The *Mycobacterium leprae* hsp65 Displays Proteolytic Activity. Mutagenesis Studies Indicate That the *M. leprae* hsp65 Proteolytic Activity Is Catalytically Related to the HslVU Protease†

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ABSTRACT: The present study reports, for the first time, that the recombinant hsp65 from *Mycobacterium leprae* (chaperonin 2) displays a proteolytic activity toward oligopeptides. The *M. leprae* hsp65 proteolytic activity revealed a trypsin-like specificity toward quenched fluorescence peptides derived from dynorphins. When other peptide substrates were used (*β*-endorphin, neurotensin, and angiotensin I), the predominant peptide bond cleavages also involved basic amino acids in P_1, although, to a minor extent, the hydrolysis involving hydrophobic and neutral amino acids (G and F) was also observed. The amino acid sequence alignment of the *M. leprae* hsp65 with *Escherichia coli* HslVU protease suggested two putative threonine catalytic groups, one in the N-domain (T^{136}, K^{168}, and Y^{264}) and the other in the C-domain (T^{375}, K^{409}, and S^{502}). Mutagenesis studies showed that the replacement of K^{409} by A caused a complete loss of the proteolytic activity, whereas the mutation of K^{168} to A resulted in a 25% loss. These results strongly suggest that the amino acid residues T^{375}, K^{409}, and S^{502} at the C-domain form the catalytic group that carries out the main proteolytic activity of the *M. leprae* hsp65. The possible pathophysiological implications of the proteolytic activity of the *M. leprae* hsp65 are now under investigation in our laboratory.

Heat shock proteins of the hsp60[1] family are molecular chaperones that guide several steps during synthesis, transport, and degradation of proteins (*1*). They are abundant in prokaryotic and eukaryotic cells and highly conserved during evolution (*2*, *3*). Under stress, the hsp60 proteins are rapidly synthesized, increasing their concentrations not only in the intracellular compartment but also on the cell surface (*4*, *5*). Both the microbial and the mammalian hsp60 belong to an important family of proteins that are major targets for the immune defense against infection (*6*). On the other hand, the microbial hsp60 have been implicated in autoimmune diseases, such as chronic inflammation and atherosclerosis (*7*−*16*).

One important application of the *Mycobacterium leprae* hsp65 is its use as an adjuvant or as a vaccine against tuberculosis. In the course of our work to determine the mechanism leading to the efficiency of the *M. leprae* hsp65 cDNA vaccine against tuberculosis (*17*, *18*), we noticed that the cytosol of the mice macrophages transfected with *M. leprae* hsp65 exhibited higher oligopeptidase activity than the control. Surprisingly, we found that the higher peptidase activity of macrophage cytosol expressing the *M. leprae* hsp65 protein was probably due to the proteolytic activity of the hsp65 itself, since this activity is displayed by the purified recombinant molecule (unpublished results).

The presented study demonstrates, for the first time, that the recombinant *M. leprae* hsp65 exhibits proteolytic activity toward polypeptides. This activity was characterized by a fluorometric assay and by the identification of the fragments generated from a number of polypeptides used as substrates. Two putative catalytic groups of the *M. leprae* hsp65 could be conceived from the alignment of its amino acid sequence with the heat shock protease HslVU of *Escherichia coli* (*19*). This hypothesis was strongly supported by site-directed mutagenesis studies of amino acid residues integrating the putative catalytic group, one of which leads to a complete loss of the proteolytic activity of the *M. leprae* rhsp65.

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[1] Abbreviations: amino acids, one letter symbols; hsp, heat shock protein; *M. leprae*, *Mycobacterium leprae*; rhsp65, recombinant *M. leprae* hsp65; qf, quenched fluorescence; Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; DMSO, dimethyl sulfoxide; ANP, human atrial natriuretic peptide; PHMB, *p*-(hydroxymercuri)benzoate; EDTA, ethylenediaminetetraacetic acid; E-64, L-*trans*-(epoxysuccinyl)leucylamido(4-guanidino)butane; PMSF, phenylmethanesulfonyl fluoride; TLCK, tosyllysine chloromethyl ketone; cFP, *N*-[1(*R*,*S*)-carboxy-3-phenylpropyl]-AAY-*p*-aminobenzoate; Z-L_3VS,)-carboxybenzyl)leucylleucylleucine vinyl sulfone; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; HPLC, high-performance liquid chromatography.

