues which appear to have distinct tissue distribution and biological functions. The Cpn60A protein is expressed in nearly all cell types and is required for viability from early embryonic stages (Kozlova, Zhimulev & Kafatos, 1997). The Cpn60B protein has 60\% sequence identity with the originally identified Cpn60 gene (Hsp60A) gene of \textit{Drosophila} (Timakov & Zhang, 2001). This protein is found within the mitochondria and in mutants lacking the protein there is a failure to generate sperm (Timakov & Zhang, 2001). The Cpn60C gene appears to be involved in the development of the trachea of the adult insect, the tissue in which this protein is mainly found. The Cpn60C mutant which showed developmental problems with the trachea also had defective sperm generation (Sarkar & Lakhota, 2005). The Cpn60D protein is mainly associated with photoreceptor cells in developing eye discs. Of note, this protein is not found to associate with the mitochondria. The key role of this Cpn60D protein appears to be the control of caspase-mediated apoptosis (Arya & Lakhota, 2008). Thus the Cpn60 proteins of \textit{Drosophila} have a bewildering range of functions and much more research is required to determine the complete roles of these proteins and whether this multiple requirement for Cpn60 proteins is the usual situation in insects.

Turning to Cpn60 proteins in mammals, almost all of these studies have been done using the human protein. There are 22 Cpn60 pseudogenes in the human genome, as well as the coding \textit{HSPD1} (\textit{cpn60}) gene. Of interest, it is proposed that HSPD1-5P and -6P, although seemingly pseudogenes, are expressed and may be functional (Mukherjee \textit{et al.}, 2010). However, this hypothesis has not been directly tested.
The moonlighting functions of human Cpn60 can be divided into: (i) those that occur within the cell; (ii) those that occur when the Cpn60 protein is attached to the plasma membrane or when the protein acts as a ligand/receptor for other molecules and (iii) the exogenous intercellular signalling actions of the secreted protein.

Within the cell, one of the most interesting actions of the human Cpn60 (Hsp60) protein is its role in controlling apoptosis. This was first identified when caspase-3 was found to form a complex with Cpn10 and Cpn60 in the Jurkat T cell line (Samali et al., 1999) and it was proposed that the maturation of the caspase 3 was due to the Cpn60 acting as a molecular chaperone (Xanthoudakis et al., 1999). However, it was found that apoptotic murine lymphoma cells expressed Cpn60 on their cell surfaces (Sapozhnikov et al., 1999) and, with tumour cells, apoptosis also resulted in surface expression of Cpn60 and the induction of anti-tumour immune responses (Feng et al., 2001). Thus the role of Cpn60 in the apoptotic process is more complex than was thought. To confuse matters, over-expression of Cpn60 in cardiac myocytes was found to inhibit apoptosis, leading to questions being raised as to the exact role of this protein in cell survival (Lin et al., 2001). Since these early studies, the literature has remained confusing, with data on tumour cells and non-tumour cells being equally conflicting, and with mammalian Cpn60 being reported to be pro- and anti-apoptotic and involved with increased cancer survival or poorer prognosis of this disease. In one study, where several apoptotic cell systems were analysed, significant heterogeneity in the cell's ability to handle Cpn60 was observed, which may go some way to explain the confusion in the literature (Chandra, Choy & Tang, 2007). The key finding was that in each apoptotic system used, Cpn60 appeared in the cell cytosol. However, this appearance of cytosolic Cpn60 can come about by two distinct mechanisms. With certain apoptotic stimuli, the cytosolic Cpn60 emanates from the mitochondria. With other apoptotic stimuli the cytosolic Cpn60 does not appear to come from the mitochondria (Chandra et al., 2007). To confuse matter further, other workers have claimed that Cpn60 interacts with Bax (Gupta & Knowlton, 2005), survivin and p53 (Ghosh et al., 2008) or cyclophilin D (Ghosh et al., 2010). The complexity of the interactions of Cpn60 with components involved in controlling apoptosis suggests that more is happening than mere protein folding and it is proposed that this key arena of cellular control is another moonlighting activity of the Cpn60 protein. In addition to endogenous Cpn60 modulating cellular apoptosis, there are reports of exogenous human Cpn60
both inhibiting B lymphocyte apoptosis (Cohen-Sfady et al., 2009) and stimulating cardiomyocyte apoptosis (Kim et al., 2009).

There are numerous reports of exogenous human or rodent Cpn60 proteins acting as potent signalling effectors with a range of leukocytes including neutrophils (Osterloh et al., 2009), monocytes (Kol et al., 2000), dendritic cells (Flohé et al., 2003), B lymphocytes (Cohen-Sfady et al., 2005; Cohen-Sfady et al., 2009; Cohen, 1992) and T lymphocytes (Osterloh et al., 2004; Zanin-Zhorov et al., 2005b). Again, the details of these biological activities are provided in abbreviated form in Table 3. One peculiar finding is that bacterial Cpn60 proteins, although they can interact and activate human and mouse monocytes, they appear unable to interact with B or T lymphocytes. The latter is purely based on lack of papers on this subject but it has been shown that the E. coli Cpn60 (GroEL) and M. tuberculosis Cpn60.2 proteins do not activate naive murine B cells, while the human protein does (Cohen-Sfady et al., 2005). Other cell populations are also targets for human and rodent Hsp60 signalling including adipocytes (Gülden et al., 2009; Gülden et al., 2008), osteoblasts (Koh et al., 2009), cardiomyocytes (Kim et al., 2009), vascular endothelial cells (de Graaf et al., 2006) and so on. There is too much information in this growing literature on the cell signalling activity of the human Cpn60 protein to cover it in any detail.

In addition to the literature on the signalling actions of the human Cpn60 protein, there are also individual reports of this protein acting as a binding ligand for a variety of components. Thus human Cpn60 has been reported to bind the HIV glycoprotein gp41 (Speth et al., 1999) and the Gram-negative bacterial pro-inflammatory component, LPS (Habich et al., 2005). The human Cpn60 protein is also found on the surface of various cells (Lin et al., 2007; Pfister et al., 2005; Piselli et al., 2000; Soltys & Gupta, 1997). There are now a number of reports that cell surface Cpn60 on human cells can act as a receptor for various ligands. These include high density lipoprotein (Bocharov et al., 2000) and the acetaldehyde alcohol dehydrogenase present on the surface of the bacterial pathogen, Listeria monocytogenes (Kim et al., 2006; Koo, Aroonnual & Bhunia, 2011; Wampler et al., 2004). This is an interesting story of the evolution of the interaction of two moonlighting proteins. One is the so-called Listeria-adhesion protein (LAP) (Jaradat, Wampler & Bhunia, 2003) which was shown to be important in the binding of this bacterium to intestinal epithelial cells. The LAP was found to be a moonlighting metabolic protein – acetaldehyde alcohol dehydrogenase (Kim et al.,
The host cell surface receptor for LAP turned out to be the human Cpn60 protein (Wampler et al., 2004). Measurement of the binding of LAP with human Cpn60, using surface plasmon resonance, revealed a Kd value in the low nanomolar range, which is a respectable binding affinity (Kim et al., 2006). The binding site in the LAP for Cpn60 is the N-terminal domain Gly_{224}^-Gly_{411} and the Kd of binding of this domain is 9.5nM (Jagadeesan et al., 2011), revealing that moonlighting interactions can be of high affinity. This resembles the situation, described earlier, where the eukaryotic moonlighting protein, PGI, binds to another moonlighting protein acting as a receptor, ubiquitin ligase. Analysis of LAP/alcohol acetaldehyde dehydrogenase binding in non-pathogenic strains of *Listeria* has found that while these strains produce this enzyme there is very little of it on the bacterial surface and so only pathogenic strains bind to target cells via LAP/Cpn60 interactions (Jagadeesan et al., 2010). As human Cpn60 is a stress protein, the role of cell stress in *Listeria* infection has been examined. Thus exposure of CaCo-2 cells, used for infection assays, to various stressors increased intracellular Hsp60 levels and enhanced the adhesion, but not invasion, of *L. monocytogenes*. Knock-down of Hsp60 with inhibitory RNA reduced the adhesion and translocation of wild-type *L. monocytogenes*, but a lap mutant showed unchanged adhesion. Overexpression of Hsp60 enhanced wild type adhesion and cellular translocation but there was no change in the lap mutant. Of importance, infection with *L. monocytogenes* increased plasma membrane expression of Hsp60. Thus there is a dynamic response between these two moonlighting proteins to enhance *L. monocytogenes* infection (Burkholder & Bhunia, 2010).

