Mycobacterium tuberculosis Chaperonin 10 Stimulates Bone Resorption: A Potential Contributory Factor in Pott's Disease

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Summary

Pott's disease (spinal tuberculosis), a condition characterized by massive resorption of the spinal vertebrae, is one of the most striking pathologies resulting from local infection with *Mycobacterium tuberculosis* (Mt; Boachie-Adjei, O., and R.G. Squillante. 1996. *Orthop. Clin. North Am.* 27:95–103). The pathogenesis of Pott's disease is not established. Here we report for the first time that a protein, identified by a monoclonal antibody to be the Mt heat shock protein (Baird, P.N., L.M. Hall, and A.R.M. Coates. 1989. *J. Gen. Microbiol.* 135:931–939) chaperonin (cpn) 10, is responsible for the osteolytic activity of this bacterium. Recombinant Mt cpn10 is a potent stimulator of bone resorption in bone explant cultures and induces osteoclast recruitment, while inhibiting the proliferation of an osteoblast bone–forming cell line. Furthermore, we have found that synthetic peptides corresponding to sequences within the flexible loop and sequence 65–70 of Mt cpn10 may comprise a single conformational unit which encompasses its potent bone-resorbing activity. Our findings suggest that Mt cpn10 may be a valuable pharmacological target for the clinical therapy of vertebral tuberculosis and possibly other bone diseases.

Tuberculosis is epidemic, accounting for 7% of the annual worldwide death toll (1). Tuberculous infections of bone, particularly of the spinal vertebrae (Pott's disease), are still common in the third world (2). It is not known how *Mycobacterium tuberculosis* (Mt)[1] infections of bone cause bone breakdown. Healthy bone is maintained by a dynamic equilibrium between the mesenchymal bone matrix–forming osteoblast cell lineage and the myeloid bone–resorbing osteoclast cell lineage (3). Mt infection of the spine obviously alters this dynamic equilibrium, resulting in the net loss of the extracellular matrix of vertebral bone and collapse of the vertebrae. Whether this loss of bone matrix is the result of the direct action of components of Mt, for example, the LPS-like cell surface molecule lipoarabinomannan (LAM) (4), on bone cells, or an indirect activation of inflammatory cells leading to bone cell activation, is not established. Evidence is appearing to suggest that molecular chaperones have biological actions in addition to their intracellular protein–folding activity (5). For example, chaperonin (cpn)10 has been found to be an essential growth and immunosuppressive factor in early pregnancy (6), and cpn60 induces cytokine synthesis (7) and resorption of bone (8).

In this study we have established that the bone resorbing activity of Mt is due to cpn10 which is as active as the most potent osteolytic cytokine, IL-1 (9, 10). Mt cpn10 also appears to induce the recruitment of osteoclasts in calvaria, and it is notable that calvarial bone resorption induced by this cpn can be completely blocked by the osteoclast-inhibiting hormone, calcitonin (11). Mt cpn10 was also found to inhibit the proliferation of cultured osteoblasts.

[1]*Abbreviations used in this paper:* cpn, chaperonin; LAM, lipoarabinomannan; Mt, *Mycobacterium tuberculosis*.

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Using a series of NH_2- and COOH-terminal truncated peptides, we have identified sites in Mt cpn10 responsible for the osteolytic activity of this molecular chaperone. We have identified the flexible loop of Mt cpn10 and the sequence 65–70 as regions most probably responsible for the bone-modulating bioactivity of this molecule.