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EXPERIMENTAL PROCEDURES

Materials. All chemicals used for polyacrylamide gel electrophoresis were from Bio-Rad (Hercules, CA), including the ready-made polyacrylamide gels. The qf substrates (qf1−7, Table 1) were synthesized and purified according to Hirata et al. (*20*). The substrates assayed (β-endorphin, neurotensin, angiotensin I, insulin β-chain, substance P, bradykinin, human ANP, and human α-casein) were from Sigma (St. Louis, MO). The purity and molecular masses of the peptides used in this study were determined by HPLC and by MALDI-TOF mass spectrometry (TofSpec-E, Micromass).

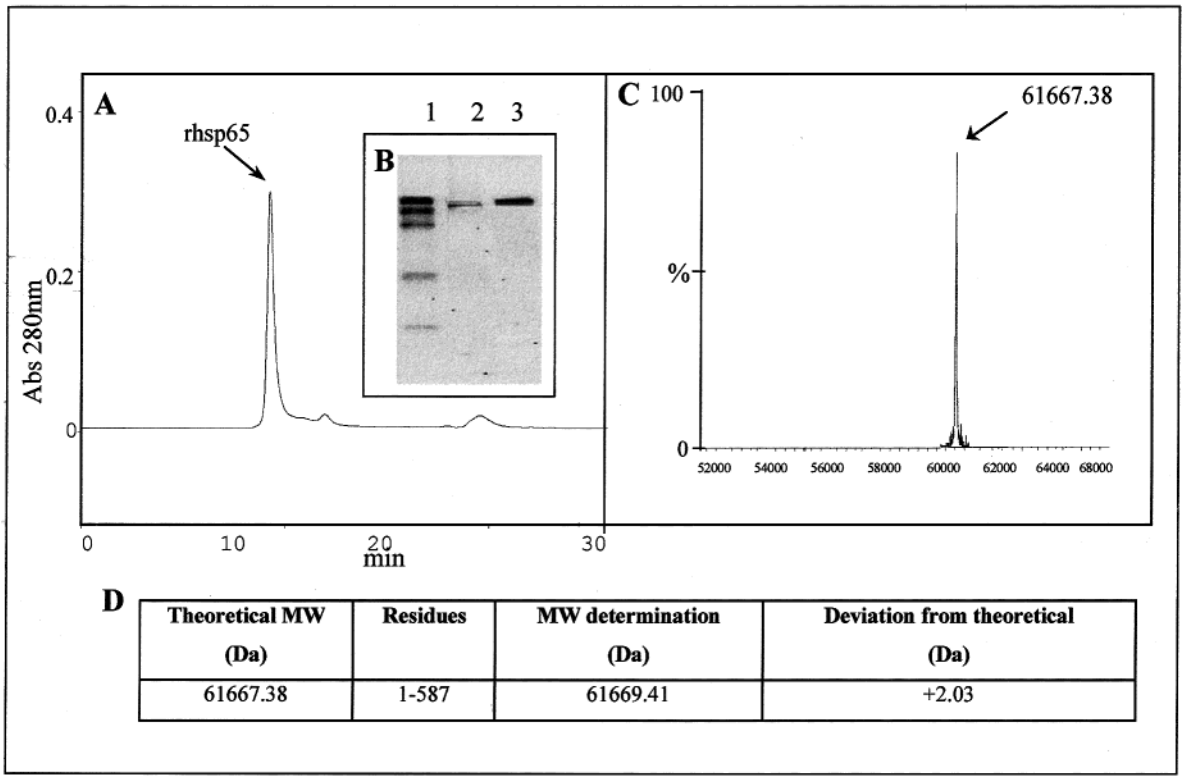
The set of inhibitors used to characterize the *M. leprae* hsp65 proteolytic activity, such as E-64, pepstatin, *o*-phenanthroline, *p*-(hydroxymercuri)benzoate, PMSF, TLCK, calpain II inhibitor, and EDTA, were purchased from Sigma Chemical Co. Dr. A. I. Smith provided the cFP-Ala-Ala-Tyr-pAb inhibitor, and Dr. H. Ploegh provided Z-L_3VS. Dr. Douglas B. Lowrie, Laboratory for Mycobacterial Research, National Institute for Medical Research, London, U.K., kindly provided the *M. leprae* hsp65 cDNA clone pIL161 (*21*).

