Characterizing the *Mycobacterium tuberculosis* Rv2707 protein and determining its sequences which specifically bind to two human cell lines

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Abstract

The Rv2707 gene encoding a putative alanine- and leucine-rich protein was found to be present in all *Mycobacterium tuberculosis* complex strains (by PCR) and its transcription was shown by RT-PCR in all but *M. bovis* and *M. microti*. Antibodies raised against Rv2707 peptides specifically recognized the native protein by Western blot and were able to locate this protein on the *M. tuberculosis* membrane by immunoelectron microscopy. A549 and U937 cells lines were used in binding assays involving synthetic peptides covering the whole Rv2707 protein. High A549 cell-binding peptide 16083 (²⁸¹QEEWPAPATHAHLGNWLKAY³⁰⁰) was identified. Peptides 16072 (⁶¹LFGPDTLPAIEKSALSTAHSY⁸⁰) and 16084 (³⁰¹RIGVGTTTTYSSTAQHSAAV³²⁰) presented high specific binding to both A549 and U937 cells. Cross-linking assays revealed that peptide 16084 specifically bound to a 40-kDa and a 50-kDa U937 cell membrane protein. High activity binding peptides (HABPs) 16083 and 16084 were able to inhibit *M. tuberculosis* invasion of A549 cells. Our results suggest that these sequences could be part of the binding sites used by the bacillus for interacting with target cells, and thus represent good candidates to be tested in a future subunit-based, multiepitope, antituberculosis vaccine.

Keywords: Rv2707; *Mycobacterium tuberculosis*; high activity binding peptide (HABP); A549 cell; U937 cell; invasion

Tuberculosis remains one of the greatest causes of morbidity and mortality worldwide; it has been calculated that one-third of the world's population is infected with *Mycobacterium tuberculosis*. There was a resurgence of

tuberculosis in the mid 1980s following a 30-yr period of decline in the disease's incidence due to improved immunoprophylactic measures and *M. tuberculosis* bacilli being highly sensitive to chemotherapy. There are many reasons for such a reversal, including the role of HIV infection (Corbett et al. 2007), the emergence of multi-drug-resistant strains of the etiological agent, and failing public health infrastructure (Harries and Dye 2006).

Even though the directly observed treatment strategy (DOTS) offers an effective control mechanism against tuberculosis, it is costly due to the type of therapy used, and also requires a combination of three antibiotics to avoid the development of drug resistance (Kaufmann

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Abbreviations: HABP, high activity binding peptide; IEM, immunoelectron microscopy; FM, fluorescence microscopy; FC, flow cytometry.

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2000). These reasons have heightened the need for an effective vaccine for controlling and preventing tuberculosis, since the BCG vaccine's protective efficacy has varied greatly in different trials (MacIntyre 2007).

The *M. tuberculosis* bacillus is a pathogen which is mainly transmitted by respiratory route, its target cells being the alveolar macrophages, as well as nonphagocytic cells, such as alveolar epithelial cells. Previous studies using the A549 alveolar epithelial and U937 monocyte cell lines have shown the utility of these cells for studying the interaction between mycobacteria and target cells (Aung et al. 1996; Bermudez and Goodman 1996; Song et al. 2003; Houben et al. 2006).

M. tuberculosis has developed evolutionary mechanisms allowing it to penetrate, survive, and replicate within the macrophage phagosomes (Houben et al. 2006; Mueller and Pieters 2006; Russell 2007). Phagocytosis is the critical step for interaction between *M. tuberculosis* and the macrophage, involving bacterial surface ligands binding to different receptors located on the phagocytic cell membrane (Pieters 2001).

The publication of the complete *M. tuberculosis* H37Rv genomic sequence (Cole et al. 1998) has provided a valuable tool for researchers in their attempt to broaden knowledge regarding the pathogen's biology, opening the gate for development of new, more efficient therapeutic and prophylactic methods for controlling this disease. Knowing the proteome has led to identifying different proteins; those found on the membrane surface and capable of acting as immunogens or subunits for vaccines being the most interesting (Chakravarti et al. 2000). Work done in this area has led to identifying a large number of *M. tuberculosis* membrane proteins whose functions remain unknown (Covert et al. 2001; Xiong et al. 2005).

This study was aimed at characterizing the *M. tuberculosis* Rv2707 gene presence in 27 different mycobacterial species as well as its transcription. Encoded protein translation and localization was assessed by Western blotting and immunoelectron microscopy (IEM), respectively. Peptide binding assays to U937 and A549 cell lines were carried out to determine those protein regions interacting with target cells. HABPs were able to inhibit *M. tuberculosis* invasion to A549 cells, suggesting their role in mycobacterial invasion and thus their potential as anti-tuberculous vaccine candidates.

