The Intercellular Signaling Activity of the Mycobacterium tuberculosis Chaperonin 60.1 Protein Resides in the Equatorial Domain*

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The major heat shock protein, chaperonin 60, has been established to have intercellular signaling activity in addition to its established protein-folding function. Mycobacterium tuberculosis is one of a small proportion of bacteria to encode two chaperonin 60 proteins. We have demonstrated that chaperonin 60.1 from this bacterium is a very active stimulator of human monocytes. To determine structure/function relationships of chaperonin 60.1 we have cloned and expressed the apical, equatorial, and intermediate domains of this protein. We have found that the signaling activity of M. tuberculosis chaperonin 60.1 resides in the equatorial domain. This activity of the recombinant equatorial domain was completely blocked by treating the protein with protease K, ruling out lipopolysaccharide contamination as the cause of the cell activation. Blockade of the activity of the equatorial domain by anti-CD14 monoclonal antibodies reveals that this domain activates monocytes by binding to CD14. Looking at the oligomeric state of the active proteins, using native gel electrophoresis and protein cross-linking we found that recombinant M. tuberculosis chaperonin 60.1 fails to form the prototypic tetradecameric structure of chaperonin 60 proteins under the conditions tested and only forms dimers. It is therefore concluded that the monocyte-stimulating activity of M. tuberculosis Cpn60.1 resides in the monomeric subunit and within this subunit the biological activity is due to the equatorial domain.

The end of the 20th century saw the discovery of the cell stress response and the crucial role of molecular chaperones, such as the prototypic tetradecameric chaperonin (Cpn)60 protein (1, 2), as protein-folding proteins vital for cell survival. In the past decade evidence has emerged that molecular chaperones are examples of moonlighting proteins with the capacity to act as intercellular signals of potential importance in controlling inflammation and immunity (3–7).

It is now established that the Cpn60 proteins from a number of bacteria, and from the mitochondrion, can activate a range of mammalian cells, including myeloid cells and vascular endothelial cells (5–7). In consequence, it is possible to hypothesize that bacterial Cpn60 proteins may be directly acting virulence factors (3). The initial observation that Cpn60 proteins could activate myeloid cells (8) used the Cpn60.2 protein from Mycobacterium tuberculosis, a protein more usually known as Hsp65 (9). It has subsequently been discovered that this bacterium contains two genes coding for Cpn60 proteins and that these proteins have ~70% sequence similarity (10). We have cloned both M. tuberculosis cnp60.1 genes and expressed and purified to homogeneity both recombinant Cnp60 proteins. Using these purified recombinant proteins we have shown that despite the substantial sequence identity there are major differences in their capacity to activate human monocytes (11). Thus the M. tuberculosis Cpn60.1 protein is 10–100 times more potent at stimulating human monocyte cytokine synthesis than is the Cpn60.2 protein and indeed may be the most potent cell signaling chaperonin 60 molecule. In addition, the monocyte-stimulating capacity of the Cpn60.1 protein is blocked by monoclonal antibodies binding to CD14. Such antibodies have no inhibitory effect with Cpn60.2. Such inhibition is not due to the blocking of contaminating lipopolysaccharide (LPS), because both recombinant proteins have equal, and biologically insignificant, levels of contaminating LPS.

Nothing is known about the relationship between the structure of Cpn60 proteins and their intercellular signaling actions. These proteins are believed to exist as oligomers, although a recent study (12) suggests that the mycobacterial proteins form dimers in vitro as well as in vivo. It is not known if their intercellular signaling capacity is due to the oligomer or the individual monomer. It is also not known if the cell-cell signaling activity requires the intact monomer or if activity resides in a smaller structural domain. To examine this we have cloned and expressed the M. tuberculosis cpn60.1 gene and examined the oligomeric status of the recombinant protein. We have also cloned, expressed, and purified the individual domains that constitute Cpn60.1 and we have tested these individual domains to identify if one or other exhibit the signaling activity of the parent protein.