Materials and Methods

Mycobacterial Sonicate. The sonicate was prepared by sonicating a suspension of viable virulent Mt (strain H37Rv) at 4°C for 1 min intervals, followed by a 1 min rest period, for a total period of 1 h. The sonicated material was then centrifuged at 100,000 *g* for 1 h, and the supernatant was filtered through a 0.22-μm membrane filter.

mAbs. Both the mAb to Mt cpn10 (SA12; 12) and the mAb to Mt cpn60 (TB78; reference 13) were obtained from murine ascites in a sufficiently high titer to bind to Mt cpn10 or cpn60 at the dilutions used in this study. SA12 is specific for mycobacterial cpn10 and TB78 is specific for mycobacterial cpn60. Neither of these mAbs are cross-reactive with any other Mt protein (14). The mAb to LAM (CS-35) of isotype IgG3 was obtained from concentrated tissue culture supernatant with a titer of 1:2,000 by Western blot analysis (Belisle, J.T., personal communication). CS-35 was raised against *Mycobacterium leprae* LAM and is cross-reactive with Mt LAM at a dilution of 1:1,000 by Western blot analysis (15). CS-35 was used at a 1:1,000 dilution in the bone resorption assay.

Mt cpn10 Peptides. r-Mt cpn10 was expressed in *Escherichia coli* and purified by reversed-phase HPLC to >97% purity as previously described (16). The synthetic peptide fragments were prepared and purified by isoelectric focusing and by reversed phase HPLC to >95% purity as previously described (17). Before addition to the bone explants, r-Mt cpn10 was passed down a Polymyxin B–agarose column (Detoxigel column; Pierce, Rockford, IL) to remove any contaminating LPS. The composition of the peptides was confirmed by amino acid analysis and mass spectroscopy. All peptides were tested for LPS using the limulus amoebocyte lysate assay (Whittaker M.A. Bioproducts, Inc., Walkersville, MD). All peptides tested negative, indicating the presence of <0.03 endotoxin U LPS.

Calcium Release and Osteoclast Recruitment in Murine Calvaria. The calvarial bone resorption assay was performed as described (18). In brief, calvaria were removed from 5-d-old MF1 mice, adherent connective tissue was dissected away, and the calvarial bone was halved, with each half being cultured separately on stainless steel grids. Calvaria were cultured in groups of 5 replicates in 30-mm dishes with 1.5 ml Biggers, Gwatkin, and Jenkins medium (ICN Biomedicals, Inc., Thame, UK) containing 5% heat-inactivated rabbit serum (GIBCO BRL, Paisley, UK) and 50 μg/ml ascorbic acid (Sigma Chemical Co., Poole, UK). After 24 h in culture, the media was replaced with media containing various concentrations of sonicated Mt, r-Mt cpn10 or Mt cpn10 peptides with or without a range of concentrations of mAb to Mt cpn10 (SA12; reference 12), mAb to Mt cpn60 (TB78; reference 13), mAb to Mt LAM at 1:1,000 dilution. Calvaria were cultured for a further 48 h and then the calcium released into the medium was measured by automated colorimetric analysis (19).

After removal of the media supporting the calvarial explants for calcium assay, the explants were then used for the measurement of osteoclast numbers by a modification of the method of Marshall et al. (20). The calvaria were fixed in 95% ethanol, 5% glacial acetic acid for 24 h, and were then washed in PBS containing 1 mg/ml BSA followed by reaction in the histochemical substrate mixture (obtained from Sigma Chemical Co., and used according to the manufacturer's instructions) for the localization of tartrate-resistant acid phosphatase (TRAP) activity, a marker enzyme for osteoclasts. Bones were then washed in PBS, decalcified, and fixed in 12.5% glutaraldehyde (BDH Chemicals, Ltd., Poole, UK) in 1 M hydrochloric acid (BDH Chemicals, Ltd.) for 5 min. Finally, the bone explants were washed and mounted in Aquamount (BDH Chemicals, Ltd.). Each calvarial explant was then scanned by transmitted light microscopy and TRAP-positive cells containing three or more nuclei were counted. The individual counting the cells was unaware of the treatment to which each explant had been exposed. Control cultures included unstimulated calvaria (to determine spontaneous release of calcium) and calvarial cultures stimulated with 1 μM prostaglandin (PG; to demonstrate that bone is responsive and to give a measure of the maximal response). r-Mt cpn10 and Mt cpn10 peptides were tested in a minimum of three experiments and gave reproducible results.

