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DOI:
10.1016/j.tube.2010.01.004

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Henderson, B, Lund, P & Coates, ARM 2010, 'Multiple moonlighting functions of mycobacterial molecular chaperones', Tuberculosis, vol. 90, no. 2, pp. 119-124. https://doi.org/10.1016/j.tube.2010.01.004

Tuberculosis 90 (2010) 119–124

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

REVIEW

Multiple moonlighting functions of mycobacterial molecular chaperones

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ARTICLE INFO

Article history:
Received 18 November 2009
Received in revised form
14 January 2010
Accepted 26 January 2010

Keywords:
Molecular chaperones
Protein moonlighting
Intercellular signalling

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[1] 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 protein[3] 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 cytokines[7] 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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doi:10.1016/j.tube.2010.01.004

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^{130}. 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.[57] 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.[58] Another example is the essential role played by murine Cpn60 in the capacitation of sperm.[59] However, human Cpn60 does not play any role in human sperm capacitation.[60] 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.[61] The immunodominant secreted antigen85 proteins of *M. tuberculosis* turn out both to be fibronectin adhesins and mycosyltransferases involved in mycolic acid synthesis.[62] 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.[63] 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[64] and the superoxide dismutase of *Mycobacterium avium* functions as an adhesin for this bacterium.[65] The most recent example is the phosphodiesterase of *M. tuberculosis* which alters cell wall permeability.[66] One recent hypothesis to explain protein moonlighting is that it is a facet of intrinsically disordered proteins.[67] Is this hypothesis is 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.[11] 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.[11] 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.[12] It was, by this time, known that *M. tuberculosis* and *M. leprae* encoded two Cpn60 proteins (termed Cpn60.1 and Cpn60.2[3]) 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.[13] In contrast, the Cpn10 protein of this organism had bone destructive activity *in vitro*.[13] 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.[14]

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.[15] The monocyte cytokine-inducing activity of Cpn60.1 could be blocked by a neutralising anti-CD14 monoclonal. This was not true of Cpn60.2.[15] 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.[16] A key question arose – what is the active Cpn60 protein – is it the oligomer, the monomer or does activity reside in some constituent sequence 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.[17]

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.[18] 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.[19] The Cpn10 protein also had some inhibitory activity but less than the Cpn60.1 protein.[19] 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.[20] 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.[21] 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.[21] This is the same profile of activity induced by the natural inhibitor of osteoclast differentiation, osteoprotegerin (OPG) in the adjuvant model.[22] 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.[21]

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.[23]

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.[2] However, when the Δ*cpn60.1* isogenic mutant was used to infect mice and guinea pigs it failed to generate a granulomatous response.[2] 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 previously been reported that the *M. tuberculosis* Cpn60 proteins did not have the classic tetradecameric structure of the prototypic *E. coli* GroEL[24] and the crystal structure of the Cpn60.2 protein was not a tetradecamer but a dimer.[25] 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.[2] 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 *cpn60* genes found in bacteria.[4]

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 KsaA, 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.[26] 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.[2] 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.[27] However, such binding obviously is not essential for survival as the *cpn60.1* gene can be inactivated without obvious phenotype in culture.[2]

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.[28] 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.[29,30] 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.[31]

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* (Figure1).

Small heat shock proteins, which are cytoplasmic proteins of molecular mass 10–20 kDa[32], and have a range of nomenclature in mycobacteria (alpha (α) crystallin, hspX (Acr1 – Rv2031c), Acr2 – Rv0251c), also 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*.[33] 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.[34]

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 of *acr1* in *M. tuberculosis* resulted in significant increases in the rate of cell division in both infected macrophages and mice[35], 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*.[36] It should be noted that upregulation of Acr1 can occur with merely standing in culture or as a result of centrifugation.[37] 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.[38] 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, the *acr2* gene can be inactivated without any *in vitro* phenotype[38] 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.[39] 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[40] as well as extracellular moonlighting functions.[20] 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