A final moonlighting activity of mammalian Cpn60 will be provided to reveal how complex the evolution of moonlighting is. It is obvious that mammalian survival depends on having functional sperm. To become functional, sperm undergo marked changes after ejaculation called capacitation. It turns out that capacitation in the mouse requires the participation of a cell surface located Cpn60 (which undergoes tyrosine phosphorylation - (Asquith et al., 2004)) plus the potential involvement of Cpn10 (Walsh et al., 2008). Given the essentiality of this capacitation process, it would have been assumed that all mammals would employ the same mechanism. However, it turns out that human sperm do not have Cpn60 on their cell surface and there is no evidence of cell surface tyrosine phosphorylation (Mitchell, Nixon & Aitken, 2007). Mice and the precursors of *Homo sapiens* diverged about 75 million years ago (Stillman & Stewart, 2004). This suggests that the line resulting in *Homo*
*sapiens* lost this particular Cpn60 moonlighting site, and its associated mechanisms, over this period, revealing a fairly rapid evolutionary dynamic in the gene(s) encoding the mammalian Cpn60 protein. In addition to the brief overview of the literature on the moonlighting actions of the human Cpn60 protein, it is important to realise that in about 50-60% of the human population it is possible to find *intact* Cpn60 in the circulation. Levels of this protein are generally measured by immunoassay and the concentration ranges from nanograms of protein per ml to tens or even hundreds of micrograms per ml of plasma (Lewthwaite *et al.*, 2002; Pockley *et al.*, 1999). Given that human Cpn60 can act as a cellular ligand at high ng/ml to low microgram/ml concentrations it is likely that there is a biological consequence to this protein being in the blood. Indeed, levels of human Hsp60 in blood have been found to correlate with measures of pathology, particularly cardiovascular pathology (Pockley *et al.*, 2000; Shamaei-Tousi *et al.*, 2007; Xu *et al.*, 2000). What controls the levels of circulating Cpn60 in humans, and why a proportion of individuals have no measurable Cpn60 in their blood is not understood.

This brief description of the moonlighting activity of eukaryotic Cpn60 proteins along with the earlier description of the moonlighting actions of secreted bacterial Cpn60 proteins, all of which are summarised in Table 3, establishes the influence evolution has had on the development of moonlighting functions in this family of proteins. The question that now needs to be addressed is – how do these various moonlighting actions of the Cpn60 protein family relate to what is thought to be the primary function of this protein – protein folding.

**V. Is Cpn60 Moonlighting Activity Due to Specific Motifs/Domains in the Protein?**

For the folding activity of Cpn60, or at least the *E. coli* GroEL oligomer, the binding site in the chaperone for client proteins (as assessed by a strong binding peptide) involves the helices H and I of the apical domain and the residues R231-T261 (Chen & Sigler, 1999). The other major controlling and highly conserved region important for protein folding is the nucleotide binding site. Conserved residues important in ATP binding include G32 [E], D87[E], T91[E], I150[A], D398[I], G415[E], N479[E], A480[E] and D495[E] (Brocchieri & Karlin, 2000; Xu & Sigler, 1998) where [A] is apical, [E] is equatorial and [I] is the intermediate domain.
What is known about the sequence:structure:function relationships of the Cpn60 molecule as a moonlighting protein? The first evidence for moonlighting sites in the Cpn60 protein came from studies of the human protein where it was shown that a Hsp60 peptide, designated p277, and in fact the equatorial domain peptide 437-460 (VLGGCALLRCIPALDSLTPANED) was a T cell modulator/antigen able to inhibit diabetes in non-obese diabetic (NOD) mice (Elias & Cohen, 1994). It was subsequently shown that in addition to being a T cell antigen, the P277 peptide could also directly signal to T lymphocytes and inhibit various aspects of the functionality of these cells (Nussbaum et al., 2006; Zanin-Zhorov et al., 2005a; Zanin-Zhorov et al., 2005b) (Table 3)). This peptide is now in phase III clinical trial for the treatment of early-onset diabetes and seems to be proving clinically effective (Tuccinardi et al., 2011). Of significance, it has been shown that this particular peptide motif (437-460) in human Cpn60 has no influence on monocytes and is, presumably, only able to bind to, and modify, lymphocyte responses (Nussbaum et al., 2006). The individual moonlighting sites in the Cpn60 protein family are shown mapped onto the *E. coli* GroEL sequence (Fig 4).

One of the bacterial Cpn60 proteins shown to stimulate human monocyte cytokine synthesis is *E. coli* GroEL (Tabona et al., 1998). For these studies use was made of a highly purified LPS-low *E.coli* expressed recombinant chaperonin in which any proteins (or other contaminants) associated with the GroEL were removed by passing the chaperone through a Reactive red column. As a control for LPS contamination, the protein was trypsinised. This is a common control in protein activity assays and it was expected that trypsinisation would completely inactivate the protein. However, the tryptic peptides still elicited significant monocyte-stimulating activity (Tabona et al., 1998). This suggested that one or more tryptic peptide contained the majority of the monocyte-activating activity and the 65 tryptic peptides produced were isolated by HPLC and tested for activity and the sequence of each peptide was determined by mass spectrometry. This revealed that there were four peptides with activity in the equatorial or intermediate domain. One of these peptides (aa471-498) overlapped with the monocyte-interacting sites aa481-500 of the human Cpn60 protein and aa460-491 of the *M. tuberculosis* Cpn60.1 protein (Henderson, unpublished data). Subsequent studies showed that proteinase K treatment of GroEL, and of other Cpn60 proteins, (which breaks the proteins into much
smaller peptides) totally inhibited their ability to stimulate monocyte cytokine synthesis (e.g. (Tormay et al., 2005); Henderson, unpublished).

