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Hu, Yanmin; Coates, Anthony R M; Liu, Alexander; Lund, Peter; Henderson, Brian

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Identification of the monocyte activating motif in *Mycobacterium tuberculosis* chaperonin 60.1

Yanmin Hu a, Anthony R.M. Coates a, Alexander Liu b, Peter A. Lund c, Brian Henderson d, * * 

a Centre for Infection and Immunity, Division of Clinical Sciences, St. George’s University of London, London, United Kingdom 
b The National Heart and Lung Institute, Imperial College London, London, United Kingdom 
c Department of Biosciences, University of Birmingham, Birmingham, United Kingdom 
d Department of Microbial Diseases, UCL Eastman Dental Institute, University College London, 256 Gray’s Inn Road, London WC1X 8JD, United Kingdom

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S U M M A R Y

Evidence is emerging that moonlighting proteins, defined as proteins with more than one biological function, play important roles in bacterial virulence. The *Mycobacterium tuberculosis* chaperone, chaperonin 60.1, is a potent stimulator of human monocyte cytokine synthesis and modulator of giant cell and osteoclast formation. Previously, we had shown that these moonlighting activities resided in the equatorial domain of this protein. In this study, through the generation of chaperonin 60.1 amino acid sequence-deletion mutants and synthetic peptides, we have identified the minimal moonlighting site in this molecular chaperone responsible for monocyte activation as peptide sequence DGSVVVNKVSEL–PAGHLNVNLTLSYGDLAAD, residues 461–491, in the equatorial domain. Modelling of this biologically active sequence in the *M. tuberculosis* chaperonin 60.1 protein reveals a surface-exposed motif with significant α-helical structure.

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1. Introduction

Protein moonlighting is an intriguing phenomenon, defined as the ability of proteins to have more than one unique biological activity.1 Most identified bacterial moonlighting proteins play a role in bacterial virulence, by functioning as virulence factors.2 Arguably, the most important of these is the *Mycobacterium tuberculosis* molecular chaperone, chaperonin (Cpn)60.1, which moonlights as a myeloid cell ‘activation and differentiation’ factor. This protein has the following actions of relevance to the pathology of tuberculosis: (i) stimulation of human monocyte pro-inflammatory cytokine synthesis3 and (ii) promotion of the formation of murine and human giant cells, a characteristic cell in the tuberculoid granuloma.4,5 Unexpectedly, this molecular chaperone is also a potent inhibitor (both in vitro and in vivo) of the formation of the natural myeloid multinucleate cell of bone, the osteoclast, which is responsible for bone matrix breakdown.6 Inactivation of the gene encoding *M. tuberculosis* Cpn60.1 abolishes the ability of this bacterium to generate the tuberculoid granuloma, the hallmark of tuberculosis, which drives much of the pathology of this disease.4 It is therefore important to understand how this protein promotes granuloma formation and to identify the active site in the protein responsible for its ability to modulate the macrophage phenotype. The chaperonin 60 protein has three structural domains: equatorial, intermediate and apical, each of which plays roles in its protein folding actions.7 In a previous study we cloned the DNA encoding each of the three individual domains of the *M. tuberculosis* Cpn60.1 protein and expressed recombinant versions of these domains. Testing each domain individually showed that the monocyte cytokine-inducing activity8 and the osteoclast-inhibiting activity (unpublished data) resided within the equatorial domain. The other two recombinant domains had only minimal activity. In the present study we have generated a series of deletion mutants of the equatorial domain of the *M. tuberculosis* Cpn60.1 protein and, in conjunction with peptide mapping studies, have identified that this protein has a single moonlighting site which is responsible for its ability to stimulate human monocyte cytokine synthesis.