Expression of the M. leprae Recombinant hsp65 by E. coli. Clone pIL161, containing the DNA coding for the *M. leprae* hsp65, was transformed into electrocompetent DH5α *E. coli* cells. The expression of the recombinant protein was performed as described previously (*21*, *22*). Briefly, DH5a *E. coli* cells containing pIL161 were grown in the presence of ampicillin to an OD_{600} of 0.6. The expression of the recombinant protein was induced by the addition of IPTG (final concentration of 0.5 mM). The induced culture was incubated for another 4 h at 30 °C and was harvested by centrifugation (5000*g*, 5 min, 4 °C). The pellet was resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) and lysed by sonication (60 Hz, two cycles of 60 s) (Tomy-Seiko, Japan). After centrifugation, the resulting pellet was washed three times with 10 mL of CE buffer (30 mM sodium citrate, 10 mM EDTA, pH 6.0). The washed pellet was resuspended in 5 mL of UPE buffer (6 M urea, 50 mM phosphate buffer, 20 mM EDTA, pH 7.0) by vortexing for 2−3 min, and the suspension was gently shaken at room temperature for 15 min. After removal of the insoluble material by centrifugation at 10000*g* for 20 min, a 0.9−1.2 M ammonium sulfate fraction was prepared by slow addition of 3.6 M ammonium sulfate stock solution, followed by incubation on ice for 30 min and centrifugation at 10000*g* for 10 min. This fraction was dissolved in 50 mM phosphate buffer, pH 6.8, dialyzed against the same buffer, and filtered (filter pore size 0.45 μm) to produce the crude fraction. The recombinant *M. leprae* hsp65 was first fractionated on a FPLC-GP-250 Plus system (MonoQ HR 5/5, Pharmacia Biotech) using 50 mM phosphate buffer, pH 6.8, containing 10 mM NaCl, and eluted with a 20−600 mM NaCl gradient under a flow rate of 1 mL/min. Subsequently, the protein solution (100 μg) was resolved on a HPLC system (Shimadzu Class VP) using a TSK-gel G3000 SW column. The buffer used was 50 mM phosphate, pH 6.8, containing 20 mM NaCl, under a flow rate of 1 mL/min. The recombinant *M. leprae* hsp65 was collected manually and submitted to mass spectrometric analyses. The homogeneity of the recombinant *M. leprae* hsp65 preparations was analyzed by polyacrylamide gel electrophoresis (*23*) followed by silver staining. Protein concentrations were determined as previously described (*24*).

Generation of the M. leprae hsp65 Mutant. Double-stranded site-directed mutagenesis of the *M. leprae* hsp65 was performed by overlap extension (*25*, *26*) on the double strand cDNA coding for *M. leprae* hsp65 (*21*). Mutagenesis was achieved by PCR using the oligonucleotide primers (sense, 5′ ggc atc gag gca gct gtc 3′, and anti, 5′ tcg aca gct gcc tcg atg 3′; sense, 5′ tat gac ggc gag aaa ctg c 3′ and anti, 5′ cag ttt ctc gcc gtc ata 3′; sense, 5′ gcg agg cac tgc agg aac 3′, and anti, 5′ tgc agt gcc tcg cgg tc 3′) that included in their sequences the mismatches coding for the substitution K168A, R407A, and K409A, respectively. Three extended PCR fragments, each one containing one of the mutations, were obtained after about 25 cycles and annealing at 60 °C, and it was incorporated into the gene construct using the restriction sites *Xba*I and *Eco*RI, naturally found in the pIL161 DNA sequence. The correct insertion of the mutation cassette and the site-directed mutations were confirmed by automatic DNA sequencing (Big Dye terminator reaction and ABI 310 analyzer, Applied Biosystems) or by double-stranded template dideoxy sequencing (*27*). The clones containing the mutated pIL161 DNA were transformed into electrocompetent DH5a *E. coli* cells, and the expression and the purification of the mutated recombinant proteins were performed as described above for the wild-type *M. leprae* hsp65 protein.

Enzymatic Assays Using qf Substrates. The hydrolysis of the qf substrates (stock solution in 10% DMSO) was conducted at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 20 mM NaCl. The hydrolysis was monitored by measuring the fluorescence at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 320$ nm in a Hitachi F-2000 spectrofluorometer, as previously described (*28*). Enzyme concentrations used ranged from 0.5 to 5 nM and qf substrates from 1/10 to 10 times the K_m values. The inner filter effect was corrected using an empirical equation as previously described (*28*). The kinetic parameters were calculated according to Wilkinson (*29*). The qf-3 substrate was used for routine measurements of *M. leprae* hsp65 peptidase activity performed in this study. One unit of recombinant *M. leprae* hsp65 activity is the amount of enzyme which hydrolyzes 1 μmol of qf-3 in 1 min. The stock solutions and the work concentrations of the inhibitors used in the characterization of the proteolytic activity of *M. leprae* hsp65 were made according to the methods described in ref *30*. The inhibitors were preincubated for 1 h at 20 °C, and the enzyme activity assays were performed as described above.