A number of predicted T cell antigenic peptides in *M. tuberculosis* Cpn60.1 were tested for their ability to stimulate human monocyte cytokine synthesis. Only peptide 195-219 [A] (KGFLASYVFTFDNQQAVLEDALIL) was active in this respect (Lewthwaite et al., 2001). The same peptides in *M. tuberculosis* Cpn60.2 (KGYISGYFVTDFPERQEAVLEDPYIL) and in GroEL (RGYLSPYFINKPETGAVELESPFIL) were inactive. Of interest, this monocyte stimulating activity of this Cpn60.1 peptide was inhibited by blocking antibodies to CD14 (Lewthwaite et al., 2001). Comparing these three sequences the most obvious difference is the presence of prolines in the inactive Cpn60.2 protein and in GroEL.

*M. tuberculosis* Cpn60.1 KGFLASYVFTFDNQQAVLEDALIL (active)
*M. tuberculosis* Cpn60.2 KGYISGYFVTDFPERQEAVLEDPYIL (inactive)
*E. coli* GroEL RGYLSPYFINKPETGAVELESPFIL (inactive)

Proline residues in the active sequence would cause alteration of the structure of the Cpn60.1 peptide potentially blocking activity. Although this data seems reasonable, there is one caveat to it. The intact *M. tuberculosis* Cpn60.1 protein stimulates purified human monocytes (which will contain some T lymphocytes) to generate a range of cytokines *but not interferon-gamma (IFN-γ)* – a T cell cytokine. However, this 195-219 [A] peptide promotes the formation of all of the cytokines generated by the parent protein but also stimulates IFN-γ synthesis, which the parent protein could not do (Lewthwaite et al., 2001). Thus it would appear that the activity of this peptide is masked in the intact protein, but it could become available if the protein was appropriately proteolysed. Modelling this peptide on the GroEL oligomer, revealed that most of its sequence would be buried in the folding cavity (Lewthwaite et al., 2001). This inappropriate activity of this Cpn60.1 peptide needs to be borne in mind when studies are made with synthetic peptides. Further study of the activity of the *M. tuberculosis* Cpn60.1 protein involved the generation of recombinant versions of the equatorial, intermediate and apical domains of this protein. Only the equatorial domain maintained the monocyte cytokine-inducing activity of the intact protein, revealing the cryptic nature of the above peptide, 195-219 [A], which is found in the intermediate domain (Tormay et al., 2005). Further
analysis of the monocyte cytokine activating binding site in the *M. tuberculosis* Cpn60,1 protein using C-terminal amino acid deletion mutation and peptide synthesis has identified this moonlighting site to be within the equatorial domain peptide 461-491 [E] (Hu et al submitted). Of interest is the fact that this abuts the putative T cell-activating moonlighting site - residues 437-460 (p277) of the human Cpn60 protein described earlier.

A number of endosymbiotic bacteria utilise their secreted Cpn60 proteins to interact with viruses as part of a mechanism of viral transmission. Of these bacteria, most attention has focused on *Buchnera* spp and it has been revealed that the interaction of the potato leafroll virus (PLRV) with this Cpn60 involves the equatorial domain (Hogenhout et al., 1998). *Buchnera* Cpn60 shares >92% amino acid sequence identity with the *E. coli* GroEL (Hogenhout et al., 1998) and GroEL also binds to this virus (Hogenhout et al, 1998). Mutational analysis has elicited the key sites on the equatorial domain responsible for viral binding as residues 9-19 [E] and 427-457 [E] (Hogenhout et al., 2000). The N-terminal residues form an alpha helix and a combination of overlapping peptides and mutant generation has identified that residues R13, K15, L17 and R18 are essential for PLRV binding to this segment of the equatorial domain, with these residues being clustered on the hydrophilic side of the proposed helix. Computer modelling and binding analysis suggests that the C-terminal binding site is probably a structural site comprising a helix composed of residues 431-459 [E] (Hogenhout et al., 2000).

The final bacterial Cpn60 proteins for which we have evidence of a moonlighting site are the proteins which elicit neurotoxicity or toxicity in insects. The first report of this was of the Cpn60 protein of *Enterobacter aerogenes* which functions as a neurotoxin. Mutating this protein and GroEL to look for loss and gain, respectively, of neurotoxic activity has identified residues, 100 [E], 101 [E], 338 [A] and 471 [E] as, individually, being responsible for this biological activity (Yoshida et al, 2001). These residues are on the surface of the protein, but apart from I100-T101, they do not seem to form a binding site. Thus it is not clear how these individual amino acids contribute to the insecticidal neurotoxicity of Cpn60. The second bacterium with an insecticidal Cpn60 protein is *Xenorhabdus nematophila* (Joshi et al., 2008). Cloning and expression of the *X. nematophila* Cpn60 protein and individual domains revealed that the apical domain retains about one third the insecticidal activity of the full length protein, and a combination of the apical and intermediate domain retained 50% activity
of the full length protein. The *E. coli* GroEL had no insecticidal activity. Sequence comparison between these two Cpn60 proteins revealed 59 substitutions – 21 in the apical domain, 6 in the intermediate and 32 in the equatorial domain. Attention was paid to surface-exposed polar residues in the *X. nematophila* protein and these were subject to alanine scanning mutagenesis. Many mutations had no effect, but T347A [A] and S356A [A] resulted in 30% and 50% loss of activity respectively. The double mutant T347A,S356A caused 80% loss of activity. There was no effect of the T347A mutation on binding to the target insect gut membrane, but the S356A mutation did abolish binding. Of interest, the binding site for this protein in the target insect gut appears to be chitin or to contain chitin (Joshi et al., 2008).

Returning to the human Cpn60 protein, and its moonlighting actions. Human Cpn60 binds to LPS and the binding site has been shown to be aa354-365 (with the motif LKGK being essential for binding) in the apical domain (Habich et al., 2005) which is an obvious site in this protein for an amphiphile to bind (see (Preuss, Hutchinson & Miller, 1999)). Christiane Habich's group have done the most work to identify the moonlighting site in human Cpn60 for the binding of this protein either to monocytes or to adipocytes. It should be noted that these studies rely on synthetic peptides which, as described earlier, have potential problems with specificity of binding. Initial studies of human Cpn60 binding to the murine monocyte cell line J774A.1 demonstrated that binding was independent of the presence of TLR4, although signalling to stimulate monocyte cytokine synthesis required the presence of TLR4 (Habich et al., 2002). This suggests TLR4 is not the primary receptor but it required for signal transduction. Competitive binding studies using a range of mammalian and bacterial Cpn60 proteins revealed that human, mouse and rat Cpn60 proteins compete, suggesting that they share a common binding site. Surprisingly, hamster Cpn60 did not compete. This is interesting as rats, mice and hamsters are all members of the superfamily, Muroidea, and will be more closely related than rats and mice are to humans. Indeed, the sequences of the human, mouse, rat and hamster Cpn60 proteins are >95% identical suggesting, if this is not simply a technical artefact, that very small sequence differences in Cpn60 proteins can have massive effects on moonlighting bioactivity. The same effect was discussed earlier with regard to the difference in activity of *M. tuberculosis* and *M. leprae* Cpn60.2 proteins in a murine model of asthma. Moreover, the Cpn60 proteins from *E. coli*, *C. pneumoniae* (presumably Cpn60.1) and *M. bovis* (presumably
Cpn60.2) also failed to block binding of human Cpn60 (Habich et al., 2003). A more detailed study of the ligand site in human Cpn60 for the macrophage J774A.1 cell surface receptor(s) used a series of overlapping 20-mer peptides which were used at a relatively high concentration to determine which peptides inhibited binding of Cpn60 to this mouse macrophage cell line. Only one peptide (aa481-500) significantly inhibited binding. Using overlapping 15-mers this inhibitory motif was further refined to residues 481-495. Modification of the 481-500 peptide at positions 490, 497 and 499 decreased the inhibition of human Cpn60 binding (Habich et al., 2004). Other studies, using N-terminal truncated human Cpn60 mutants and antibodies confirm that this 481-500 motif is the binding site in this protein for this macrophage cell line cell surface receptor (whatever this is) (Habich et al., 2004).