Osteoblast Proliferation. The measurement of cell proliferation was as previously described (21). In brief, the human osteoblast-like cell line MG63 (CRL 1427; American Type Culture Collection, Rockville, MD) was cultured at a density of 15,000 cells/well in 96-well plates and incubated overnight at 37°C in DMEM (Gibco) plus 10% FCS (Sigma Chemical Co.) in 5% CO_2/air. The media were then removed and cells were washed twice with sterile Hank's solution (Sigma Chemical Co.). To measure antiproliferative activity, various concentrations of r-Mt cpn10 or truncated peptides were added in DMEM containing 2% FCS, to the MG 63 cells. Cells were incubated for 24 h at 37°C. During the last 6 h of culture, 0.05 μCi of [^{3}H]thymidine (Amersham International plc, Amersham, UK) was added to cells. The media were then removed and the cells fixed in 5% trichloroacetic acid. 100 μl of 0.5 M NaOH was used to lyse cells, this being neutralized by an equal volume of 0.5 M HCl. Radioactivity incorporated into nuclear DNA was measured by scintillation spectrometry. The cytotoxicity of the r-Mt cpn10 was determined by lactate dehydrogenase release, measured by the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Heidelberg, Germany). Data has been generated from a minimum of three separate experiments.

Homology Modeling of Mt cpn10. The Mt cpn10 model was generated by homology modeling of the Mt cpn10 sequence onto the atomic coordinates of the GroES structure of the monomer with the flexible loop assigned. The model was energy minimized with QUANTA/CHARMm (Molecular Simulations, Inc., San Diego, CA) using a nonbonded cutoff of 14 Å and a dielectric constant distance dependence until the root mean squared deviations were <0.001 Kcal/Å. The side chains were minimized first, keeping the backbone fixed. This was followed by minimization of the whole monomer. The heptamer was generated from the monomer by the symmetry operations relating the GroES subunits. The same energy minimization procedure was repeated for the final Mt cpn10 heptamer model, which was displayed with the SYBYL molecular modeling package (Tripos UK, Milton Keynes, UK).

Results and Discussion

Sonicates of viable Mt added to explants of murine calvarial bone produced a dose-dependent stimulation of bone resorption, measured as calcium release into the tissue culture medium. Osteoclast numbers in calvarial explants were

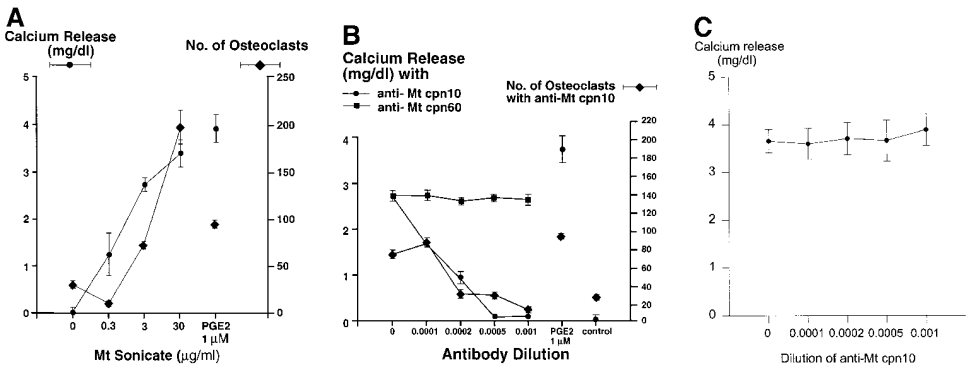

Figure 1. Effect of Mt sonicate on bone resorption. (*A*) Release of calcium from explants of murine calvaria and induction of osteoclast recruitment in the calvaria by dilutions of a sonicate of viable Mt. (*B*) The effect of adding various concentrations of mAb (12) SA12 (which recognizes Mt cpn10) or a mAb to Mt cpn60 (13) to bone explants stimulated with a fixed concentration of Mt sonicate (3 μg/ml) on the release of calcium from calvarial explants and on the numbers of osteoclasts in the calvaria. Controls with no Mt cpn10 (unstimulated calvaria) and with 1 μM PG are shown for comparison. (*C*) The effect of increasing concentrations of SA12 on the bone resorbing activity of PG. Results are expressed as the mean and standard deviation of five replicate cultures.

