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SUMMARY
Molecular chaperones and protein folding catalysts are normally thought of as intracellular proteins involved in protein folding quality control. However, in the mycobacteria there is increasing evidence to support the hypothesis that molecular chaperones are also secreted intercellular signalling molecules or can control actions at the cell wall or indeed control the composition of the cell wall. The most recent evidence for protein moonlighting in the mycobacteria is the report that chaperonin 60.2 of Mycobacterium tuberculosis is important in the key event in tuberculosis — the entry of the bacterium into the macrophage. This brief overview highlights the potential importance of the moonlighting functions of molecular chaperones in the biology and pathobiology of the mycobacteria.

The recent report that the molecular chaperones, chaperonin (Cpn)60.2 (also known as Hsp65) and Hsp70 are found on the cell surface of virulent Mycobacterium tuberculosis and that the Cpn60.2 protein acts as a major adhesin for the binding of M. tuberculosis to monocytes adds further evidence for the hypothesis that the molecular chaperone/cell stress molecules of the mycobacteria are vital moonlighting proteins (Box 1) acting outside of the cell to promote bacterial survival and virulence. This extracellular functionality may synergise with the intracellular cell stress responsiveness of these molecular chaperones to generate a novel homeostatic network of interactions. These findings are occurring at the same time as questions grow over the function of the Hsp60 proteins of the mycobacteria, with one recent study suggesting that the second Cpn60 protein of M. tuberculosis (Cpn60.1) is not a molecular chaperone.2

The identification of antibodies in patients infected with a variety of different bacteria gave rise to the concept of the ‘common antigen’ which was shown to be homologous to the, then, recently discovered Escherichia coli, GroEL protein3 which was identified as a molecular chaperone, chaperonin (Cpn)60.4 Investigations of the M. tuberculosis Cpn60.2 (Hsp65) protein led to the discovery of its pivotal role in the control of the pathogenesis of adjuvant arthritis in the rat — a model of rheumatoid arthritis.5 This pioneering work has been responsible for our current understanding, such as it is, of the potent T cell immunomodulatory activity of M. tuberculosis Cpn60.2.6 Curiously, 20 years after this seminal study there are more publications on the role of M. tuberculosis Cpn60.2 in autoimmunity than on the use of this protein as a potential vaccine for tuberculosis.

What could account for the enormously potent immunomodulatory actions of M. tuberculosis Cpn60.2? The finding in 1993 that M. tuberculosis Cpn60.2 could stimulate human peripheral blood macrophages to secrete pro-inflammatory cytokines7 suggested that this protein could induce classical activation of macrophages leading to T and B cell activation. However, more detailed analysis of macrophages exposed to M. tuberculosis Cpn60.2 revealed that they did not have enhanced expression of Fcγ receptors, MHC class II proteins or produce more oxygen-derived free radicals, which are all markers of the classically activated macrophage state.8 Classical activation is induced by factors such as interferon (IFN)γ, which is a key factor in the host response to M. tuberculosis.9 However, it is now recognised that macrophages can be induced to enter other states which have been termed alternative macrophage activation states.9 Moreover, when M. tuberculosis Cpn60.2 was incubated with cultured human vascular endothelial cells, it induced the synthesis of E-selectin, ICAM-1 and VCAM-1 by a novel, cytokine-independent, mechanism.10 These early studies revealed novel interactions of this mycobacterial Cpn60.2 protein with macrophages and vascular endothelial cells which could have consequences for the pathology of tuberculosis. For example, the ability to stimulate macrophages into a non-classical activation state will prevent antigen-presentation and stimulation of antigen-specific CD4 T lymphocytes (with concomitant IFN-γ synthesis) — perhaps accounting for the ability of M. tuberculosis to survive within the macrophage. However, the work