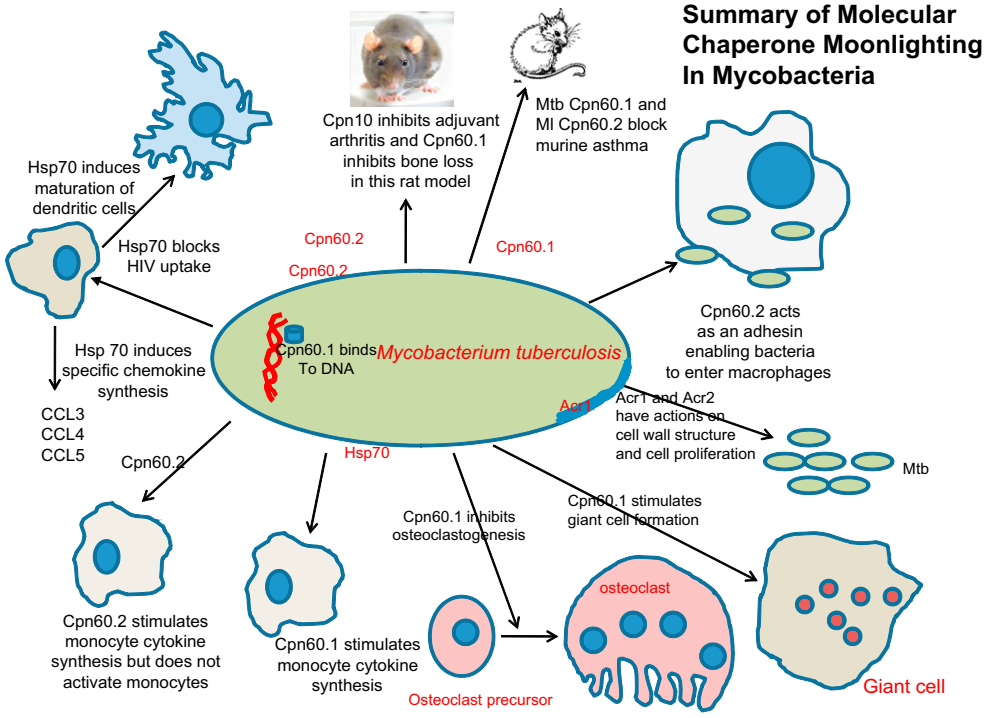

Figure 1. A summary of the moonlighting actions of the mycobacterial molecular chaperones (Ml – *Mycobacterium leprae*, Mtb – *Mycobacterium tuberculosis*).

important target for anti-tubercular therapeutics.[41] 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.[42] 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.[43] 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.[44] Various controls excluded that chemokine synthesis was due to LPS contamination[44] 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.[45] The CD40 binding active site in *M. tuberculosis* Hsp70 is within the C-terminus.[46] This distinguishes this protein from human Hsp70 whose biologically active site is within the N-terminal ATP-binding domain.[47] Curiously, the native *M. tuberculosis* Hsp70 does not stimulate IL-12 synthesis by THP1 monocytes[46] but the C-terminal domain does, suggesting that some form of proteolysis of Hsp70 is required to generate IL-12 from monocytes. Dendritic cells did respond to the native Hsp70.[46] In addition to binding to CD40, further examination of the *M. tuberculosis* Hsp70 revealed that it also bound to the HIV co-receptor and chemokine receptor CCR5.[48,49] Using inhibitors of CD40 and CCR5 revealed that both receptors are involved in the Hsp70 stimulation of dendritic cells.[48] As HIV and *M. tuberculosis* Hsp70 both bind to CCR5, could the latter prevent the former binding to CD4 T lymphocytes? Indeed, both the native *M. tuberculosis* Hsp70 and a peptide epitope 407–426 were able to dose-dependently inhibit binding of HIV to CD4 T lymphocytes, thus suggesting that this mycobacterial protein could have therapeutic properties.[50] It is also interesting in light of the known association between infection with HIV and *M. tuberculosis.* Before leaving this subject of Hsp70 signalling to monocytes it is worth noting that the human monocyte receptor for lipopolysaccharide contains human Hsp70 and Hsp90 revealing an interesting moonlighting activity of these human proteins.[51]