counted and showed a parallel increase (Fig. 1 *A*). The Mt sonicate–induced stimulation of bone resorption was dose dependently and completely inhibited by a neutralizing mAb to Mt cpn10 (SA12; reference 12), but not by a subclass-matched neutralizing mAb to Mt cpn60 (TB78; reference 13). Likewise, the mAb SA12 caused a dose-dependent decrease in the numbers of osteoclasts present in the calvarial explants (Fig. 1 *B*). In contrast, SA12 had no effect on the stimulation of bone resorption induced by PG (Fig. 1 *C*). Purified Mt LAM, added at a concentration of 1 μg/ml, had no osteolytic activity, and neutralizing mAb to LAM did not inhibit the bone resorption induced by the Mt sonicate (results not shown). Addition of polymyxin B had no effect on the bone resorbing activity of the Mt sonicate.

Purified r-Mt cpn10 caused a dose-dependent stimulation of calcium release from cultured calvaria with osteolytic activity being noted at a concentration of 1 ng/ml (equivalent to 100 pmol) that was reproducible and statistically significant ($P < 0.01$; Fig. 2 *A*). The bone resorbing activity of Mt cpn10 was dose dependently and completely inhibited by mAb SA12 (Fig. 2 *B*). The osteoclast-inactivating hormone, calcitonin, at a concentration of 10 ng/ml, also blocked r-Mt cpn10-induced bone resorption (results not shown). Addition of polymyxin B had no effect on the bone resorbing activity of r-Mt cpn10.

Addition of r-Mt cpn10 to subconfluent cultures of the human osteoblast-like cell line MG63 caused significant inhibition of cell proliferation at concentrations ⩾1 nM (Fig. 3). Inhibition of proliferation was not due to cytotoxicity of the r-Mt cpn10.

A panel of 11 NH_2- and COOH-terminal truncated peptides and short peptides (16) were used to define the specific structural features of r-Mt cpn10 responsible for its osteolytic and osteoblast antiproliferative activities. These peptides corresponded to residues 1–25, 1–58, 26–99, 46–99, 51–99, 54–99, 59–99, 65–99, 71–86, 75–99, and 91–99. Graded concentrations of each peptide were tested separately in each assay in three separate experiments. 2 of these

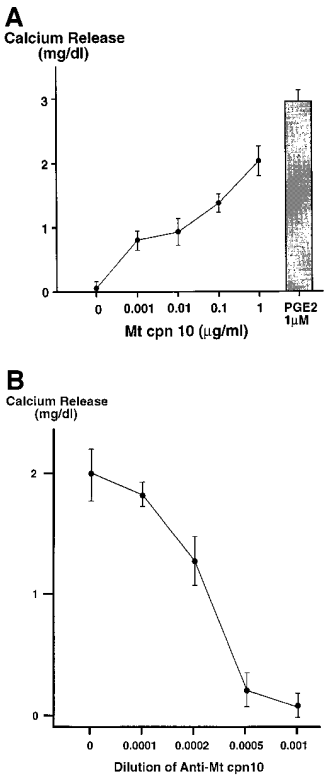

Figure 2. Effect of r-Mt cpn10 on bone resorption. (*A*) Dose-dependent stimulation of calcium release from murine calvarial explants cultured in the presence of purified r-Mt cpn10. The filled column represents the calcium released from bone explants cultured with 1 μM PG. (*B*) Effect of adding graded concentrations of mAb SA12 to calvarial bone explants stimulated with 100 ng/ml r-Mt cpn10. Results are expressed as the mean and standard deviation of five replicate cultures.