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Box 1. Protein Moonlighting

Proteins are composed of approximately 20 amino acids. If life consisted of proteins with only 100 residues the potential number of protein combinations is, 20^{100}, which is equivalent to 10^{300}. In light of the immense potential the evolutionary process has for producing different proteins it is surprising to find that a growing number of proteins, in prokaryotes and eukaryotes, have more than one function. Such proteins are termed moonlighting proteins and it is possible for one protein to have a range of different functions. An interesting facet of these proteins is that a moonlighting protein in one organism may not be a moonlighting protein in another, suggesting that small variations in protein sequence are responsible for these moonlighting actions. Thus, it has been shown that the moonlighting functions of different proteins are not conserved between different yeast species. Another example is the essential role played by murine Cpn60 in the capacitation of sperm. However, human Cpn60 does not play any role in human sperm capacitation. In addition to the cell stress proteins, Mycobacterium tuberculosis has been shown to have other moonlighting proteins such as a glutamate racemase with DNA gyrase activity. The immunodominant secreted antigen85 proteins of M. tuberculosis turn out both to be fibronectin adhesins and mycosytransferases involved in mycolic acid synthesis. The M. tuberculosis malate synthase (the first enzyme of the glyoxylate cycle) has also been found to be surface located and bind to fibronectin and laminin. In keeping with the moonlighting functions of metabolic enzymes, the TCA cycle enzyme, aconitase also functions as an iron-responsive protein responsible for posttranscriptional regulation of the expression of proteins involved in iron homeostasis and the superoxide dismutase of Mycobacterium avium functions as an adhesin for this bacterium. The most recent example is the phosphodiesterase of M. tuberculosis which alters cell wall permeability. One recent hypothesis to explain protein moonlighting is that it is a facet of intrinsically disordered proteins. Is this hypothesis correct it may open up ways of predicting the ‘moonlight-ome’ of bacteria such as M. tuberculosis. was largely ignored, possibly because there was no evidence that Cpn60.2 could be secreted by M. tuberculosis.

This pioneering work resulted in chaperonins from other bacteria being examined for cell–cell signalling activity. One such study identified a potent bone resorbing protein from an oral bacterium involved in periodontitis as the Cpn60 protein of this organism. Curiously, while the Cpn60 protein from E. coli (GroEL) was also able to promote bone matrix breakdown, neither of the Cpn60.2 proteins from M. tuberculosis or Mycobacterium leprae were able to promote bone destruction. Given the marked sequence conservation of the chaperonins, this was a completely unexpected finding, but one that, as shall be seen, repeats itself again and again. Further analysis of recombinant GroEL revealed that this protein promoted bone resorption by stimulating the formation of the multinucleate myeloid-derived cell population known as the osteoclast, which is the cell responsible for homeostatic bone breakdown. It was, by this time, known that M. tuberculosis and M. leprae encoded two Cpn60 proteins (termed Cpn60.1 and Cpn60.2) and it was thought that the Cpn60.1 protein would have bone resorbing activity. However, recombinant M. tuberculosis Cpn60.1 was also shown to be unable to stimulate bone breakdown or osteoclast proliferation. In contrast, the Cpn10 protein of this organism had bone destructive activity in vitro. This inability of the M. tuberculosis Cpn60 proteins to promote bone breakdown proved to be even more unusual when it was found that human Cpn60 (Hsp60) was also a potent stimulator of the resorption of cultured bone explants. While both M. tuberculosis Cpn60 proteins showed similar inabilities to promote bone resorption and osteoclast proliferation, comparative analysis of these two very similar proteins (with 61% sequence identity) has begun to reveal significant differences in terms both of protein folding activity and extracellular signalling capacity. Direct comparison of the human monocyte cytokine-inducing ability of these two Cpn60 proteins showed that the recombinant Cpn60.1 protein was both more potent and more efficacious than the Cpn60.2 protein. The monocyte cytokine-inducing activity of Cpn60.1 could be blocked by a neutralising anti-CD14 monoclonal. This was not true of Cpn60.2. Now this neutralising activity does not mean that Cpn60.1 binds to CD14, but only that CD14 is part of some, as yet, undiscovered receptor complex through which Cpn60.1 signals. Affinity proteomic analysis of the human monocyte proteins that bound to M. tuberculosis Cpn60.1 failed to identify CD14 as a cognate ligand. A key question arose—what is the active Cpn60 protein—is it the oligomer, the monomer or does activity reside in some constituent subunit of the monomer. The three individual domains (equatorial, axial and apical) of M. tuberculosis Cpn60.1 were cloned and expressed and all monocyte cytokine stimulating activity was found to reside in the equatorial domain. The cell signalling activities of the M. tuberculosis Cpn60 proteins have been derived from in vitro experiments which might be viewed as artefactual. However, there is now evidence emerging for the moonlighting actions of these proteins being important in the living animal and for major differences to exist between Cpn60 proteins from different species. Thus administration of the Cpn60 proteins from M. tuberculosis, M. leprae, bacillus Calmette-Guérin (all Cpn60.2 proteins), Streptococcus pneumoniae, or Helicobacter pylori to mice with developing experimental allergic asthma revealed that only the M. leprae Cpn60.2 protein was able to block the signs and symptoms of asthma including the inhibition of local inflammation and of bronchial hyperreactivity. Using the identical model to compare the effect of acute administration of M. tuberculosis Cpn60.1, Cpn60.2 or Cpn10 the results concurred with the earlier study in respect of the lack of activity of the Cpn60.2 protein. However, low doses of Cpn60.1 were able to block both lung inflammation and bronchial hyperreactivity. The Cpn10 protein also had some inhibitory activity but less than the Cpn60.1 protein. The surprising aspect of this finding is that the M. tuberculosis and M. leprae Cpn60.2 proteins exhibit around 95% sequence identity. For most bioinformaticists these would be the same protein. Yet as far as the mouse is concerned it sees two very different biological signals in these Cpn60.2 proteins—one which is potently anti-inflammatory and one that is not.