2. Materials and methods

2.1. Cloning and expression of full length Cpn60.1 and amino acid-deleted Cpn60.1 mutants

To amplify the full-length cpn60.1 gene coding for 538 amino acids, PCR was performed using *M. tuberculosis* genomic DNA as a
template and primers 60.1F (5'-ACTAGTGAGGGCAGCTCCTCCGTCGAG-3') and 60.1R (5'-AAATGATCCTCCGTCGAGCCCTCGTGCTGGTG-3') which were designed to include the start codon but not the stop codon. The PCR product was cloned into the BamHI restriction site of the pQE60 vector (Qiagen), resulting in the generation of a gene encoding the recombinant version of the Cpn60.1 protein with six histidine residues at the C-terminus. This protein has been termed Cpn60.1WT. The individual mutated Cpn60.1 proteins were all expressed in E. coli as inclusion bodies. In order to refold the proteins, the inclusion bodies were incubated at 4 °C for 1 h followed by dilution in a 10-fold volume of PBS. Refolding of the proteins was carried out at 4 °C overnight. The recombinant proteins were purified using a BioCad Sprint chromatography system (Applied Biosystems, Warrington, UK). The proteins were bound to Ni-NTA Agarose (Qiagen) and were washed with 5 column volumes of polymyxin B (Sigma) at 2 mg/ml in PBS to remove contaminating LPS and other hydrophobic cytokine-inducing components. The recombinant proteins were eluted from the column by addition of 10 column volumes of a 10–300 mM imidazole gradient. The fractions containing the recombinant proteins were further purified by anion exchange chromatography using a Porous HQ column on the BioCad Sprint chromatography system. Care was taken to ensure that all chemicals, solutions, water and column apparatus had minimal contamination with LPS.

2.3. Examination of protein purity

The purity of the full length and mutated proteins was monitored by SDS-PAGE. 1–5 μg of each purified recombinant protein was analysed on a 4–12% Bis-Tris gradient gel (Invitrogen). The proteins were detected by staining with Simply Blue Safe Stain (Invitrogen).

2.4. Assay of LPS contamination

LPS contamination of the purified recombinant M. tuberculosis Cpn60.1 recombinant proteins was determined using the Limulus amoebocyte lysate assay (Associates of Cape Cod, Liverpool, UK) according to the manufacturer’s instructions. All purified chaperonins contain approximately 5–10 pg of LPS/μg of recombinant protein.

2.5. Isolation of human monocytes

Human monocytes were prepared from the mixed buffy coat blood from multiple healthy donors (from the Blood Transfusion Centre, London, UK) by density gradient centrifugation and differential adherence as described. The isolated monocytes were seeded at 2 × 10⁶ cells/well in 24 well plates and incubated at 37 °C with 5% CO₂ for 1 h. The cells were washed twice with PBS to remove non-adherent cells, followed by addition of 1 ml of RPMI medium containing 2% foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Invitrogen).

2.6. Induction and measurement of cytokines

Different concentrations of the full length Cpn60.1 protein and the recombinant mutated proteins were incubated with the human monocytes for 24 h in the presence of 20 μg/ml polymyxin B to ensure that activity measured was not due to contaminating LPS, and the medium was removed and stored at -70 °C until analysed. Cytokine levels were determined by two-site enzyme-linked immunosorbent assay (ELISA) as described by Tormay et al. Results are means of three replicate wells for each stimulus using cells from the same buffy coat residue. The experiments were repeated twice more with monocyte isolated from different batches of buffy coat blood, with reproducible results.

2.7. Peptide synthesis and assay of peptide cytokine stimulating activity

Peptide 461–491 (DGSVVVKVSPELAGHGLNVNTSLYGDLAAD) and a series of internal peptides: (461–476) DGSVVVKVSPELAGH, (477–491) GLNVNTSLYGDLAAD, (461–470) DGSVVVKVS, (471–