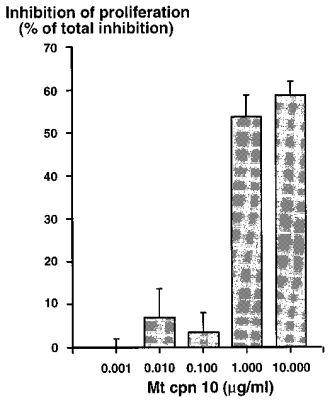

Figure 3. Mt cpn10–induced inhibition of osteoblast proliferation. The effect of purified r-Mt cpn10 on the proliferation of the human osteoblast–like cell line MG 63. Inhibition of proliferation is measured as the percentage of total inhibition (no thymidine incorporation) calculated from the percentage of decrease in thymidine incorporation for a control cell culture.

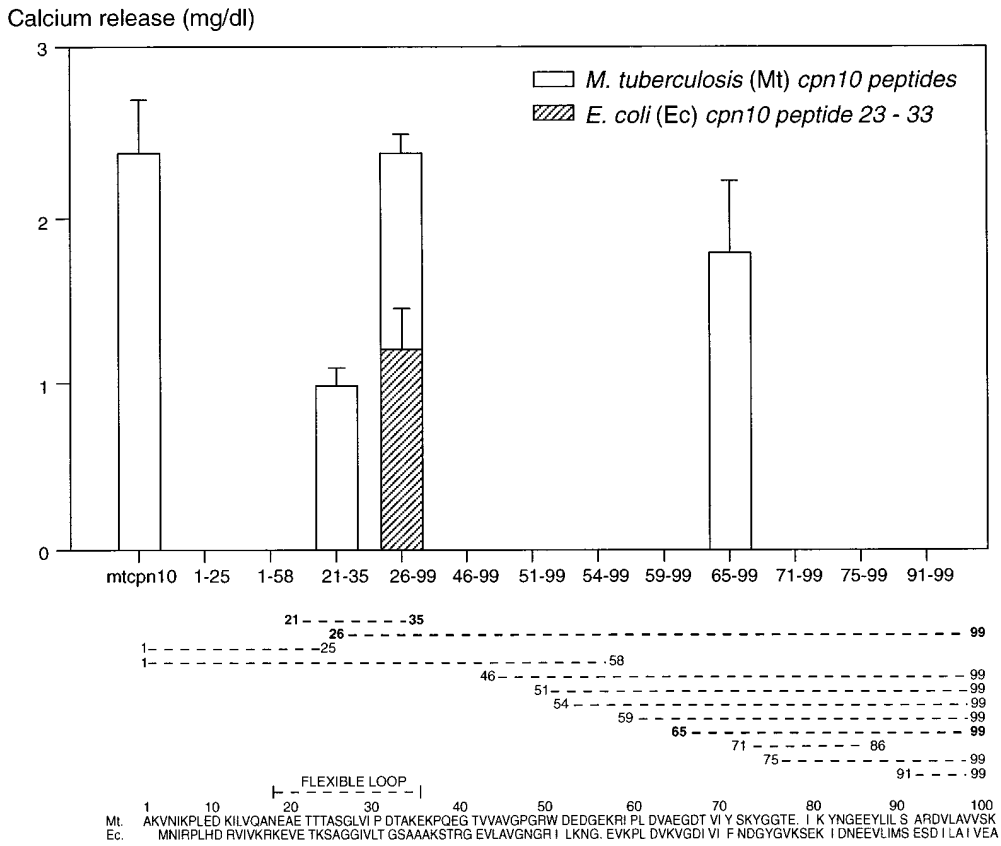

Figure 4. Bone resorbing activity of NH_2- and COOH-terminal truncated Mt cpn10 peptides compared to full-length Mt cpn10. The top graph shows the amount of calcium released, in one typical experiment, from calvarial explants cultured with r-Mt cpn10 or truncated synthetic Mt cpn10 peptides at a concentration of 1 μg/ml. The bottom section shows the sequences of the various Mt cpn10 peptides and the sequences of Mt and *E. coli* cpn10. A peptide GGIVLTGSAAA which spans residues 23–33 in the mobile loop of *E. coli* cpn10 also demonstrated osteolytic activity (*shaded column*). The background release of calcium has been subtracted. Results are expressed as the mean and standard deviation of five replicate cultures.