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.[52]

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 *groEL2* promoter which controls Cpn60.2 transcription.[53] This did not appear to be the case in *M. tuberculosis*.[2] 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.[54] 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.[55] 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 participants 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 osteoclast formation by inhibiting NFATc1, but not NF-κB[21], 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.[56] 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.

Funding: None.

Competing interests: None declared.

Ethical approval: Not required.

References

1. Hickey TB, Thorson LM, Speert DP, Daffé M, Stokes RW. *Mycobacterium tuberculosis* Cpn60.2 and DnaK are located on the bacterial surface, where Cpn60.2 facilitates efficient bacterial association with macrophages. *Infect Immun* 2009;**77**:3389–401.
2. Hu Y, Henderson B, Lund PA, Tormay P, Ahmed MT, Gurcha SS, et al. A *Mycobacterium tuberculosis* mutant lacking the groEL homologue cpn60.1 is viable but fails to induce an inflammatory response in animal models of infection. *Infect Immun* 2008;**76**:1535–46.
3. Shinnick TM, Vodkin MH, Williams JC. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. *Infect Immun* 1988;**56**:446–51.
4. Lund PA. Multiple chaperonins in bacteria – why so many? *FEMS Microbiol Rev* 2009;**33**:785–800.
5. van Eden W, Thole JE, van der Zee R, Noordzij A, van Embden JD, Hensen EJ, et al. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature* 1988;**331**:171–3.
6. van Eden W, Wick G, Albani S, Cohen I. Stress, heat shock proteins, and autoimmunity: how immune responses to heat shock proteins are to be used for the control of chronic inflammatory diseases. *Ann N Y Acad Sci* 2007;**1113**:217–37.
7. Friedland JS, Shattock R, Remick DG, Griffin GE. Mycobacterial 65-kDa heat shock protein induces release of proinflammatory cytokines from human monocytic cells. *Clin Exp Immunol* 1993;**91**:58–62.
8. Peetermans WE, Raats CJ, Langermans JA, van Furth R. Mycobacterial heat-shock protein 65 induces proinflammatory cytokines but does not activate human mononuclear phagocytes. *Scand. J Immunol* 1994;**1993**(39):613–7.
9. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* 2009;**27**:451–83.
10. Verdegaal ME, Zegveld ST, van Furth R. Heat shock protein 65 induces CD62e, CD106, and CD54 on cultured human endothelial cells and increases their adhesiveness for monocytes and granulocytes. *J Immunol* 1996;**151**:369–76.
11. Kirby A, Meghji S, Nair SP, White P, Reddi K, Nishihara T, et al. The potent bone resorbing mediator of *Actinobacillus actinomycetemcomitans* is homologous to the molecular chaperone GroEL. *J Clin Invest* 1995;**96**:1185–94.