These findings used the mouse macrophage cell line J774A.1, which is often used in place of primary mouse macrophages, which are more difficult to obtain and maintain. Surprisingly, when these experiments were repeated with primary bone marrow cells from the C57BL/6J mouse, an entirely different set of results were obtained. The original results with J774A.1 cells identifying the 481-500 motif as the ligand binding site in human Cpn60 were re-confirmed, but with the primary mouse bone marrow cells the peptides inhibiting human Cpn60 binding were aa241-260 [A], aa391-410 [I] and aa461-480 [E]. Both aa241-260 and aa461-480 were significantly more inhibitory to human Cpn60 binding than the aa391-410 peptide. The aa461-480 peptide is contained within the active moonlighting site of the M. tuberculosis Cpn60.1 protein (461-490 - see above). Competition experiments with mouse, rat, hamster and Histoplasma capsulatum Cpn60 proteins revealed that all eukaryotic Cpn60 proteins inhibited binding of human Cpn60, but, again, the bacterial Cpn60 proteins did not. Again, this contradicts the findings with the J774A.1 cells, in which hamster Cpn60 failed to compete with human Cpn60. It is suggested, based on use of human Cpn60 N-terminal truncation mutants (Aa1-137, aa1-243 and aa1-359), that all three peptide motifs identified by competition experiments are required for the binding of human Cpn60 to primary murine macrophages. All three motifs are accessible when modelled onto GroEL (Habich et al., 2006). This study reveals the unexpected finding that the receptors for human Cpn60 on a macrophage cell line and on primary macrophages are distinct and, unfortunately, still undefined.
In more recent years Habich and co-workers have switched to studying the influence of human Cpn60 on murine adipocytes. These fat cells are now recognised to be important controllers of inflammation as well as body weight and the signals controlling their function need to be defined (Ouchi et al., 2011). Initial studies revealed that human Cpn60 induced a time- and concentration-dependent production of interleukin(IL)-6, the chemokine, CXCL1 and monocyte chemoattractant protein (MCP)-1 (now termed CCL2) from cells of the adipocyte line 3T3-L1 and from adipocytes of obese mice (Gulden et al, 2008, 2009). Analysis of the ligand binding site in human Cpn60 for adipocyte cell lines or primary cells has revealed yet another site in this protein for cell binding and, presumably, cell activation - namely aa1–50 [E] and aa91–110 [E]. These results were obtained by using overlapping peptides to compete with human Cpn60 and by showing that none of the human Cpn60 N-terminal truncation mutants were active. Both these regions are in the equatorial domain of GroEL (Gülden et al., 2009; Gülden et al., 2008; Märker et al., 2010).

It appears that evolution has allowed a range of peptide motifs in the Cpn60 protein to develop moonlighting activities. From the very limited information currently at our disposal, this seems to range from the N-terminus of the equatorial domain all the way through to the C-terminal segment of the equatorial domain and including the apical and intermediate domains. Interestingly, with the human Cpn60 and M. tuberculosis Cpn60.1 proteins their ability to activate myeloid cells involves the region 460-500 which includes key parts of the nucleotide binding domain. It is not known if residues such as N479, A480 and D495 in GroEL (important for nucleotide binding) are also involved in the moonlighting mechanism by which this protein can induce cell activation. More detailed analysis of the moonlighting sites in the Cpn60 protein superfamily is needed to fully understand how the moonlighting activity has evolved.

VI. Evolution of Functional Promiscuity in Cpn60

The question asked throughout this review has been - why is Cpn60 such a functionally promiscuous protein? There are two possible, non-mutually exclusive, hypotheses to explain the origin and preservation of promiscuity of Cpn60: (i) the various functions of Cpn60 in the cell were present at the time of its origination and; (ii) Cpn60 has undergone amino acid evolutionary changes that generated substantial leaps in protein functionality.
The first hypothesis, according to which the different Cpn60 functions were present at the time of its origin, is difficult to reconcile with Darwinian theory of evolution, as functions can only be preserved if these are constrained by natural selection. One possibility is that the different functions coded in Cpn60 overlap to such an extent that performing one or another function requires only subtle evolutionary change. For example, in such a conserved protein, small amino acid differences can switch the protein sequence from coding one function to coding another. Under this view, the fitness landscape of Cpn60 is smooth, with low-fitness valleys being rapidly crossed to climb one of the alternative adaptive peaks, each one favouring an alternative function (Figure 5A).

Importantly, in contrast to most proteins from other heat-shock families, Cpn60 is a general folding machine able to bind and fold a wide range of proteins, also known as clients. These range in size from between 20 kDa (below which proteins can fold spontaneously) to 80 kDa (above which proteins can hardly fit into the Cpn60 central cavity). This range includes most proteins with structural and regulatory functions and, consequently, Cpn60 is indirectly involved in a large set of different processes in the cell. Under this scenario, changes in the subset of client proteins could change the processes in which Cpn60 participates - that is, the apparent difference in the function of Cpn60 is a by-product of the difference in the set of clients with which it interacts. This scenario is also supported by the fact that bacteria have very plastic proteomes, with a significant proportion (up to 30%) of the protein-coding genes being horizontally transferred between species. The continuous horizontal gene transfer between bacteria would impose therefore a selective pressure to preserve Cpn60 functions, as these functions would be alternatively needed according to the set of invading genes. In support of this, the most dramatic changes in Cpn60 functions or protein structures occurred in organelles, such as mitochondria, and endo-cellular symbiotic bacteria, both of which are housed in cells that reduce their potential for interchanging genes with other bacteria. Cpn60 can also change the set of its clients by being horizontally transferred to other organisms. Horizontal transfer of Cpn60 is a likely event (Zauner et al., 2006), as many archaea has been found to have Cpn60 like proteins (Williams et al., 2010). Other chaperonins has been also reported to be horizontally transferred from an ancient archaeon to bacteria and have been named Group III chaperonins (Techtmann & Robb, 2010).