MATERIALS AND METHODS

Source of Proteins—The recombinant M. tuberculosis Cpn60.1 protein was produced and purified as described previously (11). To prepare the individual domains the complete coding sequences of the M. tuberculosis cpn60.1 gene was amplified by PCR using the Epicenter FailSafe PCR system (Cambio, Cambridge, United Kingdom) and cloned into the expression vector pBAD/Myc-HisB (Invitrogen). The resulting plasmid was designated pBTBc601. This construct expresses the full-length protein containing an additional 21 amino acids comprising the myc epitope and six histidine residues at the C terminus of the protein. The respective protein was designated HcCpn60.1. The individual protein
domains were all based on this His-tagged version of the full-length protein. The equatorial domain as well as the equatorial domain in conjunction with the intermediate domain of Cpn60.1 was cloned by inverse PCR using the plasmid pBTBc601 as template. The resulting plasmids were called pBTBc601a and pBTBc601ab. The resulting proteins were designated HcCpn60.1a (equatorial domain of Cpn60.1) and HcCpn60.1ab (equatorial and intermediate domain of Cpn60.1). The apical domain of Cpn60.1 was obtained by amplifying the respective fragment by PCR and cloning the PCR product into the vector pBAD/Myc-HisB to produce the protein designated HcCpn60.1c.

All recombinant proteins were expressed in the Escherichia coli strain, Top10. HcCpn60.1a, HcCpn60.1a, and HcCpn60.1ab were all formed as inclusion bodies. To produce folded proteins the inclusion bodies were re-suspended in 6 M guanidine hydrochloride, and this was quickly diluted by adding a 10-fold volume of PBS. Refolding was continued overnight at 4 °C, and the protein was clarified by centrifugation at 30,000 × g and subsequent filtration of the cleared protein through a 0.25-μm filter. The recombinant protein was then bound to Probond nickel-chelating resin (Invitrogen), and bound protein was washed with 5 column volumes of a 2 mg/ml solution of polymyxin B (Sigma) in PBS to remove any contaminating LPS. Protein was eluted from the column by application of a 10-300 mM imidazole gradient in 10 column volumes, and fractions containing the recombinant chaperonin were pooled. The eluted protein was then dialyzed against 20 mM sodium phosphate buffer, pH 6.8. Recombinant proteins were further purified by anion exchange chromatography using a Poros HQ column on a BioCad Sprint chromatography system (Applied Biosystems, Warrington, UK). The purification of HcCpn60.1c was essentially the same as described except for the fact that no renaturation was required, and soluble protein was directly bound to the nickel-nitrolotriacetic acid matrix. E. coli GroEL and GroES were purchased from Stressgen (Bioquote, York, UK).

Analysis of Protein Purity—Protein purity was analyzed by SDS-PAGE. To do this 50 pmol of each purified recombinant protein (1.1–2.9 μg, dependent on protein) was run on a 4–12% Bis-Tris gradient gel (Invitrogen), and the gel was subsequently stained with Simply Blue Safe Stain (Invitrogen).}

FIG. 1. SDS-PAGE of purified recombinant M. tuberculosis chaperonin 60.1 proteins. Full-length Cpn60.1 protein (lane 1), equatorial domain of Cpn60.1 (lane 2), equatorial domain plus intermediate domain of Cpn60.1 (lane 3), and apical domain of Cpn60.1 (lane 4). 50 pmol of each protein was loaded per lane. All proteins contain an additional 21 amino acids at their C terminus, including 6-His residues.

RESULTS

Physicochemical Characteristics of Recombinant Cpn60 Variants—All the recombinant proteins were analyzed by SDS-PAGE and stained with a sensitive commercially available Coomassie Blue stain. All proteins were of the expected protein mass. Even at high protein loadings there were no visible contaminating bands (Fig. 1). The predicted structure of the intact mycobacterial Cpn60.1 monomer and its constituent domains are shown in Fig. 2.