12. Reddi K, Meghji S, Nair SP, Arnett TR, Miller AD, Preuss M, et al. The *Escherichia coli* chaperonin 60 (groEL) is a potent stimulator of osteoclast formation. *J Bone Miner Res* 1998;**13**:1260–6.
13. Meghji S, White PA, Nair SP, Reddi K, Heron K, Henderson B, et al. *Mycobacterium tuberculosis* chaperonin 10 stimulates bone resorption: a potential contributory factor in Pott's disease. *J Exp Med* 1997;**186**:1241–6.
14. Meghji S, Lillicrap M, Maguire M, Tabona P, Gaston JS, Poole S, et al. Human chaperonin 60 (Hsp60) stimulates bone resorption: structure/function relationships. *Bone* 2003;**33**:419–25.
15. Lewthwaite JC, Coates AR, Tormay P, Singh M, Mascagni P, Poole S, et al. *Mycobacterium tuberculosis* chaperonin 60.1 is a more potent cytokine stimulator than chaperonin 60.2 (hsp 65) and contains a CD14-binding domain. *Infect Immun* 2001;**69**:7349–55.
16. Henderson B, Mesher J. The search for the chaperonin 60 receptor. *Methods* 2007;**43**:223–8.
17. Tormay P, Coates AR, Henderson B. Structure:function relationships of *Mycobacterium tuberculosis* chaperonin 60 proteins: the cell signalling activity of *M. tuberculosis* chaperonin 60.1 resides in the equatorial domain. *J Biol Chem* 2005;**280**:14272–7.
18. Rha YH, Taube C, Haczku A, Joetham A, Takeda K, Duez C, et al. Effect of microbial heat shock proteins on airway inflammation and hyperresponsiveness. *J Immunol* 2002;**169**:5300–7.
19. Riffo-Vasquez Y, Spina D, Page C, Desel C, Whelan M, Tormay P, et al. Differential effects of *Mycobacterium tuberculosis* chaperonins on bronchial eosinophilia and hyperresponsiveness in a murine model of allergic inflammation. *Clin Exp Allergy* 2004;**34**:712–9.
20. Novartis Foundation Symposium 291. The biology of extracellular molecular chaperones. Wiley: Chichester, 2008.
21. Winrow VR, Mesher J, Meghji S, Morris CJ, Fox S, Coates ARM, et al. The two homologous chaperonin 60 proteins of *Mycobacterium tuberculosis* have distinct effects on monocyte differentiation into osteoclasts. *Cell Microbiol* 2008;**10**:2091–104.
22. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;**402**:304–9.
23. Khan N, Alam K, Mande SC, Valluri VL, Hasnain SE, Mukhopadhyay S. *Mycobacterium tuberculosis* heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages. *Cell Microbiol* 2008;**10**:1711–22.
24. Qamra R, Srinivas V, Mande SC. *Mycobacterium tuberculosis* GroEL homologues unusually exist as lower oligomers and retain the ability to suppress aggregation of substrate proteins. *J Mol Biol* 2004;**342**:605–17.
25. Qamra R, Mande SC. Crystal structure of the 65-kilodalton heat shock protein, chaperonin 60.2, of Mycobacterium tuberculosis. *J. Bacteriol* 2004;**186**:8105–13.
26. Ojha A, Anand M, Bhatt A, Kremer L, Jacobs Jr WR, Hatfull GF. GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. *Cell* 2005;**123**:861–73.