In concert with the second hypothesis, according to which subtle functional changes in Cpn60 are likely produced by substantial sequence variation, Cpn60 carries on functions in some bacteria that
are completely disengaged from its original folding purpose (for example, in pathogens, the expression of the different cpn60 gene copies varies along the different cycles of infection, hinting different roles for the different duplicates). In this case, leaping from one function to another would require more substantial amino acid changes than anticipated by gradual evolution - the adaptive landscape for the various functions would be much more abrupt or rugged (Figure 5B). Because Cpn60 is essential for cell viability, such punctuated amino acid changes would not be possible unless favoured by mechanisms of mutational robustness. One such mechanism is gene duplication, highly recurrent in the evolutionary history of Cpn60 (Lund, 2009). Previous theory predicts that after gene duplication, one of the gene copies, freed from selection pressures, can neutrally explore a wider spectrum of phenotypes while the other copy performs the ancestral function (Ohno, 1970). While non-functionalisation is the most likely fate for the freely evolving copy, owing to the stochastic nature of mutations, in a small proportion of cases mutations can form novel functions that can become fixed under particular ecological conditions. Indeed, preservation of additional Cpn60 copies resulting from gene duplication would make it possible to explore a wider spectrum of mutations. A change in the ecological conditions of bacteria – for example, the establishment of an infection or shifting to an intra-cellular lifestyle - may uncover phenotypic traits that were cryptic in the genetic background of the duplicated cpn60 of free-living bacteria but that became advantageous in the new intra-cellular environment. Because of the enormous variety of functions of cpn60, greater mutational robustness in this protein can ensure the establishment and persistence of novel ecological adaptations if the appropriate conditions are met. As a case in point, Cpn60 became duplicated twice at the origin of the Chlamydiae group of bacteria yielding two additional gene copies, cpn60.2 and cpn60.3 (Karunakaran et al, 2003). Interestingly, these copies are differently and independently expressed during the cycle of this pathogen: cpn60.2 is highly expressed during the infectious cycle of the Chlamydiae while cpn60.3 is highly expressed during persistent chlamydial infections (Gérard et al., 2004). The difference in the cpn60 gene expression levels at any point of the Chlamydiae life cycle suggests different roles for their coded proteins, despite the high sequence similarity among them. Indeed, evolutionary analyses on cpn60 gene copies put forward the conclusion that these genes diversified their functions after gene duplication (McNally & Fares, 2007).
VII. Conclusions

Type I chaperonin 60 is essential for the survival of virtually all bacteria, and all animals and plants and mutations in this protein are now being found to be deleterious in humans. Thus a V72I substitution results in a form of hereditary spastic paraplegia (Hansen et al., 2002) and a separate mutation, D29G causes another neurological condition, called Mit-CHAP60 disease (Magen et al., 2008). The significant conservation of the Cpn60 protein sequence has been recognised since the discovery of this protein as a molecular chaperone (Zeilstra-Ryalls, Fayet & Georgopoulos, 1991). It is this conservation of sequence which makes it so difficult to explain why this protein should have evolved so many moonlighting functions. This paradox is not explainable by gene duplication as the Chlamydiae paralogues do not exhibit the moonlighting actions of the parent gene product (Cpn60.1). So there appears to be an unknown mechanism working on certain genes to allow the evolution of additional biologically active sites. If this is true, then it certainly has been active with the Cpn60 protein which has developed a bewildering array of additional biological actions. This mechanism seems to work over fairly short time scales as judged by the plethora of distinct biological actions attributable to Cpn60 paralogues. Moreover, the loss of the role of Cpn60 in sperm capacitation between mouse and man also sets a limit to the time required for the de-evolution of this particular biological activity. It will require much bioinformatic and functional analysis of the Cpn60 protein to explain its massive propensity for moonlighting but understanding this mechanism could have potential for the development of novel biological activities in a world crying out for new therapeutic and other biological reagents.

Acknowledgements

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References


stimulator than chaperonin 60.2 (hsp 65) and contains a CD14-binding domain. *Infect Immun* **69**, 7349-55.


### Table 1. Oligomeric Structure of the Chaperonin 60 and Chaperonin 10 Proteins

<table>
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<th>Chaperonin</th>
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<th>Reference</th>
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<td>GroEL</td>
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<td>(Fukami et al., 2001)</td>
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<td>(Shimamura et al., 2004)</td>
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<td>(De Bruyn et al., 2000)</td>
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<td>(Qamra et al., 2004)</td>
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<td></td>
<td>(Tormay et al., 2005)</td>
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</tr>
<tr>
<td>Lactobacillus johnsonii</td>
<td>Cpn60</td>
<td>Mucin</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>Cpn60</td>
<td>?</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td>Cpn60</td>
<td>Yeast invertase</td>
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<tr>
<td>Legionella pneumophila</td>
<td>Cpn60</td>
<td>?</td>
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<tr>
<td>Leptospira interrogans</td>
<td>Cpn60</td>
<td>?</td>
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<tr>
<td>Mycobacterium avium</td>
<td>Cpn60</td>
<td>αβ3</td>
</tr>
<tr>
<td>Mycobacterium bovis BCG</td>
<td>Cpn60.1</td>
<td>DC-SIGN</td>
</tr>
<tr>
<td>Mycobacterium leprae</td>
<td>Cpn60.2</td>
<td>?</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>Cpn60.2</td>
<td>CD43</td>
</tr>
<tr>
<td>Mycobacterium smegmatitis</td>
<td>Cpn60</td>
<td>?</td>
</tr>
<tr>
<td>Plesiomonas shigelloides</td>
<td>Cpn60</td>
<td>?</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Cpn60</td>
<td>?</td>
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<tr>
<td>Salmonella typhimurium</td>
<td>Cpn60</td>
<td>Mucus</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>Cpn60</td>
<td>?</td>
</tr>
<tr>
<td>Streptococcus suis</td>
<td>Cpn60</td>
<td>?</td>
</tr>
</tbody>
</table>
## Table 3. Summary of the Moonlighting Actions of Chaperonin 60 Proteins