HPLC Analysis of Peptides Hydrolyzed by the Recombinant M. leprae hsp65. The peptide solutions (20−50 μM) in 50 mM sodium phosphate buffer, pH 6.8, containing 20 mM NaCl were incubated with the recombinant *M. leprae* hsp65 (5 nM) at 37 °C for 4 h. Samples (100 μL) were periodically taken for HPLC analysis. The hydrolysis products were separated by reverse-phase HPLC (Class VP, Shimadzu), collected manually, and submitted to amino acid analysis and mass spectrometry. The scissile bonds were deduced from the amino acid compositions of the fragments. The HPLC conditions used for the analytical procedure were 0.1% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile−solvent A (9:1) as solvent B. The separations were performed at a flow rate of 1 mL/min using a J. T.



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Theoretical MW (Da)	Residues	MW determination (Da)	Deviation from theoretical (Da)
61667.38	1-587	61669.41	+2.03

FIGURE 1: Purification of *M. leprae* rhsp65. (Panel A) Purification of *M. leprae* hsp65 by gel filtration. The *M. leprae* hsp65-containing fractions, collected from the MonoQ column, were pooled and submitted to a TSK-gel G3000 SW filtration step. The eluted proteins in 50 mM phosphate buffer, pH 6.8, with 20 mM NaCl, at a flow rate of 1 mL/min, were monitored by measuring absorbance at 280 nm. (Panel B) *M. leprae* hsp65 SDS−PAGE analysis: molecular weight standard (lane 1); 1 and 2 μg of *M. leprae* rhsp65, collected from gel filtration chromatography steps (lanes 2 and 3, respectively). (Panels C and D) Mass spectrometry analysis of pure *M. leprae* rhsp65.

Baker C-18 column (4.6 × 300 mm) and a 10−70% gradient of solvent B over 30 min. Analytical HPLC was performed using an SPD-10AV Shimadzu UV/vis detector and an RF-10Ax fluorescence detector. The same HPLC apparatus, equipped with a J. T. Baker C-8 column (4.6 × 300 mm), was used for the hydrolytic studies of human α-casein. In all cases, elution was followed by ultraviolet absorption (214 nm) and by fluorescence monitoring (λ_{em} = 420 nm and λ_{ex} = 320 nm).

Amino Acid Analysis. The amino acid composition of the purified recombinant *M. leprae* hsp65 and the concentration of the substrates stock solutions were determined as follows: after lyophilization, protein or peptide samples were hydrolyzed for 22 h, at 110 °C, in 6 N HCl containing 1% phenol, in vacuum-sealed tubes and then subjected to amino acid analysis using a pico Tag station (*31*).

Mass Spectrometry Analyses. (a) Molecular Mass Determination of M. leprae hsp65. The homogeneity of the protein preparation and peptides and the identification of the fragments generated by the hydrolytic activity of the recombinant *M. leprae* hsp65 were performed by mass spectrometry using the following adaptations to the system as described by Chassaigne and Lobisnki (*32*). Samples were analyzed on a triple quadrupole mass spectrometer, model Quattro II, equipped with a standard ES probe (Micromass, Altrinchan), adjusted to ca. 4 μL/min. During all experiments the source temperature was maintained at 80 °C and the needle voltage at 3.6 kV, applying a drying gas flow (nitrogen) of 200 L/h and a nebulizer gas flow of 20 L/h. The mass spectrometer was calibrated with intact horse heart myoglobin and its typical cone-voltage induced fragments. The molecular mass of *M. leprae* hsp65 was determined by ES−MS, adjusting the mass spectrometer to give a peak width at half-height of 1 mass unit, and the cone sample to skimmer lens voltage controlling the ion transfer to the mass analyzer was set to 60 V. About 50 pmol (10 μL) of each sample was injected into electrospray transport solvent. The ES spectra were obtained in the multichannel acquisition mode, scanning from *m*/*z* 500 to *m*/*z* 1800 at scan time of 5 s. The mass spectrometer data acquisition and treatment system was equipped with MassLynx and MaxEnt software for handling spectra.

(b) Determination of Cleavage Sites of Peptide Substrates by Recombinant M. leprae hsp65. The peptide fragments were detected by scanning from *m*/*z* 50 to *m*/*z* 2000 at 6 s/scan, with 31 V cone. Product ions from MS/MS experiments were detected during several scannings through the appropriated mass range for each situation, using high energy (25 eV) for single-charged and low collision energy (15 eV) for multiple-charged precursor ions. No tandem MS was recorded for peptides smaller than four amino acid residues.