11 peptides, 26–99 and 65–99, exhibited reproducible osteolytic activity (Fig. 4). Polymyxin B had no inhibitory effects on the activity of these peptides. Peptide 26–99 contains sequences that are within the flexible loop region of Mt cpn10 (residues 16–35). To determine if this flexible loop contributed to the osteolytic activity, two short peptides within the flexible loop in Mt cpn10 (21–35: TTTASGLVIPDTAKE) and in the *E. coli* cpn10 (GroES residues 23–33: GGIVLTGSAAA) were synthesized and were also found to have osteolytic activity in the calvarial assay (Fig. 4). Mt, unlike *E. coli*, is able to secrete extracellular cpn10 (22), which has important implications for the pathogenic effects of Mt cpn10 in vivo.

All 12 Mt cpn10 peptides were repeatedly tested for antiproliferative activity but even at very high concentrations, none showed any ability to inhibit osteoblast proliferation.

Peptide 1–58 was inactive in the bone resorption assay, although it contains the predicted flexible loop. The most likely explanation is that the structure of 1–58 differs from that of whole protein, because peptide 1–58 is a dimer (16), the aggregation of which is unusual as it occurs via the NH_2-terminal region in contrast to the whole protein in which contact between two neighboring protomers involves the COOH-terminal tail of one protomer and the NH_2-terminal region of the other (23).

Peptides 46–99, 51–99, 54–99, and 59–99 were also inactive, although they contain the active 65–99 sequence, and again, structural differences are the likely explanation for this discrepancy. For example, the structure of the inactive peptide 59–99 has been assigned to that of four antiparallel β strands (24), but circular dichroism spectroscopy data with the active peptide 65–99 (data not shown) indicate that the latter is mainly composed of the random coil conformation. Peptide 26–99 is active in the bone resorption assay since most of the mobile loop is part of its NH_2-terminal tail and is probably accessible to solvent (and hence a receptor) as often happens to the NH_2-terminal and COOH-terminal regions of polypeptides and proteins. For the same reason, amino acids 65–70 would be considered the active sequence in peptide 65–99.

A molecular model of heptameric Mt cpn10 was derived from the *E. coli* cpn10 crystal structure (reference 23; Fig. 5). The sequences derived from the peptide data which contribute to the osteolytic activity are colored red and correspond to the flexible loop (21–35) at the bottom outer edge of the heptamer which is in close proximity to the sequence 65–70. Although the flexible loop is exposed in the heptameric model, the sequence 65–70 is inaccessible at the subunit interface. Furthermore, based on studies with GroES, which dissociates to monomers <1 μM (25), Mt cpn10 would be expected to dissociate at the concentrations used in all these biological assays. This suggests that an alternative oligomeric form of Mt cpn10 may be required for osteolytic activity. A tetrameric form of Mt cpn10 has been reported (16), and this may be the osteolytically active form. Alternatively, Mt cpn10 might assemble as a hep-