LPS Contamination of Recombinant Proteins—Using polymyxin B to wash the recombinant proteins on the nickel affinity columns lowered the concentrations of LPS in the Cpn60 protein preparations to <2 pg/μg protein.

Oligomeric Structure of the Recombinant Proteins—Chaperonin 60 proteins are known to form higher oligomeric quaternary structures (1). To determine the oligomeric structure of the M. tuberculosis Cpn60.1 protein, as well as of the subdomains, proteins were first run on a native polyacrylamide gel. The result is shown in Fig. 3A. GroEL and GroES from E. coli were included as controls. As expected GroEL forms a large complex, whereas GroES forms the characteristic heptamer. In contrast, neither the full-length protein nor the protein domains form such higher molecular mass complexes. In relation to GroEL and GroES, the M. tuberculosis Cpn60 full-length proteins, the apical domain, and the apical plus intermediate domain seem to be in the dimeric form (110, 60, and 80 kDa, respectively). The apical domain seems to only form monomers (~23 kDa). Because native gel electrophoresis does not allow for an accurate mass prediction, proteins were incubated with the protein cross-linking reagent, disuccinimidyl suberate, to

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M. tuberculosis H9262

14273

188

17

16

15

14

13

12

11

10

9

8

7

6

5

4

3

2

1

0

100 μg/ml streptomycin, and 2 mM glutamine (all from Invitrogen). The cells were counted and diluted to 2 × 10⁶ cells/ml, and 1-ml aliquots were dispensed into 24-well tissue culture plates. Cells were incubated for 1 h at 37 °C and then washed with PBS to remove non-adherent cells. Finally, cells were resuspended in RPMI medium containing 2% fetal calf serum 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (all from Invitrogen).

Induction and Measurement of Cytokines—Human monocytes were exposed to graded concentrations of the various recombinant Cpn60.1 proteins for 16 h, and the medium was removed and frozen to −70 °C until analysis. The concentration of cytokines, namely IL-1β, IL-6, and tumor necrosis factor α, in the medium supporting the cells was assessed by enzyme-linked immunosorbent assay using paired antibodies to these cytokines from R&D Systems (Abingdon, UK). In most experiments the activating protein was incubated with cells in the presence of 20 μg/ml polymyxin B to ensure that activity recorded was not due to LPS. To ascertain the role of CD14 in cell activation, neutralizing antibodies to CD14 (R&D Systems) or the appropriate isotype antibody control were added to the activated cell culture at a concentration of 10 μg/ml. Results are means of three independent stimulation experiments using cells from the sameuffy coat residue. In different experiments different buffy coat residue was used, resulting in some variation of the results.

RESULTS
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FIG. 2. Structural model of the *M. tuberculosis* heat shock protein Cpn60.1. The structural model was obtained by sequence alignment of *M. tuberculosis* Cpn60.1 with GroEL using the ExPASy modeling server. A, three-dimensional representation of the monomer structure of the Cpn60.1 protein. The different domains are indicated by different shading. B, the sequence of Cpn60.1 is provided with the domain structure outlined underneath. Arrows and boxes indicate β-sheets and helical regions, respectively. The domains are again indicated by different shading of the arrows and boxes (crosshatch, equatorial domain; 45° hatch, intermediate domain; vertical hatch, apical domain).

FIG. 3. Native gel electrophoresis and cross-linking of *M. tuberculosis* Cpn60.1 and the subdomains. A, native gel electrophoresis was performed with 5 µM protein. Proteins were incubated in 50 mM Tris/Cl, 5 mM MgCl₂, 150 mM KCl, 20% (v/v) glycerol for 2 h and subsequently resolved on 6% Tris/glycine native gel. The positions of GroEL and GroES from *E. coli* are marked by arrows. Lane 1, non-His-tagged parent protein; lane 2, His tag full-length protein; lane 3, equatorial domain; lane 4, equatorial plus intermediate domain; lane 5, apical domain; lane 6, GroEL and GroES from *E. coli* as positive control. B, each protein at 8 µM was incubated with 400 µM disuccinimidyl suberate and subsequently resolved on SDS-PAGE and visualized with Simply Blue Safe Stain. Lane 1, non-His-tagged parent protein; lane 2, His tag full-length protein; lane 3, equatorial domain; lane 4, equatorial plus intermediate domain; lane 5, apical domain; lane 6, GroEL from *E. coli* as positive control.