RESULTS

Purification of M. leprae rhsp65. The *M. leprae* rhsp65 used in this study was purified as described in Experimental Procedures. After the ion-exchange chromatography, the pool containing *M. leprae* rhsp65 showed minor contaminants, being subsequently purified by chromatography on a TSK-gel G3000 SW column (Figure 1A). The absence of contaminating proteins was assured by subjecting two different concentrations of the purified *M. leprae* rhsp65

Table 1: Specificity for qf Dynorphin-Derived Substrates by the *M. leprae* rhsp65 and Sites of Cleavage[a]

	substrates (Abz-...-EDDnp)										K_m	k_{cat}	k_{cat}/K_m
no.	P_7	P_6	P_5	P_4	P_3	P_2	$P_1\downarrow$	P'_1	P'_2	P'_3	(μM)	(s^{-1})	$(s\cdot mM)^{-1}$
1				G	F	L	R	R	V		20 ± 2	4.8 ± 0.6	237
2			G	G	F	L	R	R			12 ± 1	0.31 ± 0.02	26
3			G	G	F	L	R	R	V		1.3 ± 0.3	0.96 ± 0.05	740
4			G	G	F	I	R	R	Q		7.5 ± 0.6	0.37 ± 0.01	49
5			G	G	F	L	R	R	V	E	4.0 ± 0.5	1.4 ± 0.1	350
6		D	A	G	F	L	R	R	V		1.1 ± 0.1	1.0 ± 0.2	909
7	D	A	G	G	F	L	R	R	V		0.9 ± 0.1	1.1 ± 0.1	1222

[a] Assays were carried out in 50 mM phosphate buffer, pH 6.8, containing 20 mM NaCl, at 37 °C. The parameters were calculated as the mean value (±SD). All peptides were cleaved only at peptide bond R↓R, which were determined by mass spectrometry analyses (see Experimental Procedures for details). All enzymatic assays were made in triplicate.

preparation to SDS−PAGE. Figure 1B, lanes 2 and 3, clearly shows that the *M. leprae* rhsp65 used in this study was homogeneous. The mass spectrometry analysis (Figure 1C,D) demonstrated that the purified *M. leprae* rhsp65 is a 61667.38 Da molecule composed of 587 amino acid residues. The specific activity of the *M. leprae* rhsp65 was 1.4 units/mg.

Analysis of the Proteolytic Activity of M. leprae rhsp65. (a) Hydrolysis of qf Peptides. Table 1 shows the K_m, k_{cat}, and k_{cat}/K_m values determined for the hydrolysis of the qf substrates derived from dynorphin by *M. leprae* rhsp65. A series of qf peptides, from six to nine amino acid residues, was hydrolyzed at the R−R bond by the *M. leprae* rhsp65. The catalytic efficiency (k_{cat}/K_m) varied from 26 $(s\cdot mM)^{-1}$ for the hexapeptide (qf-2) to 1222 $(s\cdot mM)^{-1}$ for the nonapeptide (qf-7). The qf-7 substrate was hydrolyzed with the highest k_{cat}/K_m value, mainly due to its lowest K_m value, followed by the qf-6 substrate. The qf-3 substrate was also well hydrolyzed, although it seems that the presence of an E residue at position P'_3 [according to the nomenclature of Schechter and Berger (*33*)], as in qf-5, increased the K_m value by 3-fold. The substrates containing six amino acid residues showed the highest K_m values (qf-1 and qf-2 substrates). On the other hand, the presence of I, a β-branched amino acid, at position P_2, seemed to hinder the proteolytic attack on the susceptible peptide bond, thus resulting on a higher K_m value.

(b) Hydrolysis of Bioactive Peptides and α*-Casein.* Six biologically active peptides of various sizes and amino acid sequences and one protein were assayed. Hydrolyses were monitored by HPLC, and the results are summarized in Table 2. The most susceptible of the substrates used was β-endorphin, presenting two major cleavage sites ($K_{19}\downarrow N_{20}$ and $K_{28}\downarrow_{29}$) and one minor cleavage site ($G_2\downarrow G_3$). Neurotensin was well hydrolyzed at the $R_8\downarrow R_9$ peptide bond. The *M. leprae* rhsp65 showed preference for hydrolysis involving basic amino acid residues in the P_1 position, although a minor hydrolysis (9%) occurred at the G−G bond of the β-endorphin. Another exception was the hydrolysis of angiotensin I, which occurred at position $F_8\downarrow H_9$, releasing angiotensin II. No significant hydrolysis products of bradykinin, substance P, atrial natriuretic peptide, β-chain of insulin, and α-casein were detected.