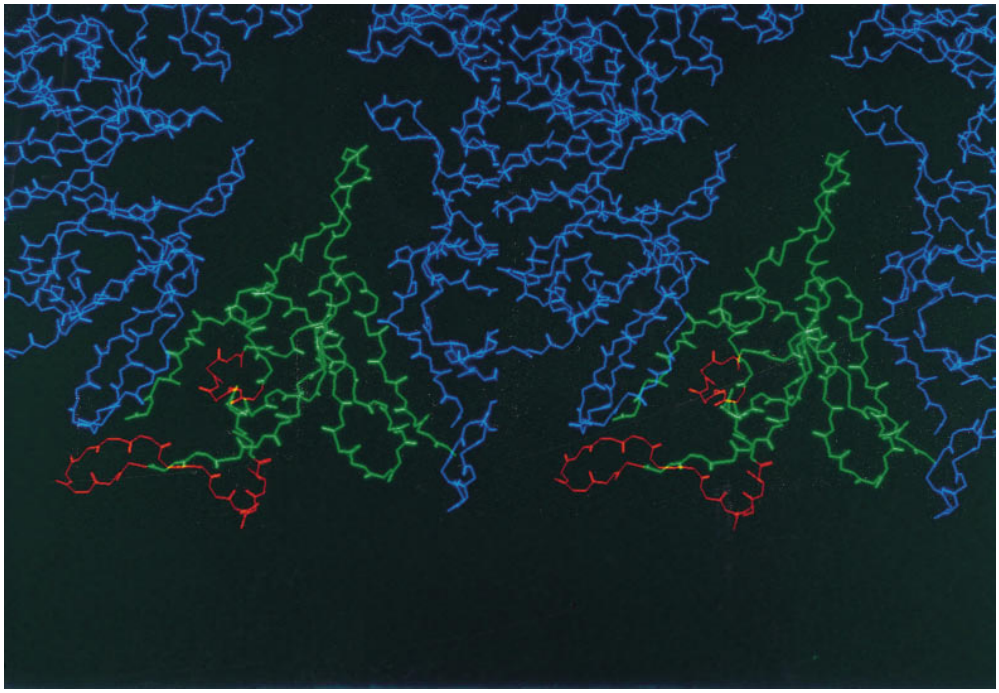

Figure 5. Locations of sequences on the Mt cpn10 structure that are likely to be important in bone resorption. Stereo view of polypeptide backbone of Mt cpn10 heptamer model viewed from the top down the sevenfold axis with one subunit highlighted in green. Peptide sequences with osteolytic activity are highlighted in red.

tamer only at the putative cell receptor. The flexible loop also binds to cpn60 in the cpn60–cpn10 protein-folding complex (26), suggesting that the putative cell receptor for Mt cpn10 has some structural homology with cpn60. If this structural homology is significant, it would require the Mt cpn10 to assemble as a heptamer on the receptor. Considering the gross structural rearrangements that occur in the cpn10 subunits of the heptamer when it binds to cpn60 (27), it may be possible for the two active sequences in Mt cpn10 to make contact with the receptor. None of the peptide fragments containing the active sequences appear to be involved in the interaction of Mt cpn10 with the human osteoblast-like cell line MG63, possibly due to their inability to assemble as a heptamer. In this regard, it may be important to note that mitochondrial cpn60 is expressed on the surface of human cells (28). These receptors are likely to be of therapeutic importance in the treatment of bone tuberculosis and possibly in other bone diseases.

Based upon the peptide activity data, the Mt cpn10 structure model gives an approximate guide to the location of the osteolytically-active sequences on the Mt cpn10 structure. The precise molecular structure accounting for the bioactivity of Mt cpn10 will be obtained by ongoing work on solving the Mt cpn10 structure by x-ray crystallography (Roberts, M.M., A. Coker, G. Fossati, P. Mascagni, A.R.M. Coates, and S.P. Wood, unpublished data) and the use of site-directed mutagenesis.

It is not known which Mt strains are associated with Pott's disease. In this study we have tested the Mt strain H37Rv, which is a virulent strain commonly used in research into tuberculosis. We have shown that the obligate protein, Mt cpn10, is the osteolytically-active component produced by this organism. All strains of Mt must contain this protein and therefore have the potential to induce bone disease. There may be additional factors to consider in the propensity of Mt to cause Pott's disease, and further studies into this area are clearly necessary.

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