<table>
<thead>
<tr>
<th>Species</th>
<th>Chaperonin</th>
<th>Biological Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BACTERIA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Cpn60.2</td>
<td>Stimulates monocyte cytokine synthesis without classically activating the monocytes</td>
<td>Friedland et al 1993; Peetermans et al 1994</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.2</td>
<td>Stimulates vascular endothelia cell adhesion protein expression by novel mechanism not involving IL-1β or TNFα</td>
<td>Verdegaal et al 1996</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.2</td>
<td>Fails to stimulate bone breakdown or osteoclast formation</td>
<td>Kirby et al 1995; Meghi et al 1997</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.2</td>
<td>Binds to macrophage cell surface CD43 and acts as an adhesin to allow the bacterium to invade macrophages</td>
<td>Hickey et al 2009, 2010</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.2</td>
<td>Fails to stimulate bone breakdown or osteoclast formation</td>
<td>Rha et al 2002; Riffo-Vasquez et al 2004</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.1</td>
<td>More potent monocyte cytokine stimulator than Cpn60.2</td>
<td>Lewthwaite et al 2001</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.2</td>
<td>Fails to inhibit murine allergic arthritis</td>
<td>Riffo-Vasquez et al 2004</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.1</td>
<td>Potent inhibitor of murine allergic asthma</td>
<td>Winrow et al 2008</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.1</td>
<td>Inhibits human monocyte cytokine synthesis induced by PPD</td>
<td>Khan et al 2008</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.1</td>
<td>Inhibits leukocyte diapedesis in allergic lung model</td>
<td>Riffo-Vasquez et al 2012</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.1</td>
<td>Binding protein for single stranded, not double stranded DNA</td>
<td>Basu et al 2009</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>Cpn60.2</td>
<td>Protein has a protease active site capable of cleaving oligo-peptides</td>
<td>Portaro et al 2002</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>Cpn60.1</td>
<td>Essential for biofilm formation</td>
<td>Ojha et al 2005</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Cpn60.1</td>
<td>Not essential for biofilm formation</td>
<td>Hu et al 2008</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Cpn60.1</td>
<td>Changes in cell surface mycolic acids and less persistent in infected animals. Massive upregulation of secreted Cpn60.2</td>
<td>Wang et al 2011</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Cpn60</td>
<td>Stimulates monocyte cytokine synthesis</td>
<td>Tabona et al 1998</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Cpn60</td>
<td>Stimulates formation of osteoclasts and activates these cells</td>
<td>Reddi et al 1998</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Cpn60</td>
<td>Regulates error-prone DNA polymerase IV</td>
<td>Layton and Foster 2005</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Cpn60</td>
<td>Stimulates cytokine synthesis by HeLa cells transfected with CD36 or class B scavenger receptor types I and II</td>
<td>Baranova et al 2011</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>Cpn60</td>
<td>Stimulates bone breakdown <em>in vitro</em></td>
<td>Kirby et al 1995</td>
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<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>Cpn60</td>
<td>Promotes cell migration</td>
<td>Zhang et al 2004a</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>Cpn60</td>
<td>Long term exposure to this Cpn60 inhibits cultured epithelial cell viability</td>
<td>Zhang et al 2004b</td>
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<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>Cpn60</td>
<td>Inhibits epithelial cell apoptosis through activation of ERK and inhibition of caspase 3</td>
<td>Zhang et al 2004c</td>
</tr>
<tr>
<td><em>Rhizobium leguminosarum</em></td>
<td>Cpn60.1</td>
<td>Fails to stimulate human monocytes to synthesise cytokines</td>
<td>Lewthwaite et al 2002</td>
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<tr>
<td><em>R. leguminosarum</em></td>
<td>Cpn60.3</td>
<td>Stimulates human monocytes to synthesise cytokines</td>
<td>Lewthwaite et al 2002</td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td>Cpn60.1</td>
<td>Stimulates macrophage cytokine and metalloproteinase synthesis</td>
<td>Kol et al 1998</td>
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<tr>
<td><em>C. pneumoniae</em></td>
<td>Cpn60.1</td>
<td>Stimulates murine bone marrow-derived dendritic cell maturation</td>
<td>Costa et al 2002</td>
</tr>
<tr>
<td><em>C. pneumoniae</em></td>
<td>Cpn60.1</td>
<td>Stimulates human monocyte-derived dendritic cell maturation</td>
<td>Ausiello et al 2006</td>
</tr>
<tr>
<td><em>C. pneumoniae</em></td>
<td>Cpn60.1</td>
<td>Activates human vascular endothelial cells</td>
<td>Bulut et al 2002</td>
</tr>
</tbody>
</table>
C. pneumoniae Cpn60.1 Promotes the oxidation of low-density lipoprotein (LDL) Kalayoglu et al 2000
C. pneumoniae Cpn60.1 Stimulates proliferation of vascular smooth muscle cells Sasu et al 2001
C. pneumoniae Cpn60.1 Inhibits vascular endothelial cells NO synthesis Chen et al 2009
C. pneumoniae Cpn60.1 In vivo administration induces local accumulation of neutrophils Da Cost et al 2004
C. pneumoniae Cpn60.1 Local administration induces inflammation in the lungs Bulut et al 2009
C. pneumoniae Cpn60.1 Functions as a cell surface adhesin for binding to host cells Wuppermann et al 2008
C. pneumoniae Cpn60.2, Cpn60.3 Neither of these proteins functions as a cell adhesin Wuppermann et al 2008
C. trachomatis Cpn60.1 Stimulates Lox-1 synthesis in VECs and promotes atherogenesis Lin et al 2011
C. trachomatis Cpn60.2 Transcription responsive to low environmental iron concentration LaRue et al 2007
Helicobacter pylori Cpn60 Cpn60 cell surface located and antibodies to this protein inhibit growth of the bacterium Yamaguchi et al 1997
H. pylori Cpn60 Functions as cell surface adhesin for bacterial binding to host cells Kaniya et al 1998
H. pylori Cpn60 Recombinant protein stimulates monocyte cytokine synthesis via involvement of Toll-like receptor (TLR2) Takenaka et al 2004
H. pylori Cpn60 Non-recombinant Cpn60 stimulates monocyte cytokine synthesis with no involvement of TLR2 or TLR4 Gobert et al 2004
H. pylori Cpn60 Stimulates epithelial cell cytokine synthesis via TLR2 Zhao et al 2007
H. pylori Cpn60 Stimulates IL-8 synthesis by gastric epithelial cells Yamaguchi et al 1999
Legionella pneumophila Cpn60 Invasin protein which also functions to recruit mitochondria within invaded cells and modulate actin cytoskeleton Chong et al 2009
E. coli Cpn60 E. coli GroEL could not replicate the above effects of the L. pneumophila Cpn60 protein Chong et al 2009
Porphyromonas gingivalis Cpn60 Stimulates macrophage NF-κB Argueta et al 2006
Bartonella bacilliformis Cpn60 Promotes apoptosis of cultured vascular endothelial cells Smitherman and Minnick 2005
Lactobacillus johnsonii Cpn60 Stimulates epithelial cell pro-inflammatory cytokine synthesis Bergonzelli et al 2006
Lactobacillus johnsonii Cpn60 Aggregates Helicobacter pylori but not other intestinal bacteria Bergonzelli et al 2006
Helicobacter pylori Cpn60 Failed to aggregate H. pylori Bergonzelli et al 2006
Helicobacter pylori Cpn60 Protects mononuclear cells from apoptosis induced by dexamethasone Ortega-Ortega et al 2011
Francisella tularensis Cpn60 More active stimulator of monocyte cytokine synthesis than the LPS from this bacterium and synergises with this LPS to activate monocytes Noah et al 2010
Enterobacter aerogenes Cpn60 Potent insect neurotoxin Yoshida et al 2001
Xenorhabdus nematophila Cpn60 Insect toxin acting by binding to intestinal cells Joshi et al 2008
E. coli Cpn60 No activity as insect neurotoxin unless single residues mutated Yoshida et al 2001
Pea Aphid endosymbiont Cpn60 Functions as a histidine kinase Moricka et al 2004
Endosymbiotic bacteria Cpn60 Believed to be involved in the evolution of endosymbiosis in insects Fares et al 2005, Williams et al 2010
Buchnera sp Cpn60 Secreted Buchnera Cpn60 is required to stabilise potato leafroll virus in the insect haemolymph so allowing for viral transmission Hogenhout et al 1998
Endosymbiotic bacteria Cpn60 Involved in the transmission of various viruses from insects to plants due to ability of Cpn60 to bind various viruses Gottlieb et al 2010 Edelbaum et al 2009 Akad et al 2007 Banerjee et al 2004
Mollicutes Cpn60 A proportion of mycoplasmas do not have cpn60 genes. In those that have the Cpn60 protein appears to be a cell surface protein involved in adhesion of the bacteria to the cells of their host Clark and Tillier 2010
**Fungi**