Investigation of the Enzymatic Mechanism of the rhsp65 of M. leprae: Identification of the Putative Amino Acid Residues Involved in the Catalytic Activity of the rhsp65 of M. leprae. The proteolytic activity of the *M. leprae* rhsp65 was not affected by the classical inhibitors of metallopeptidase [metal chelating compounds, EDTA and *o*-phenanthroline, or active site-directed oligopeptidase inhibitor cFP (*34*)], serine peptidase (PMSF, TLCK), thiol peptidase (E-64, PHMB, calpain II), or aspartic peptidase (pepstatin A) inhibitors. The threonine peptidase inhibitor $Z\text{-}L_3VS$ [proteasome inhibitor (*35*)] also had no inhibitory effect.

Table 2: Hydrolysis of Bioactive Peptides and Human α-Casein by the *M. leprae* rhsp65[a]

assayed substrates	rates of hydrolyses (nmol μg^{-1} min^{-1})	cleavage site(s)
β-endorphin	135	$(G_2\downarrow G_3)/(K_{19}\downarrow N_{20})/(K_{28}\downarrow K_{29})$
$neurotensin_{1-13}$	102	$R_8\downarrow R_9$
angiotensin I	79	$F_8\downarrow H_9$
bradykinin	nh[b]	
substance P	nh[b]	
ANP	nh[b]	
β-chain of insulin	nh[b]	
human α-casein	nh[b]	

[a] Assays were carried out in 1 mL of 50 mM phosphate buffer, pH 6.8, containing 20 mM NaCl, at 37 °C, using 20−100 μM peptides and 5 nM *M. leprae* rhsp65. Control samples were identical, except that *M. leprae* rhsp65 was omitted. The velocity of hydrolysis was determined by comparing the peak areas of untreated substrates versus samples digested by *M. leprae* rhsp65. [b] nh = no hydrolysis detected.

The possible catalytic groups responsible for the proteolytic activity of the *M. leprae* rhsp65 were suggested by the alignment of the amino acid sequences of the *M. leprae* rhsp65 and the heat shock proteolytic enzyme HslVU (Figure 2). Although the HslVU and the *M. leprae* hsp65 show low amino acid sequence similarity, the reactive amino acids of the HslVU (T^1, K^{33}, and S^{124}) align with T^{136}, K^{168}, and Y^{264} at the N-domain and with T^{375}, K^{409}, and S^{502} at the C-domain of the *M. leprae* hsp65 molecule (Figure 2). These two putative catalytic sites of *M. leprae* hsp65 were subjected to mutagenesis. The best results were obtained by mutations performed at the putative catalytic triad at the C-domain (Table 3). Thus, the substitution of K^{409}/A led to a complete loss of the proteolytic activity, whereas the substitution of R^{407}/A was less effective (72% activity loss). On the other hand, the substitution of K^{168}/A at the putative triad at the N-domain was much less effective (25% activity loss).

DISCUSSION

Although the family of the hsp60 has been subject of intense investigation, no description of an intrinsic proteolytic activity has yet been reported. In the course of our investigation on the mechanism leading to the efficiency of the *M. leprae* hsp65 cDNA vaccine against tuberculosis (*17*, *18*,

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HSP65   MPGRDGETQP ASCGRPSRAL HPASVSNGGC RHPVTLASFL IRRNHFAMAK TIAYDEEARR   60

HSP65   GLERGLNSLA DAVKVTLGPK GRNVVLEKKW GAPTITNDGV SIAKEIELED PYEKIGAELV  120

HSP65   KEVAKKTDDV AGDGTTTATV LAQALVKEGL RNVAAGANPL GLKRGIEKAV DKVTETLLKD  180
HslVU                   TTIVS VRRNGHVVIA GDGQATLGNT VMKGNVKKVR RLYNDKVIAG   45
                          **                          *

HSP65   AKEVETKEQI AATAAISAGD QSIGDLIAEA MDKVGNEGVI TVEESNTFGL QLELTEGMRF  240
HslVU   FAGGTADAFT LFELFERKLE MHQGHLVKAA VELAKDWRTD RMLRKLEALL AVADETASLI  105

HSP65   DKGYISGYFV TDAERQEAVL EEPYILLVSS KVSTVKDLLP LLEKVIQAGK SLLIIAEDVE  300
HslVU   ITGNGDVVQP ENDLIAIGSG GPYAQAAARA LLENTELSAR EIAEKALDIA GDICIYTNHF  165