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**Table 1**

<table>
<thead>
<tr>
<th>Primer sequences</th>
<th>Deletion (aa)</th>
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<tr>
<td>5'-ACTAGTGAGGGCAGCTCCTCCGTCGAG-3'</td>
<td>538</td>
</tr>
<tr>
<td>5'-AAATGATCCTCCGTCGAGCCCTCGTGCTGGTG-3'</td>
<td>514–539</td>
</tr>
<tr>
<td>5'-ACTAGTGAGGGCAGCTCCTCCGTCGAG-3'</td>
<td>514–524</td>
</tr>
<tr>
<td>5'-AAATGATCCTCCGTCGAGCCCTCGTGCTGGTG-3'</td>
<td>524–538</td>
</tr>
</tbody>
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**Figure 1.** Diagram of the full length Cpn60.1 protein and the position of the individual deletions in the mutant proteins generated for this study.
ELPAGHGLNV and NTLSYGDLAAD were synthesised by Cambridge Peptides UK. Graded concentrations of each peptide were incubated with the human monocytes for 24 h and the medium was removed and cytokine levels were determined by ELISA. Results are means of three replicate wells for each experiment using cells from the same buffy coat residue. The experiments were repeated twice more with monocyte isolated from different batches of buffy coat blood with reproducible results.

3. Results

3.1. Generation of amino acid-deletions mutants of the Cpn60.1 equatorial domain

It has previously been established that the equatorial domain of the M. tuberculosis Cpn60.1 protein contains the monocyte-modulating active site of this protein. In order to identify the sequence of this moonlighting site, a series of cpn60.1 expression constructs, bearing different deletions in the C-terminal end of the equatorial domain, were generated (Figure 1). The resultant proteins were missing adjacent segments of 23–31 amino acids, starting at the C-terminus, in succession (Figure 1). All mutant proteins were expressed at significant levels and at the correct molecular mass as judged by SDS-PAGE (Figure 2). The lipopolysaccharide (LPS) contamination of the individual recombinant proteins was measured using a commercial Limulus assay. Each recombinant protein contained approximately the same low level of LPS contamination (5–10 pg LPS/µg protein).

3.2. Assay of mutant proteins for the stimulation of human monocyte cytokine synthesis

The wild type protein and the truncated derivatives were incubated at various concentrations with human peripheral blood monocytes, and, after 24 h in culture, the levels of the pro-inflammatory cytokines, IL-1β, IL-6 and TNFα, were measured. As shown in Figure 3, all of the mutant proteins, with the exception of Cpn60.1Δ461–491, were as active, or more active, than the parental Cpn60.1 protein. In contrast, the mutant Cpn60.1Δ461–491 protein was virtually inactive. As all recombinants. As all recombinants contained the same level of LPS contamination the differences in activity cannot be due to this potentially bioactive contaminant.

Figure 2. SDS-PAGE of the purified recombinant M. tuberculosis full length or amino acid-deleted chaperonin 60.1 proteins. Full-length Cpn60.1 protein (lane 1), Δ376–405 of Cpn60.1 (lane 2), Δ406–432 of Cpn60.1 (lane 3), Δ433–460 of Cpn60.1 (lane 4), Δ461–491 of Cpn60.1 (lane 5), Δ492–514 of Cpn60.1 (lane 6) and Δ515–539 of Cpn60.1 (lane 7).

Figure 3. Comparison of the cytokine-stimulating activity of two independently generated batches of the recombinant wild type Cpn60.1 and the various amino acid-deletion mutants (left- and right-hand graphs). The proteins were examined over a range of concentrations for their ability to induce the synthesis and release of: TNF-α (A), IL-6 (B) and IL-1β (C). Polymyxin B was added 20 µg/ml in all assays. The data shown are the means of three replicate wells for each concentration of recombinant all from one experiment with each batch. To make the diagram clearer the error bars (which were below 10%) have been omitted. The results show good reproducibility and have been repeated a further two times with consistent results using blood from different sets of donors.
3.3. Cytokine-inducing activity of peptide 461–491

To verify that these residues (461–491, DGSVVNKSEL-PAGHGLNVNNTLSYGDLAAD) constituted the biologically active motif in the M. tuberculosis Cpn60.1 equatorial domain, a synthetic peptide with this sequence was synthesised, as were a number of internal peptides. Graded concentrations of these peptides were incubated with human peripheral blood monocytes for 24 h and the release of pro-inflammatory cytokines assayed. Only peptide 461–491 demonstrated the ability to induce human monocytes to generate cytokines (Figure 4). To determine the minimal peptide within the 461–491 motif responsible for biological activity we made a series of internal peptides, specifically: (461–476) DGSVVNKSEL-PAGH, (477–491) GLNVNNTLSYGDLAAD, (461–470) DGSVVNVKS, (471–480) ELPAGHGLNV and (481–491) NTLSYGLDLAAD. These covered the complete 461–491 sequence and would have identified a smaller active peptide if it existed. However, none of these internal peptides showed any ability to activate human monocyte cytokine synthesis (results not shown).