Histoplasma capsulatum  Cpn60  Binds to CD11/CD18 on target cells and antibodies to this protein prolong survival of mice infected with this fungus  Long et al 2003

Saccharomyces cerevisiae  Cpn60  Mitochondrial DNA binding protein  Smiley et al 1992

E. coli  Cpn60  Not a mitochondrial DNA binding protein  Kaufman et al 2003

**Plants**

Chlamydomonas reinhardtii  Cpn60  Group II intron-specific RNA-binding protein  Balczun et al 2006

Arabidopsis thaliana  Cpn60 α and β  Required for plastid division  Suzuki et al 2009

**Insects**

Drosophila melanogaster  Cpn60A  Involved in embryogenesis  Baena-Lopez et al 2008

Drosophila melanogaster  Cpn60B  Only expressed in testis and responsible for spermatid individualisation process  Timakov and Zhang 2001

Drosophila melanogaster  Cpn60C  Responsible for development of insect trachea and for the fertility of Drosophila  Sarkar and Lakhotia 2005

Drosophila melanogaster  Cpn60C  Present in ovarioles where it associates with actin cytoskeleton And is associated with oogenesis  Sarkar and Lakhotia 2008

Drosophila meanogaster  Cpn60D  Essential factor for caspase-induced apoptosis  Arva and Lakhotia 2008

Drosophila melanogaster  Cpn607  Forms a complex with the insecticidal compound azadirachtin a tetranortriterpenoid  Robertson et al 2007

Bombus mori  Cpn60  N-terminus of Cpn60 binds to 14-3-3 proteins potentially important in the cellular function of this insect  Tabunoki et al 2008

**Mammals**

Endogenous Hsp60 with intracellular moonlighting functions

**Homo sapiens**  Hsp60(HSPD1)  Endogenous Hsp60 stimulates maturation of pro-caspase 3  Xanthoudakis et al 1999

**Homo sapiens**  Hsp60  VitD3-induced sensitivity of melanoma cells to NK cell killing is due to Hsp60-induced expression of Fas  Lee et al 2011

**Homo sapiens**  Hsp60  Endogenous Hsp60 activates hepatitis B virus polymerase and promotes virion growth by a specific pattern of binding  Park et al 2002

**Homo sapiens**  Hsp60  Endogenous Hsp60 forms association with cell surface αβ-integrin required for allowing integrin signalling  Barazi et al 2002

**Homo sapiens**  Hsp60  Cytosolic accumulation of endogenous Hsp60 in cells can have pro- or anti-apoptotic effects  Chandra et al 2007

**Homo sapiens**  Hsp60  Endogenous Hsp60 inhibits vascular endothelial cell apoptosis induced by digoxin through inhibition of caspase-3  Qiu et al 2008

**Homo sapiens**  Hsp60  Endogenous Hsp60 in tumour cells interacts with survivin and with p53 and inhibits tumour cell apoptosis  Ghosh et al 2008

**Homo sapiens**  Hsp60  Upregulation of intracellular Hsp60 results in interaction with B-catenin and enhanced propensity for cellular metastasis  Tsai et al 2009

**Homo sapiens**  Hsp60  Endogenous cytoplasmic Hsp60 stimulates NF-κB  Chun et al 2010

**Homo sapiens**  Hsp60  Binds to clusterin to form a complex which is tumour-suppressive  Chaiwatanasirikul & Sala 2011

**Homo sapiens**  Hsp60  Component of a mitochondrial membrane permeability transition pore in tumour but not normal cells  Ghosh et al 2010

**Homo sapiens**  Hsp60  Interaction of hepatitis C virus core protein with intracellular Hsp60 results in the production of oxygen-derived free radicals and enhances TNF-alpha-mediated apoptosis.  Kang et al 2009

**Homo sapiens**  Hsp60  Endogenous Hsp60 reported to be involved in intracellular cholesterol transport  Olvera-Sanchez et al 2011

**Homo sapiens**  Hsp60  Endogenous Hsp60 is a high affinity binding protein for the Immunosuppressant mizoribine  Itoh et al 1999
### Hsp60 as soluble or cell surface ligand

<table>
<thead>
<tr>
<th>Species</th>
<th>Hsp60</th>
<th>Exogenous Hsp60 binds to HIV glycoprotein gp41</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Cell surface Hsp60 binds to high density lipoprotein</td>
<td>Speth et al 1999</td>
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<tr>
<td>Mus musculus</td>
<td>Hsp60</td>
<td>Cell surface protein on sperm essential for capacitation</td>
<td>Asquith et al 2004</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Human sperm do not have cell surface Hsp60 and this protein is not required for human sperm capacitation</td>
<td>Mitchell et al 2007</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Binds to lipopolysaccharide through aa 354-365 with LKGK motif being critical for binding</td>
<td>Habich et al 2005</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Cell surface receptor on human cells for the acetaldehyde alcohol dehydrogenase present on the cell surface of Listeria monocytogenes</td>
<td>Koo et al 2011</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Present at cell surface in apoptotic cells and functions to enhance macrophage phagocytic activity</td>
<td>Goh et al 2011</td>
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</tbody>
</table>

### Hsp60 as Exogenous Cell Signalling Ligand with Myeloid, Lymphoid and Other Cells

#### Macrophage/Dendritit Cells

<table>
<thead>
<tr>
<th>Species</th>
<th>Hsp60</th>
<th>Exogenous Hsp60 stimulates human and mouse macrophages to produce NO, TNF, IL-12, IL-15</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 stimulates mouse macrophage cytokine synthesis</td>
<td>Ohashi et al 2000</td>
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<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 stimulates human monocyte cytokine synthesis</td>
<td>Kol et al 2000</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 activate the stress-activated protein kinases p38 and JNK1/2, the mitogen-activated protein kinases ERK1/2 and I-kappaB kinase after uptake into macrophages</td>
<td>Vabulas et al 2001</td>
</tr>
<tr>
<td>Homo sapiens and Mus musculus</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 proteins from both species stimulated myeloid cells but with different time course etc. Of note was the fact the mouse Hsp60 did not bind to CD14</td>
<td>Breloer et al 2002</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Analysis of binding of exogenous Hsp60 to murine macrophages has identified peptide regions aa241-260, aa391-410 and aa461-480 as being responsible for binding</td>
<td>Habich et al 2006</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 induces maturation of dendritic cells kinases ERK1/2, and the I-kappaB kinase (IKK) in monocytes.</td>
<td>Flohe et al 2003</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 induces self- tolerance in monocytes</td>
<td>Kilmartin and Reen 2004</td>
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<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 binds to TREM-2 on microglia</td>
<td>Stefano et al 2009</td>
</tr>
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</table>

#### B Lymphocytes

<table>
<thead>
<tr>
<th>Species</th>
<th>Hsp60</th>
<th>Exogenous Hsp60 inhibits B lymphocyte apoptosis</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 activates B cells via TLR4</td>
<td>Cohen-Sfady et al 2009</td>
</tr>
</tbody>
</table>