27. Basu D, Khare G, Singh S, Tyagi A, Khosla S, Mande SC. A novel nucleoid-associated protein of *Mycobacterium tuberculosis* is a sequence homolog of GroEL. *Nucleic Acids Res* 2009;**37**:4944–54.
28. Retzlaff C, Yamamoto Y, Hoffman PS, Friedman H, Klein TW. Bacterial heat shock proteins directly induce cytokine mRNA and interleukin-1 secretion in macrophage cultures. *Infect Immun* 1994;**62**:5689–93.
29. Ragno S, Winrow VR, Mascagni P, Lucietto P, Di Pierro F, Morris CJ, et al. A synthetic 10-kD heat shock protein (hsp10) from *Mycobacterium tuberculosis* modulates adjuvant arthritis. *Clin Exp Immunol* 1996;**103**:384–90.
30. Agnello D, Scanziani E, Di GM, Leoni F, Modena D, Mascagni P, et al. Preventive administration of *Mycobacterium tuberculosis* 10-kDa heat shock protein (hsp10) suppresses adjuvant arthritis in Lewis rats. *Int. Immunopharmacol* 2002;**2**:463–74.
31. Vanags D, Williams B, Johnson B, Hall S, Nash P, Taylor A, et al. Therapeutic efficacy and safety of chaperonin 10 in patients with rheumatoid arthritis: a double-blind randomised trial. *Lancet* 2006;**368**:855–63.
32. Jee B, Katoch VM, Awasthi SK. Dissection of relationship between small heat shock proteins and mycobacterial diseases. *Indian J Lepr* 2008;**80**:231–45.
33. Lee BY, Hefta SA, Brennan PJ. Characterisation of the major membrane protein of virulent *Mycobacterium tuberculosis*. *Infect Immun* 1992;**60**:2066–74.
34. Cunningham AF, Spreadbury CL. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog. *J Bacteriol* 1998;**180**:801.
35. Hu Y, Movahedzadeh F, Stoker NG, Coates AR. Deletion of the *Mycobacterium tuberculosis* alpha-crystallin-like hspX gene causes increased bacterial growth in vivo. *Infect Immun* 2006;**74**:861–8.
36. Yuan Y, Crane DD, Barry CE. Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. *J Bacteriol* 1996;**178**:4484–92.
37. Kendall SL, Movahedzadeh F, Rison SC, Wernisch L, Parish T, Duncan K, et al. The *Mycobacterium tuberculosis* dosRS two-component system is induced by multiple stresses. *Tuberculosis (Edinb)* 2004;**84**:247–55.
38. Wilkinson KA, Stewart GR, Newton SM, Vordermeier HM, Wain JR, Murphy HN, et al. Infection biology of a novel alpha-crystallin of *Mycobacterium tuberculosis*: Acr2. *J Immunol* 2005;**174**:4237–43.
39. Stewart GR, Newton SM, Wilkinson KA, Humphreys IR, Murphy HN, Robertson BD, et al. The stress-responsive chaperone alpha-crystallin 2 is required for pathogenesis of *Mycobacterium* tuberculosis. *Mol Microbiol* 2005;**55**:1127–37.
40. Meimaridou E, Gooljar SB, Chapple JP. From hatching to dispatching: the multiple cellular roles of the Hsp70 molecular chaperone machinery. *J Mol Endocrinol* 2009;**42**:1–9.
41. Méndez-Samperio P. Expression and regulation of chemokines in mycobacterial infection. *J Infect* 2008;**57**:374–84.