HSP65   GEALSTLVVN KIRGTFKSVA VKAPGFGDRR KAMLQDMAIL TGAQVISEEV GLTLENTDLS  360
HslVU   HTIEELSYKA EFHHHHHH                                               183

HSP65   LLGKARKVVM TKDETTIVEG AGDTDAIAGR VAQIRTEIEN SDSDYDREKL QERLAKLAGG  420
HslVU                   TTIVSV RRNGHVVIAG DGQATLGNTV MKGNVKKVRR LYNDKVIAGF   46
                          *** **

HSP65   VAVIKAGAAT EVELKERKHR IEDAVRNAKA AVEEGIVAGG GVTLLQAAPA LDKLKLTGDE  480
HslVU   AGGTADAFTL FELFERKLEM HQGHLVKAAV ELAKDWRTDR MLRKLEALLA VADETASLII  106

HSP65   ATGANIVKVA LEAPLKQIAF NSGMEPGVVA EKVRNLSVGH GLNAATGEYE DLLKAGVADP  540
HslVU   TGNGDVVQPE NDLIAIGSGG PYAQAAARAL LENTELSARE IAEKALDIAG DICIYTNHFH  166

HSP65   VKVTRSALQN AASIAGLFLT TEAVVADKPE KTAAPASDPT GGMGGMDF               588
HslVU   TIEELSYKAE FHHHHHH                                                183
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FIGURE 2: Alignment of the amino acid sequences of hsp65 from *M. leprae* and HslV from *E. coli*. The conserved threonine residues, participating at the catalytic triads, are indicated by open boxes; the charged residues (basic amino acids), putatively important for the hydrolytic activity, are underlined and in bold letters. Asterisks indicate identical amino acid residues. Note that the catalytic triad is present twice in the *M. leprae* rhsp65 amino acid sequence.

Table 3: Residual Peptidase Activity of Wild-Type and Mutant *M. leprae* rhsp65 Using qf-3 as Substrate[a]

enzyme	residual activity (%)
WT	100
K168A	75
R407A	28
K409A	nh

[a] Fluorometric assays were carried out in 1.0 mL of 50 mM phosphate buffer and 20 mM NaCl, pH 6.8, at 37 °C, containing 5 μM substrate qf-3 and 5 nM *M. leprae* rhsp65. The residual hydrolytic activities of mutant *M. leprae* hsp65 (K168/A, R407/A, and K409/A) were compared to the activity of the wild-type *M. leprae* rhsp65 enzyme. Each value represents the mean of three individual assays.

36), we noticed that the cytosol of the mice macrophages transfected with cDNA of the *M. leprae* hsp65 displayed higher proteolytic activity than the control macrophage cytosol. The proteolytic activity was determined by using the qf-3 as substrate. We subsequently discovered that the purified recombinant *M. leprae* hsp65, by itself, displayed proteolytic activity toward qf-3 (unpublished results). The present work reports the characterization of the *M. leprae* hsp65 proteolytic activity.

The qf dynorphin-related peptides are suitable substrates for characterization of protease specificity since they offer a number of peptide bonds to be hydrolyzed by a wide range of proteolytic enzymes. In addition, they are convenient for determination of the kinetic parameters since the hydrolysis of any peptide bond of the qf substrates can easily be monitored (*37*). In this work, we used a series of qf dynorphin-related oligopeptides of six to nine amino acid residues to characterize the specificity of the proteolytic activity of *M. leprae* hsp65. All peptides were hydrolyzed at the R−R bond, with preference for large qf substrates (catalytic efficiencies 50-fold higher) as compared to the small ones.

Besides the dynorphin-related peptides, *M. leprae* hsp65 was able to convert angiotensin I into angiotensin II and to hydrolyze β-endorphin and neurotensin, but it failed to hydrolyze bradykinin, the β-chain of insulin, and α-casein. The hydrolysis of the dynorphin-related peptides, β-endorphin and neurotensin, indicated that *M. leprae* hsp65 has a clear preference for basic amino acids in P_1 (*33*). This enzymatic profile suggests that the recombinant *M. leprae* hsp65 displays a trypsin-like specificity with a narrow selectivity for its substrates, since polypeptides such as the β-chain of insulin and α-casein were not significantly hydrolyzed, even after extensive incubation times. Several classical inhibitors that block serine protease, thiol protease, metalloprotease, and aspartyl protease failed to inhibit the proteolytic activity of *M. leprae* hsp65.