#### T Lymphocytes

<table>
<thead>
<tr>
<th>Species</th>
<th>Hsp60</th>
<th>Exogenous Hsp60 stimulates cytotoxic T lymphocytes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous LPS-free Hsp60 stimulates T lymphocytes</td>
<td>Osterloh et al 2004</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 stimulates T lymphocytes in a TLR4-independent manner</td>
<td>Osterloh et al 2008</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 has inhibitory effects on leukocytes and administration to Th1-mediated hepatitis model in mice blocks tissue pathology</td>
<td>Zanin-Zhorov et al 2005b</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 up-regulates suppressor of cytokine signalling (SOCS)3 expression via TLR2 and STAT3 activation in human T cells</td>
<td>Zanin-Zhorov et al 2003</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 functions as a co-stimulator of regulatory T lymphocytes</td>
<td>Zanin-Zhorov et al 2005a</td>
</tr>
</tbody>
</table>

#### Neutrophils

<table>
<thead>
<tr>
<th>Species</th>
<th>Hsp60</th>
<th>Exogenous Hsp60 promotes human and murine neutrophils to increase their anti-bacterial activities such as enhanced free radical formation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 induces maturation of dendritic cells kinases ERK1/2, and the I-kappaB kinase (IKK) in monocytes.</td>
<td>Flohe et al 2003</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 inhibits B lymphocyte apoptosis</td>
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</tr>
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<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 activates B cells via TLR4</td>
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</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 stimulates cytotoxic T lymphocytes</td>
<td>More et al 2001</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous LPS-free Hsp60 stimulates T lymphocytes</td>
<td>Osterloh et al 2004</td>
</tr>
<tr>
<td>Homo sapiens</td>
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<td>Homo sapiens</td>
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<td>Homo sapiens</td>
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<td>Homo sapiens</td>
<td>Hsp60</td>
<td>Exogenous Hsp60 promotes human and murine neutrophils to increase their anti-bacterial activities such as enhanced free radical formation</td>
<td>Osterloh et al 2009</td>
</tr>
</tbody>
</table>
**Other Cell Populations**

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Activity</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><em>Homo sapiens</em></td>
<td>Hsp60</td>
<td>Exogenous Hsp60 stimulates proliferation of vascular smooth muscle cells</td>
<td>de Graaf et al 2006</td>
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<tr>
<td><em>Homo sapiens and rattus rattus</em></td>
<td>Hsp60</td>
<td>Exogenous Hsp60 Induces TLR4-dependent apoptosis of cardiomyocytes</td>
<td>Kim et al 2009</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>Hsp60</td>
<td>Exogenous Hsp60 induced IRAK-1 in experimental myocardial ischaemia model in the mouse</td>
<td>Li et al 2011</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Hsp60</td>
<td>Exogenous (soluble) Hsp60 binds to ATP synthase (β-subunit) on vascular endothelial cells</td>
<td>Alard et al 2011</td>
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<tr>
<td><em>Homo sapiens</em></td>
<td>Hsp60</td>
<td>Exogenous Hsp60 stimulates apoptosis in osteoblasts</td>
<td>Kim et al 2009</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Hsp60</td>
<td>Exogenous Hsp60 stimulates ERK1/2 and IL-1β synthesis in an oral epithelial cell line</td>
<td>Pleguezuelos et al 2005</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Hsp60</td>
<td>Exogenous Hsp60 binds to murine adipocytes and stimulates production of pro-inflammatory cytokines. Residues aa1-50 and aa91-110 have been identified as being responsible for cell binding</td>
<td>Gulden et al 2008, 2009 Marker et al 2010</td>
</tr>
</tbody>
</table>

*Species names in bold denote Cpn60 proteins that fail to exhibit a biological activity that is possessed by a homologue/paralogue.
Figure 1. The oligomeric structure of *E. coli* GroEL (A) and, for comparison, the dimeric structure of the chaperonin 60.2 protein of *Mycobacterium tuberculosis* (B). In addition, this figures shows the domain structure of the individual Cpn60 monomer.
Figure 2. Comparison of the evolutionary changes in the structures of the three Cpn60 paralogues of the chlamydiae. The three-dimensional structure of one of the monomers of the *Escherichia coli* GroEL (PDB accession number: 1SS8) is shown. Sites under adaptive evolution and functional divergence are highlighted as space-filled structures. Yellow, red and blue spheres label sites under adaptive evolution and/or functional divergence in the ATP binding/hydrolysis sites, sites pointing to the central cavity of the homo-tetradecamer GroEL complex and sites involved in substrate binding, respectively (reproduced from McNally D and Fares MA (2007) *BMC Evol Biol* 7:81.)
Figure 3. A schematic diagram showing a number of the activities attributed to the Cpn60 protein when present: (i) intracellularly; (ii) on the cell surface and; (iii) after secretion from cells. A full list of the moonlighting properties of this protein is found in Table 3.
Enterobacter aerogenes

Xenorhabdus nematophilae

Buchnera aphidicola_YP_0045898

Homo_sapiens_Hsp60_NP_002147.2

M_tuberculosis_cpn602

M_tuberculosis_cpn601

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M_tuberculosis_cpn602

M_tuberculosis_cpn601

**Chaperonin**  | Moonlighting Function | Sequence Involved | Reference |
---|---|---|---|
**Human** | T cell immunomodulator | VLGGGCALLRCPALDSLTPANED (437-460) | Zanin-Zhorov et al (2005a,b) |
**Binding to lipopolysaccharide** | | DAMLLKGGKGDK (354-365) | Habich et al (2005) |
**Binding to cell surface of J77A.1 cells** | | KNAGVEGLKNGSSS (481-500) | Habich et al (2003) |
**Binding to mouse adipocytes** | | MRLPTVRFQMRPVSRVLAPHTRAYAKDVKGADARALMQGGGLADA (1-50) | Golden et al (2008/9) |
**Buchnera aphidicola** | Binding to potato leafroll virus | KFGNEARIKM (9-19) | Hogenhout et al (1998) |
**Xenorhabdus nematophilae** | Insecticidal toxin | T347 and T356 | Joshi et al (2008) |
**Enterobacter aerogenes** | Insecticidal neurotoxin | aa 100, 101, 336, 471 | Yoshida et al (2001) |
**M. tuberculosis Cpn60.1** | Monocyte/T cell activator | KOFLSAYFVTFDNQAOVLEDALIL (195-219) | Lewthwaite et al (2001) |
**M. tuberculosis Cpn60.1** | Monocyte activity modulation | DGSVVNVKSELPAGHGLNNTLSYGLDAAD (461-491) | Hu et al (submitted) |

Residues in **bold and blue** in the *E. aerogenes* sequence are the GDGTTT ATPase motif

Residues in **bold and red** are those in the apical domain that show no GroES binding or polypeptide folding in the Fenton et al paper.

Top five antigenic regions in Cpn60.1 as predicted by “antigenic” at [http://bips.unistra.fr/EMBOSS/](http://bips.unistra.fr/EMBOSS/) are shown in **bold, italics**.
Figure 5. Fitness landscape for Cpn60 functions. In this landscape, adaptive functions—that is, functions conferring high biological fitness—are represented by peaks while maladaptive functions are valleys. A ball, whose color is indicative of it conferring a fitness advantage or disadvantage, represents the function of cpn60 in the landscape. A) Smooth fitness landscape in which transition between alternative adaptive functions requires little changes at the molecular level; B) rugged landscape in which crossing valleys is precluded by the low fitness of the mutation-induced changes of cpn60 functions.