determine if protein subunits were forming complexes in solution (Fig. 3B). The *E. coli* GroEL clearly shows the presence of higher oligomers. However, it is evident that the *M. tuberculosis* Cpn60.1 proteins, under the conditions tested, do not form higher oligomeric structures. The full-length His-tagged protein, the equatorial domain, and the equatorial plus intermediate domain show faint bands at a molecular mass that approximates a dimeric structure. However, no higher oligomeric structures were evident. The apical domain was wholly monomeric.

Effect of Introducing the His Tag on the Cytokine-inducing Activity of Cpn60 Proteins—In all previous studies we have used recombinant *M. tuberculosis* Cpn60.1 proteins lacking a His tag and purified by conventional chromatographic techniques. In this study the full-length Cpn60.1 protein was expressed from a plasmid construct that generated a fusion protein containing 21 extra amino acids at the C terminus, including 6-terminal histidine residues. Comparison of these two forms of the recombinant protein revealed that the His-tagged protein was an active cytokine-inducing protein, although it was somewhat less active than the non-fusion version of the protein (Fig. 4).

Comparison of the Cytokine-inducing Activity of the Recombinant Cpn60.1 Domains—Human monocytes were incubated with a range of molar concentrations of the parent Cpn60.1 fusion protein or with the various recombinant Cpn60.1 domains and the synthesis of cytokines analyzed. The parent protein (HcCpn60.1), the equatorial domain (HcCpn60.1a), and the equatorial plus intermediate domain (HcCpn60.1ab) all demonstrated the capacity to induce pro-inflammatory cytokine synthesis. In most experiments the equatorial domain and the equatorial domain linked to the intermediate domain showed higher potency and efficacy than the full-length parent protein. This was particularly marked with the induction of IL-1β synthesis. In contrast, in none of the many separate samples of human monocytes tested did the apical domain demonstrate any capacity to induce cytokine synthesis (Fig. 5).

Effect of Proteinase K Treatment on Cytokine-stimulating Activity of Cpn60.1 Variants—We have used proteinolysis in previous studies as a control for LPS contamination and to check on the possible activity of internal peptides (e.g. Ref. 11). The various recombinant Cpn60.1 proteins/domains were digested with proteinase K prior to adding to cells, and the complete proteolysis of each recombinant protein is shown in Fig. 6A. Exposure to this proteinase obliterated the cytokine inducing activity of the full-length protein and the equatorial and equatorial plus intermediate domain proteins (Fig. 6B). This effect was seen in the presence as well as the absence of polymyxin B. As expected, proteinase K treatment had no effect on the cytokine-stimulating activity of LPS (data not shown).

CD14 Dependence of Recombinant Cpn60 Proteins—The full-length recombinant Cpn60.1 and the protein domain were in-
cubated with human monocytes that had been pre-treated with either the non-fusion protein version of M. tuberculosis Cpn60.1 or the recombinant Cpn60.1 containing myc-6-His at the C terminus. The wild type protein lacking the C-terminal tag (●) and the C-terminal His-tagged version (■) were tested at a range of molar concentrations from 20 to 160 nM (1.15–11.8 μg/ml) for their ability to induce IL-6. Polymyxin B was added at 20 μg/ml with all concentrations.