Thus far the writing has suggested that secreted molecular chaperones are pro-inflammatory, cell activating, proteins. However, this is not the case and more molecular chaperones show anti-inflammatory actions than pro-inflammatory activity. More detailed analysis of the effect of the M. tuberculosis Cpn60 proteins on bone and bone cells has revealed that the Cpn60.2 protein has no positive or negative effects on bone remodelling dynamics. In contrast, recombinant Cpn60.1 is a potent inhibitor of agonist-induced bone resorption and osteoclast proliferation in vitro. The activity of this protein is uniquely long-lived and appears to function, at least over the first few days of interaction, by blocking transcription of the major osteoclast transcription factor Nfatc1. Administration of Cpn60.1 also completely blocks the massive osteoclastic bone destruction seen in the joints of rats with...
adjuvant arthritis. Curiously, this inhibition of bone destruction occurs without any diminution of joint inflammation. This is the same profile of activity induced by the natural inhibitor of osteoclast differentiation, osteoprotegerin (OPG) in the adjuvant model. However, the M. tuberculosis Cpn60.1 protein does not induce the synthesis of OPG and has no sequence homology to OPG. It must therefore be classified as a bacterial OPG mimic (or vice versa) — a very unusual finding in bacteriology. In this study it was also shown that M. tuberculosis Cpn60.1 is present in the blood of patients with tuberculosis.

Another example of the inhibitory actions of M. tuberculosis Cpn60.1 is its effect on the biological activity of purified protein derivative (PPD) an admixture of pro-inflammatory mycobacterial components. It is known that responsiveness to this material is suppressed in tuberculosis. It has been reported that M. tuberculosis Cpn60.1 inhibits PPD-induced expression of IL-12p40 by macrophages via a mechanism involving induction of TLR2 and binding of the Cpn60.1 protein to this pattern recognition receptor to downregulate nuclear c-rel and, in consequence, block IL-12p40 transcription.

To explore more fully the role of the M. tuberculosis chaperonins, attempts were made to inactivate each of the three genes. It proved impossible to inactivate the chromosomal copies of cpn60.2 or cpn10 unless plasmid-encoded copies were supplied, suggesting that these genes are essential. However, the cpn60.1 gene could be inactivated (in the virulent strain H37Rv) without obvious phenotype and the isogenic mutant responded to a wide variety of stresses in an identical manner to the wild type organism. However, when the Δcpn60.1 isogenic mutant was used to infect mice and guinea pigs it failed to generate a granulomatous response. The complemented strain gave an in vivo response identical to the wild type organism showing that the effect of gene inactivation is not due to polar effects. It had been previously reported that the M. tuberculosis Cpn60 proteins did not have the classic tetracamer structure of the prototypic E. coli GroEL and the crystal structure of the Cpn60.2 protein was not a tetramer-camer but a dimer. This raised questions about the role of these mycobacterial proteins as molecular chaperones. Using a cpn60/ cpn10 (groEL/groES) conditional mutant to determine complementation it was found that the cpn60.2 and cpn10 genes could complement this E. coli mutant. However, and this is an important finding, the cpn60.1 gene would not complement. This suggests that the cpn60.1 gene has evolved to lose its protein folding activity but gain the activity profile of a myeloid cell-modulating mediator able to inhibit osteoclast production but promote giant cell/granuloma formation. The development of non-folding actions has recently been suggested to be the evolutionary fate of all the extra genes found in bacteria.