3.4. Modelling the 461–491 peptide motif on the M. tuberculosis Cpn60.1 structure

As the structure of M. tuberculosis Cpn60.1 has not been solved, we used the protein homology/analogy recognition engine PHyre9 to model a protomer of the protein. Because of the high degree of similarity between Cpn60.1 and its homologues, the model has a good chance of being reasonably accurate. Two images of the protein are shown in Figure 5, with the peptide from 461 to 491 coloured in red. This clearly reveals that peptide 461–491 is largely surface exposed as would be expected for a biologically active site. We also used the antigenic programme in the EMBoss suite to predict the degree of antigenicity in the Cpn60.1 sequence. This identified the peptide 461–483 as being the second most antigenic region in the protein.

4. Discussion

It is well established that the Cpn60 molecular chaperone is an indispensible protein both in bacteria and eukaryotes.10 The prototypic Cpn60 protein, E. coli GroEL, is a tetradecamer formed of two back-to-back seven-membered rings. These rings form folding cavities within which a proportion of the cellular proteins can be correctly folded.10 Up to 30% of bacteria encode more than one Cpn60 protein, raising the possibility that these molecular chaperones may possess other (moonlighting) properties.11 M. tuberculosis encodes two Cpn60 proteins, termed Cpn60.1 and Cpn60.2.11 While GroEL is a tetradecamer, it is surprising to find that the M. tuberculosis Cpn60 proteins fail to form the classical tetradecameric folding oligomer, as assessed by physicochemical analysis.12,13 Moreover, the M. tuberculosis Cpn60.2 protein crystallises as a dimer.13,14 Despite the reported inability of M. tuberculosis Cpn60 proteins to form tetradecamers under standard conditions, we have reported that the Cpn60.2 protein can effectively replace E. coli GroEL, and partially assembles into tetradecamers under specific conditions,15 while the Cpn60.1 protein is unable to support the growth of E. coli,15 suggesting this latter protein does not function as a molecular chaperone.

Chaperonin 60 proteins exhibit a wide variety of moonlighting functions,1,16 of which the first to be reported was the ability of the M. tuberculosis Cpn60.2 protein to stimulate human macrophages to generate pro-inflammatory cytokines.17 Many Cpn60 proteins, from bacteria and mammals, can function as extracellular signals for leukocytes.18 It is important to realise that although members of the Cpn60 protein family have significant sequence similarity, small changes in sequence can have marked effects on their biological activities. For example, the Cpn60.2 protein of Mycobacterium leprae is a potent inhibitor of allergic asthma in mice, whereas the homologue from M. tuberculosis (which is approximately 95% identical at the amino acid level) has no effect in this experimental inflammatory model.19,20 This makes the finding that the two
Cpn60 proteins of *M. tuberculosis* have different patterns of moonlighting activity, for example in terms of inhibiting osteoclast formation,6 more explicable. The finding that the two *M. tuberculosis* Cpn60 proteins do not compete for binding to the human monocyte cell surface,5 supports the hypothesis that these two proteins bind to different receptors or receptor complexes.

A key question needing answer is - what is the agonist binding site on the mycobacterial Cpn60 proteins that allows them to modulate myeloid cell functionality? As described, the Cpn60 protein monomer contains three protein domains termed the equatorial, intermediate and apical domains. Generation of recombinant versions of these three domains from the *M. tuberculosis* Cpn60.1 protein revealed that the ability to stimulate human monocyte cytokine release6 and inhibit osteoclast formation (Henderson, Meghji and Coates, unpublished) resides in the equatorial domain, with the other domains demonstrating minimal activity.5 The biologically active site in a protein can be determined by generating overlapping synthetic peptides which cover the whole length of the protein, or expressing recombinant truncation mutants of the protein of interest. Both methods have been used with the human Cpn60 homologue more generally known as Hsp60.21 However, synthetic peptides can exhibit biological actions not found in the parent protein. For example, we have found that a specific peptide (residue 195–219) from the apical domain of *M. tuberculosis* Cpn60.1 was a fairly potent monocyte stimulator,5 yet no monocyte-stimulating activity was found when the recombinant apical domain was tested by itself.