42. Lehner T, Bergmeier LA, Wang Y, Tao L, Sing M, Spallek R, et al. Heat shock proteins generate beta-chemokines which function as innate adjuvants enhancing adaptive immunity. *Eur J Immunol* 2000;**30**:594–603.
43. Vabulas RM, Ahmad-Nejad P, Ghose S, Kirschning CJ, Issels RD, Wagner H. Hsp70 as endogenous stimulus of the toll/interleukin-1 receptor signal pathway. *J Biol Chem* 2002;**277**:15107–12.
44. Wang Y, Kelly CG, Karttunen JT, Whittall T, Lehner PJ, Duncan L, et al. CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. *Immunity* 2001;**15**:971–83.
45. Lazarevic V, Myers AJ, Scanga CA, Flynn JL. CD40, but not CD40L, is required for the optimal priming of T cells and control of aerosol *M. tuberculosis* infection. *Immunity* 2003;**19**:823–35.
46. Wang Y, Kelly CG, Singh M, McGowan EG, Carrara AS, Bergmeier LA, et al. Stimulation of Th1-polarizing cytokines, C–C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. *J Immunol* 2002;**169**:2422–9.
47. Becker T, Hartl FU, Wieland F. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. *J Cell Biol* 2002;**158**:1277–85.
48. Whittall T, Wang Y, Younson J, Kelly C, Bergmeier L, Peters B, et al. Interaction between the CCR5 chemokine receptors and microbial HSP70. *Eur. J. Immunol* 2006;**36**:2304–14.
49. Floto RA, MacAry PA, Boname JM, Mien TS, Kampmann B, Hair JR, et al. Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5. *Science* 2006;**314**:454–8.
50. Babaahmady K, Oehlmann W, Singh M, Lehner T. Inhibition of human immunodeficiency virus type 1 infection of human CD4+ T cells by microbial HSP70 and the peptide epitope 407–426. *J Virol* 2007;**81**:3354–60.
51. Triantafilou K, Triantafilou M, Dedrick RL. A CD14-independent LPS receptor cluster. *Nat Immunol* 2001;**2**:338–45.
52. Xolalpa W, Vallecillo AJ, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, et al. Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium* tuberculosis. *Proteomics* 2008;**7**:3332–41.
53. Aravundham V, Christy AJ, Roy S, Ajitkumar P, Narayanan PR, Narayanan S. *Mycobacterium tuberculosis groE* promoter controls the expression of the bicistronic *groESL1* operon and shows differential regulation under stress conditions. *FEMS Microbiol Lett* 2009;**292**:42–9.
54. Canova MJ, Kremer L, Molle V. The *Mycobacterium tuberculosis* GroEL1 chaperone is a substrate of Ser/Thr protein kinases. *J Bacteriol* 2009;**191**:2876–83.
55. Cumar CM, Khare G, Srikanth CV, Tyagi AK, Sardesai AA, Mande SC. Facilitated oligomerisation of mycobacterial GroEL: evidence for phosphorylation-mediated oligomerization. *J Bacteriol*; 2009 [Epub ahead of print].
56. Lukacs KV, Lowrie DB, Stokes RW, Colston MJ. Tumor cells transfected with a bacterial heat-shock gene lose tumorigenicity and induce protection against tumors. *J Exp Med* 1993;**178**:343–8.
57. Jeffery CJ. Moonlighting proteins – an update. *Mol Biosyst* 2009;**5**:345–50.
58. Gancedo C, Flores CL. Moonlighting proteins in yeast. *Microbiol Mol Biol Rev* 2008;**72**:197–210.
59. Asquith KL, Baleato RM, McLaughlin EA, Nixon B, Aitken RJ. Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition. *J Cell Sci* 2004;**117**:3645–57.
60. Mitchell LA, Nixon B, Aitken RJ. Analysis of chaperone proteins associated with human spermatozoa during capacitation. *Mol Hum Reprod* 2007;**13**:605–13.
61. Sengupta S, Ghosh S, Nagaraja V. Moonlighting function of glutamate racemase from *Mycobacterium tuberculosis*: racemization and DNA gyrase inhibition are two independent activities of the enzyme. *Microbiology* 2008;**154**:2796–803.
62. Kremer L, Maughan WN, Wilson RA, Dover LG, Besra GS. The *M. tuberculosis* antigen 85 complex and mycosyltransferase activity. *Letts Appl Microbiol* 2002;**34**:233–7.
63. Kinhikar AG, Vargas D, Li H, Mahaffey SB, Hinds L, Belisle JT, et al. *Mycobacterium tuberculosis* malate synthase is a laminin-binding adhesin. *Mol Microbiol* 2006;**60**:999–1013.
64. Banerjee S, Nandyala AK, Raviprasad P, Ahmed N, Hasnain SE. Iron-dependent RNA-binding activity of *Mycobacterium tuberculosis* aconitase. *J Bacteriol* 2007;**189**:4046–52.
65. Reddy VM, Suleman FG. *Mycobacterium avium*-superoxide dismutase binds to epithelial cell aldolase, glyceraldehyde-3-phosphate dehydrogenase and cyclophilin A. *Microb Pathog* 2004;**36**:67–74.
66. Podobnik M, Tyagi R, Matange N, Dermol U, Gupta AK, Mattoo R, et al. A mycobacterial cyclic AMP phosphodiesterase that moonlights as a modifier of cell wall permeability. *J Biol Chem* 2009;**284**:32846–57.
67. Hazy E, Tompa P. Limitations of induced folding in molecular recognition by intrinsically disordered proteins. *Chemphyschem* 2009;**10**:1415–9.