DISCUSSION

It has been established for some years that, despite the significant sequence conservation, molecular chaperones such as Cpn60 from bacterial and eukaryotic pathogens are potent immunogens and modulators of immunity (15, 16). One explanation for the ability of these proteins to activate innate and acquired immunity is that they have the capacity to act as intercellular signals for eukaryotic cells (4–7). The past decade has seen a growing number of reports of the ability of molecular chaperones, such as Cpn60, to activate monocytes, macrophages, and vascular endothelial cells (4–6, 17). In this respect, molecular chaperones could be thought of as pathogen-associated molecular patterns; that is, as highly conserved components of pathogens for which the host has evolved recognition systems in the form of pathogen recognition receptors (18). An obvious difference between pathogen-associated molecular patterns such as LPS, peptidoglycan, and lipoarabinom
mannan and the molecular chaperones is the fact that the latter are also produced by the host and there is substantial evidence that host molecular chaperones, such as human Cpn60 (Hsp60), can stimulate immune cells (19–21).

The authors’ interest in the signaling actions of bacterial chaperonins arose from the discovery that the potent bone-resorbing mediator exported by the oral bacterium, Actinobacillus actinomycetemcomitans was the chaperonin 60 protein of this organism. We then showed that the E. coli Cpn60 protein, GroEL, was a potent stimulator of bone resorption. However, neither the M. tuberculosis nor the M. leprae Cpn60.2 protein was able to induce bone resorption (22). Further investigation of the mycobacterial chaperonins revealed that neither of the two Cpn60 proteins produced by M. tuberculosis could stimulate bone resorption. In fact, the major bone-resorbing moiety of this bacterium turned out to be the co-chaperone, Cpn10 (23). This was the first evidence that the chaperonin 60 protein was not a unitary molecule and that different Cpn60 proteins from different sources could express different patterns of biological activity.

To investigate the M. tuberculosis Cpn60 proteins in more detail we cloned and expressed both genes as non-fusion proteins and purified them to homogeneity. Despite >70% sequence similarity between both proteins, it was found that Cpn60.1 was significantly more potent and efficacious than Cpn60.2. Indeed, this mycobacterial protein appears to be the most biologically active form of Cpn60 (6, 11). A major difference between these two proteins was that Cpn60.1 was blocked by neutralizing antibodies to CD14 implicating that this protein activates monocytes via the CD14/TLR4 receptor complex. In contrast, Cpn60.2 was unaffected by antibodies to CD14 (11). This suggests that these two proteins bind to different receptors or to different parts of the CD14/TLR4 receptor complex. This has been confirmed by studies of competitive binding of Cpn60 proteins from different species to macrophages (24).

There is much that is not understood about the interaction of Cpn60 proteins and immune cells. To investigate the structure-function relationships of the M. tuberculosis Cpn60.1 protein we have developed a structural model based on the crystal structure of GroEL. This has allowed the development of a strategy for cloning and expressing the individual domains of this protein. Examination of the structure of GroEL reveals the presence of three domains in the monomer: (i) the equatorial domain, which makes contact with the corresponding equatorial domain in the oligomeric structure; (ii) the intermediate domain; and (iii) the apical domain (Fig. 1). To simplify the purification of the individual domains it was decided to produce all recombinant domains as C-terminal myc-6-His-tagged fusion proteins, and the parent protein was also recloned into the same vector. This allowed the production of: (i) the intact parent protein; (ii) the equatorial domain; (iii) the equatorial domain linked to the intermediate domain; and (iv) the apical domain. Some of these recombinant domain proteins formed inclusion bodies, but it proved possible to obtain them in soluble form.

The first finding from this study was that addition of the myc epitope and 6-His residues to the C terminus of Cpn60.1 still allowed the generation of proteins with biological activity. Comparison of the fusion protein form of Cpn60.1 with the recombinant non-fusion protein form revealed that there had been some loss of potency. This may suggest that the C terminus of Cpn60.1 is also involved in the cell-cell signaling activity of this protein. With an active parent molecule it was now possible to determine which of the domains of Cpn60.1 contributed to the activity of this protein. This revealed that the equatorial domain and the equatorial domain plus the intermediate domain retained biological activity with the former being as active, or even more active, on a molar basis, as the parent molecule. The recombinant apical domain was inactive under the conditions tested. We have previously reported that the apical domain contained a peptide, residues 195–219, which had cytokine-stimulating activity, and it was surprising that this domain had no biological activity. It should be noted that this peptide was used at much higher molar concentration in the previous study (11) compared with the apical domain in this study. Moreover, the model we present here indicates that this particular sequence forms part of a β-sheet with a large loop in contrast to the helical structure prediction for the peptide. It also cannot be ruled out that the addition of the C-terminal His tag has a negative effect on any residual cytokine stimulating activity of the apical domain.