Is this finding with the Cpn60.1 protein of M. tuberculosis just a ‘one off’? The answer is no, as it has been reported that inactivation of the cpn60.1 gene in Mycobacterium smegmatis results in a mutant that grows normally in planktonic culture but which fail to form biofilms. This is due to the association of Cpn60.1 with KsA, a protein involved in the type II fatty acid synthase which generates cell surface mycolic acids. To generate biofilms, the bacterium must produce elevated levels of short-chain fatty acids and failure to do so results in altered cell surface properties and an inability to form a biofilm.

As the reader may have begun to expect, when it comes to protein moonlighting, the inactivation of the cpn60.1 gene in M. tuberculosis does not alter mycolic acid synthesis or the ability to form biofilms. Surprise-follows-surprise in moonlighting, with the recent report that M. tuberculosis Cpn60.1 binds to DNA with high affinity but with low specificity. Such binding is protective of the DNA when it is exposed to DNase I or hydroxyl radicals. However, such binding obviously is not essential for survival as the cpn60.1 gene can be inactivated without obvious phenotype in culture.

The function of the 10 kDa mycobacterial Cpn10 protein is less clear. Initial studies of the monocyte activating capacity of a variety of M. tuberculosis molecular chaperones suggested the only one without activity was Cpn10. This suggests the possibility that this Cpn10 protein has anti-inflammatory properties. Two studies have shown that M. tuberculosis Cpn10, when administered acutely, can inhibit the severity of adjuvant arthritis in the rat. In such experiments, the Cpn10 is acting as a direct modulator of leukocyte, or other cell population, function and not as a vaccine. These studies support the more recent findings with human recombinant Cpn10, which is currently in clinical trial as a therapeutic agent for inflammatory diseases such as rheumatoid arthritis. Thus a picture is emerging of a moonlighting landscape for the mycobacterial chaperonins, particularly the Cpn60 proteins, with a growing range of biological actions being ascribed to these proteins which appear to be linked to the virulence of M. tuberculosis (Figure 1).

Small heat shock proteins, which are cytoplasmic proteins of molecular mass 10–20 kDa, and have a range of nomenclature in mycobacteria (alpha (α) crystallin, hspX (Acr1 – Rv2031c), Acr2 – Rv0251c), all appear to have moonlighting functions. The α-crystallins are large polydisperse oligomers of two isoforms, αA- and αB-crystallins whose function is to prevent protein aggregation and to disaggregate protein aggregates.

An early finding was the identification of α-crystallin as the major membrane protein in the virulent Erdman strain of M. tuberculosis. A later study revealed that M. tuberculosis and Mycobacterium bovis, if grown under low oxygen conditions, which can induce dormancy, increased the thickness of their cell walls and these cell walls contained large amounts of α-crystallin. It is unclear what this small heat shock protein is doing within the cell wall. It may be functioning to prevent protein misfolding in the cell wall milieu or possibly it is having some unexpected moonlighting function.

If α-crystallin (Acr1) is a molecular chaperone then inactivation of the gene coding for this protein would be assumed to affect cell behaviour, particularly cell growth. Unexpectedly, an unmarked deletion ofacr1 in M. tuberculosis resulted in significant increases in the rate of cell division in both infected macrophages and mice, suggesting this protein is, somehow, involved in the control of cell division rate. As a corollary, overexpression of the acr1 gene from the strong cpn60 promoter resulted in reductions in the growth rates of both M. smegmatis and M. tuberculosis. It should be noted that upregulation of Acr1 can occur with merely standing in culture or as a result of centrifugation. The other α-crystallin-like protein, Acr2 also has unusual behaviour. Within 1 h of infection of monocytes/macrophages there is increased expression of M. tuberculosis Acr2 reaching a peak of 18–55-fold within 24 h. This is one of the most induced proteins in M. tuberculosis and also one of the most immunogenic — possibly suggesting release from cells in vivo. However, theacr2 gene can be inactivated without any in vitro phenotype and the only change in vivo is a slowing of disease progression, possibly as a result of diminished recruitment of leukocytes to the infected lungs. These findings suggest that Acr1 and Acr2 should be studied for additional moonlighting functions.