The catalytic groups of the *M. leprae* hsp65 were identified by site-directed mutagenesis of amino acid residues integrating the putative catalytic triads, deduced from the amino acid sequence alignment with the heat shock protease HslVU of *E. coli* (Figure 2). For this protein, the triad consists of a T^1 nucleophile residue, activated by a basic amino acid (K^{33}), and a hydrogen-bonding residue (S^{124}) (*19*). Coincidentally, despite the low similarity between the *M. leprae* hsp65 and

the heat shock protease HslVU (less than 20% similarity), the alignment of these proteins indicates a conservation of the same amino acid residues that constitute the catalytic triad of the HslVU in two distinct regions of the *M. leprae* hsp65 molecule. This coincidence suggests two putative catalytic triads in the *M. leprae* rhsp65 sequence, one at the N-domain, consisting of $T^{136,137}$, K^{168}, and Y^{264}, and another at the C-domain, consisting of $T^{375,376}$, R^{407} or K^{409}, and S^{502}. Another protease, the β-subunit of the 20S proteasome, displays the same alignment coincidence. The amino acid sequence data indicate that the HslVU displays only 18% identity with the β-subunit of the 20S proteasome. Nevertheless, structural features of these proteases allow interactions of T^{1}, K^{33}, and S^{124} to form the catalytic triad (*35*, *38*, *39*). However, unlike HslVU and the β-subunit of the 20S proteasome, the putative hydroxyl group of the nucleophile threonine in the *M. leprae* hsp65 is not at the N-terminus but at positions 136 and 375. All of this information was used to generate a working hypothesis both to suggest a mechanism for the proteolytic activity of the *M. leprae* rhsp65 and to perform the site-directed mutagenesis of the molecule.

Similarly to the HslVU mutagenesis studies, we did not include mutations of the threonine residues, because it had been shown for the HslVU that mutations of T^{1}/A or T^{2}/A cause either a small reduction of enzymatic activity or drastic structural alterations (*40*). Mutation of the K^{33}/A catalytic residue of the 20S proteasome was more effective, in that the mutant polypeptide no longer showed proteolytic activity, although its tertiary structure remained unaltered (*39*). The same rationale was applied to the *M. leprae* hsp65. In fact, the mutation of K^{409}/A caused a complete loss of its proteolytic activity, while CD studies revealed no difference between the structures of the mutant and the wild-type proteins (data not shown). Another basic amino acid, R^{407}, was shown to exert an additional influence on the catalytic activity, since the mutation R^{407}/A strongly affected the proteolytic activity of *M. leprae* hsp65. This was not the case for the K^{168}/A mutation, which only caused a 25% loss of the proteolytic activity of *M. leprae* hsp65. This result suggests that the mutation of the basic residue of the putative triad at the N-domain probably reduced the enzyme activity by affecting structural parameters that could influence the catalytic efficiency. Taken together, these results strongly suggest that the amino acid residues T^{375}, K^{409}, and S^{502} are the triad that mainly performs the proteolytic activity of the *M. leprae* hsp65.

The family of the hsp60 molecules is considered to be located intracellularly in the mitochondria of eukaryotic cells, where they are suggested to assist the folding and the assembly of oligomeric complexes (*41*). However, hsp60 also exists in other cell compartments, possibly fulfilling other functions. Several studies indicate that hsp60 molecules are also located at the cytoplasmic membrane of endothelial and mononuclear cells (*7*, *42*, *43*, *44*) and in serum (*10*, *11*, *45*). They are highly expressed in cardiovascular tissues (*46*), either in healthy individuals or in those subjected to stress stimuli, including high temperature, mechanical stress, infections, surgical stress, and oxidant and cytokine stimulation (*7*, *47*). Since the eukaryotic and bacterial hsp60 share extensive homology (*5*), the bacterial hsp65 is suggested to stimulate autoimmune reaction, thus contributing to chronic inflammation, hypertension, and atherosclerosis (*11*, *45*, *48*). On the other hand, it is worth considering that the mycobacterium hsp65 is among the most effective antigens of mycobacterium to confer resistance against mice tuberculosis (*18*, *49*). Moreover, it was demonstrated that the *M. leprae* hsp65 stimulates detectable humoral and cell-mediated immunity in guinea pigs infected with *Mycobacterium tuberculosis* (*50*).

The results presented here clearly opened new approaches for the participation of this stress protein in a number of pathophysiological processes. This class of bacterial proteins, besides representing important antigens, should be regarded as a peptidase, which is able to generate or destroy other biological active molecules possibly involved in those processes. The possible pathophysiological implications of the proteolytic activity of the *M. leprae* hsp65 are now under investigation in our laboratory.

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