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\[ \text{Fig. 7. CD14 dependence of the cytokine-inducing activity of the M. tuberculosis Cpn60.1 proteins. Human monocytes were either pre-treated with 10 µg/ml of a neutralizing anti-CD14 (●) or with 10 µg/ml of an isotype control (♦) antibody before addition of a range of concentrations of recombinant full-length Cpn60.1 protein or the various recombinant domains. IL-6 production (A–C), IL-1β production (D–F) Full-length protein (A and D), equatorial domain (B and E), and equatorial domain plus intermediate domain (C and F).} \]
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diate domain to this construct does not alter the activity. We therefore conclude that the sequence 195–219 is not responsible for the activity of the full-length protein and that the primary cytokine stimulating activity is associated with the equatorial domain. Recent studies with the human mitochondrial Cpn60 protein also implicate the equatorial domain as the primary domain for interaction with and activation of cells of myeloid origin. We have shown that deletion mutants from the C-terminal end of the human mitochondrial Cpn60 protein resulted in the loss of ability to induce bone resorption (25). Another study has identified that an epitope important for receptor binding on macrophages mapped to the C terminus of the equatorial domain (26).

The current paradigm is that the folding activity of chaperonin 60 proteins requires them to form higher oligomeric structures. GroEL, the prototypic member of this family, forms a tetradecamer consisting of two seven membered rings (1, 2). It is not known if the cell-cell signaling activity of these proteins depends on the formation of such oligomeric structures. Studies with GroEL, in which trypsinization failed to block activity (27), and the finding that boiling the mycobacterial Cpn60 proteins did not inhibit activity (11) suggest that the cell-cell signaling activity of Cpn60 proteins resides in the monomer and in submonomeric species. Here we show that even at high concentration neither the full-length M. tuberculosis Cpn60.1 protein nor its active subdomains form larger oligomers but exist as dimers. Thus under the conditions used for stimulating peripheral blood mononuclear cells the proteins are either in the dimeric or monomeric form. This is further evidence that the cell signaling activity is not associated with protein oligomerization. Indeed, it has recently been reported that the mycobacterial proteins reside as dimers in the cell and that the folding properties of M. tuberculosis Cpn60.1 do not require this protein forming a higher oligomeric structure (12).

There is still debate in the literature about the meaning of the cell-cell signaling activity of Cpn60 proteins, and one recent paper has attributed the bioactivity of human Cpn60 to contaminating LPS (28). In another study the bioactivity of Hsp70 was also attributed to LPS contamination (29). We have developed methods for removing both LPS and contaminating proteins from recombinant chaperonin 60 proteins (27, 30), and all samples of methods for removing both LPS and contaminating proteins from all samples of these proteins are either in the dimeric or monomeric form. This is further evidence that the activity of these proteins is not due to contaminating LPS or other non-proteinaceous contaminants.

The activity of the non-fusion protein version of Cpn60.1 was completely inhibited by neutralizing antibodies to CD14 (11). The activity of the intact fusion protein version of Cpn60.1, the equatorial domain, and the equatorial and intermediate domain versions was also blocked by such antibodies indicating that cell activation is CD14-dependent.

The experimental data presented in this report allow the following conclusions to be reached. The biological activity of the M. tuberculosis Cpn60 proteins resides in the individual monomers. This does not rule out the possibility that oligomerization enhances activity. The equatorial domain contains the binding site for human myeloid cells. The recombinant Cpn60.1 equatorial domain is therefore an excellent starting point for defining the exact residues and structure conferring the potent cell signaling activity of this molecular chaperone.

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