The final mycobacterial molecular chaperone found to have moonlighting actions is the Hsp70 (DnaK) protein. In the human, there are many Hsp70 proteins and it is emerging that they have a range of intracellular functions as well as extracellular moonlighting functions. However, much less is known about the moonlighting functions of bacterial Hsp70 proteins. There is growing evidence that the generation of CC and CXC chemokines in tuberculosis drives the process of granuloma formation and may be an
important target for anti-tubercular therapeutics. However, there has been little work done to establish the factors in *M. tuberculosis* that drive granuloma formation. The finding that recombinant *M. tuberculosis* Hsp70 dose-dependently stimulated CD8-enriched T cells from naive non-human primates to secrete the CC chemokines: CCL3 (Mip1α), CCL4 (Mip1β) and CCL5 (RANTES) was therefore both unexpected and of significant interest. Of importance for a later part of this article is the fact that CCL4 and CCL5 bind to the chemokine receptor CCR5. The human Hsp70 protein was reported to bind to the toll-like receptors (TLR)2 and TLR4. However, a detailed study of the binding of *M. tuberculosis* Hsp70 to a number of monocytic and dendritic cells and cell lines revealed that the induction of the formation of the above CC chemokines required the binding of Hsp70 to CD40, a tumour necrosis factor receptor superfamily member. Various controls excluded that chemokine synthesis was due to LPS contamination and this finding supports the hypothesis that *M. tuberculosis* Hsp70 is a major inducer of CC chemokines required for granuloma formation. In an entirely separate study, it was found that CD40-/- mice were extremely susceptible to aerosolised infection by *M. tuberculosis* and that this was due to inadequate priming of interferon (IFN)γ-producing T cells consequent on a failure to secrete sufficient IL-12. Unexpectedly, CD40L-/- mice were resistant to infection with *M. tuberculosis*, suggesting an alternative ligand was interacting with CD40. It was shown that *M. tuberculosis* Hsp70 was such an alternative CD40 ligand in these infected mice. The CD40 binding active site in *M. tuberculosis* Hsp70 is a major inducer of CC chemokines and cell proliferation. In *M. tuberculosis* Hsp70, CCL3 (Mip1α), CCL4 (Mip1β) and CCL5 (RANTES) were therefore both unexpected and of significant interest. This binding could play an important role in the formation of granulomas.

Widening the moonlighting actions of *M. tuberculosis* Hsp70 is the report that it also binds to plasminogen, although the role that this binding could play is not yet known.

### 1. Conclusions

It is becoming clear that we still understand little about the chaperonins of the mycobacteria. Thus the recently described *groE* promoter which regulates expression of Cpn10 and Cpn60.1 in *M. tuberculosis* and *M. smegmatis* is claimed to be more active in the latter organism than the *groEL* promoter which controls Cpn60.2 transcription. This did not appear to be the case in *M. tuberculosis*. It has recently been reported that *M. tuberculosis* Cpn60.1 is phosphorylated on residues Thr25 and Thr54 by the serine/threonine protein kinase, PknF. In contrast, the *M. smegmatis* Cpn60.1 is not phosphorylated by its cognate PknF. The role of such phosphorylation on the biological actions, including the moonlighting actions, of *M. tuberculosis* Cpn60.1 needs to be defined. The potential importance of the phosphorylation of the mycobacterial chaperonins is further emphasised by the report that double ring formation in *M. tuberculosis* Cpn60.1 is dependent upon phosphorylation at Ser393. Thus not only are the moonlighting actions of the mycobacterial chaperonins unclear, even their biochemical/mechanistic actions are unexpected.
The mycobacteria, like other bacteria, have a range of cell stress proteins including molecular chaperones, protein folding catalysts and redox-active proteins. It is clear that the major molecular chaperones such as Cpn10, Cpn60.1, Cpn60.2, Hsp70 and the small heat shock proteins have important moonlighting functions both at the cell surface and as secreted proteins and are clearly participating in the disease processes induced by infection with M. tuberculosis. An important question that has not been addressed is whether these various cell stress proteins interact to generate emergent properties. Thus these proteins could show antagonism, synergy or some more complex interactional behaviour. The functions of these known moonlighting molecular chaperones and cell stress proteins need to be examined in more detail and all other similar proteins in the mycobacteria should be analysed for moonlighting activity. The evidence at the present time points to these proteins being potential therapeutic targets for the treatment of tuberculosis. Furthermore the ability of M. tuberculosis Cpn60.1 to inhibit osteoclaster formation by inhibiting NAPAC1, but not NF-kB33, could have important therapeutic consequences for diseases such as rheumatoid arthritis and osteoporosis where osteoclast over-production causes pathology. Finally, a very early report revealed that cultured macrophage tumour cells (J774) transfected with the gene encoding M. tuberculosis Cpn60.2 lost their ability to form tumours. This was suggested to be due to increased immunological recognition of the tumour cells, but with our increased understanding of the moonlighting actions of this protein other explanations may be likely.

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