In the present study we generated recombinant versions of the *M. tuberculosis* Cpn60.1 protein in which small peptides of between 23 and 31 residues were removed in turn from the protein, generating a series of internal C-terminal deletion mutants. This approach clearly has the potential to be more informative than truncation mutagenesis. Extensive washing of the recombinant proteins, with polymyxin B, while they were bound to a Ni-NTA column, was used to remove contaminating LPS and other hydrophobic bacterial lipoproteins and peptides. Comparisons of the activities of the different proteins showed that only the deletion mutant lacking residues 461–491 failed to promote significant cytokine synthesis. The synthetic peptide (DGSVVNVKSELPGHGLNVNTLSYGDLAAAD) corresponding to these residues, produced a full agonist response for the generation of the pro-inflammatory cytokines: IL-1β, TNFα and IL-6. This confirms that it is this peptide motif in the intact Cpn60.1 protein that contains the monocyte cytokine-inducing agonist site. Computer modelling and antigenicity prediction show this region is highly likely to be surface exposed when in the full-length protein. Attempts to identify a smaller active unit within the 30 amino acid residue, 461–491 motif, using a range of internal peptides including 15-mers and 10-mers, failed to generate a peptide with any activity. This suggests that this external structure on the protein surface is the minimum required to interact with the cognate receptor or receptors on the macrophage cell surface to induce cytokine synthesis.

The only other example of a Cpn60 protein whose agonist site has been partially mapped is the human mitochondrial protein, normally referred to as Hsp60. This has been achieved using a different approach to that used for study of the mycobacterial protein including, namely (i) the binding/competition of overlapping human Cpn60 peptides and (ii) the use of a small number of monoclonal antibodies with known binding sites in the human Cpn60 protein. Using these approaches with the mouse monocyte cell line, J774A, the agonist site in human Cpn60 was defined as residues 481–500 (KNAGVEGSLIVEKIMQSSSE).21 Surprisingly, when the same experiments were repeated with primary bone marrow macrophages from the mouse (whose Cpn60 protein has identical sequence to the human), three binding peptides were identified: DAYVLLSEKKISSQVIPA (aa241–260), NERLKLSDLGAVLUKVGTTS (aa391–410) and QKIGIEIKRTLKIPMTIA (aa461–480).22 These peptides have not been tested for their ability to activate monocytes and so the correspondence between these data and the results reported in this present study needs further analysis. However, it is striking that in both of the studies with human Cpn60, peptides within the C-terminal part of the equatorial domain containing residues 461–500 have been identified. Also of interest is the finding that the well-known human Cpn60 peptide p277, which has T cell immunomodulatory actions,23 and is in clinical trial for treating early-onset diabetes,24 is actually the human Cpn60 peptide, 437–460 (VLGCCCALLRCIPALDIPSTPANED), a peptide adjacent to the 461–491 peptide identified by the authors. Of interest, this 437–461 human peptide has no influence on human monocytes.25

In conclusion, we have shown by direct experimentation that the agonist site in the *M. tuberculosis* Cpn60.1 protein responsible for the ability of this molecule to stimulate human monocytes to generate pro-inflammatory cytokines is the equatorial domain C-terminal peptide 461–491 (DGSVVNVKSELPGHGLNVNTLSYGDLAAAD). Having defined this as the agonist site on this molecular chaperone it may be possible to use this information to develop antagonists of this site to prevent the pro-tubercular actions of this *M. tuberculosis* molecular chaperone.

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**Competing interests:** None declared.

**Ethical approval:** Not required.

**References**