Results

Bioinformatics analysis

The Rv2707 gene consists of 975 bp encoding a hypothetical 324 amino acid alanine- and leucine-rich transmembrane protein, having a theoretical 35.05 kDa weight. The in silico analysis used for determining protein cell

localization did not reveal a signal peptide, but did determine that the Rv2707 protein had a 0.516 hydropathy index, as well as the presence of six transmembrane helices from amino acids 41–62, 104–125, 146–168, 187–205, 218–241, and 250–272. The results obtained with these two parameters suggested the presence of the protein in the *M. tuberculosis* H37Rv membrane. Likewise, PSORT software predicted the localization of the protein on the bacterial membrane. Membrane localization was later experimentally confirmed by both Western blot and IEM, as will be shown below. Blast analysis of the protein revealed that it presented identity with other mycobacteria, i.e., *M. avium* (83%), *M. ulcerans* (80%), *M. leprae* (75%), and *M. smegmatis* (69%). Likewise, it was found to be related to the RNase BN family (Zuo and Deutscher 2001), having 72% identity. This class of RNase is the only one which has not had homologs identified in eukaryotes. The members of this family are hydrophobic proteins, predicted to have six transmembrane helices by secondary structure prediction, a similar pattern to that being here described for Rv2707.

PCR assay

PCR experiments with genomic mycobacterial DNA showed the presence of the Rv2707 gene in all *M. tuberculosis* complex strains. Figure 1A and B shows that the gene encoding the Rv2707 protein was exclusive to *M. tuberculosis* H37Rv, H37Ra, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti* strains, due to the presence of a 203-bp amplification signal. It was also observed that this gene was not present in mycobacteria other than tuberculosis (MOTT).

RT-PCR assay

cDNA was synthesized as described in Materials and Methods. RT-PCR was performed on all PCR positive strains on genomic DNA to determine whether the Rv2707 gene was transcribed in 7H9 culture medium conditions. There was evidence of the 203-bp amplification fragment having similar intensity in cDNA from *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. africanum* strains. There was no trace of transcription in *M. bovis* or in *M. microti* (Fig. 1C). *rpoB* constitutive gene was used as a positive control for transcription assays for each mycobacterial strain (360-bp fragment) (Fig. 1D). It is worth noting that *rpoB* is transcribed in *M. bovis* and *M. microti*, suggesting cDNA integrity in these two species but the Rv2707 gene is not transcribed in either.

Rv2707 protein recognition by Western blot

Figure 2 shows Western blotting with preimmune and 20 d post-third inoculation sera from two rabbits injected with synthetic polymerized peptide 25680 and 25684,

Figure 1. Molecular assays. Panels A and B show a 203-bp PCR product from the gene encoding the *M. tuberculosis* H37Rv Rv2707 protein, amplified on DNA from *M. tuberculosis* complex strains and MOTT. 1. MWM 123 bp (Gibco); 2. *M. tuberculosis* H37Rv; 3. *M. tuberculosis* H37Ra; 4. *M. bovis*; 5. *M. bovis* BCG; 6. *M. africanum*; 7. *M. microti*; 8. *M. flavescens*; 9. *M. fortuitum*; 10. *M. szulgai*; 11. *M. peregrinum*; 12. *M. phlei*; 13. *M. scrofulaceum*; 14. *M. avium*; 15. *M. smegmatis*; 16. MWM 123 bp (Gibco); 17. *M. nonchromogenicum*; 18. *M. simiae*; 19. *M. intracellulare*; 20. *M. gastri*; 21. *M. kansasii*; 22. *M. diernhoferi*; 23. *M. gordonae*; 24. *M. marinum*; 25. *M. terrae*; 26. *M. chelonae-chelonae*; 27. *M. xenopi*; 28. *M. vaccae*; 29. *M. triviale*; 30. PCR negative control. In panel C, a Rv2707 203-bp RT-PCR product obtained from cDNA from *M. tuberculosis* complex strains. 1. MWM 123 bp (Gibco); 2. *M. tuberculosis* H37Rv; 3. *M. tuberculosis* H37Ra; 4. *M. bovis*; 5. *M. bovis* BCG; 6. *M. africanum*; 7. *M. microti*; 8. Negative control; 9. PCR positive control; 10. PCR negative control. In panel D, a 360-bp PCR product from the same strains, but amplifying the *Mycobacterium rpoB* gene.

respectively, corresponding to the polymerized amino acid sequences of Rv2707-derived peptides 16070 and 16085 following a 0, 20, and 40-d inoculation schedule. Western blotting showed that none of the preimmune sera recognized any *M. tuberculosis* sonicate proteins (lanes 1 and 2). The rabbit immunized with 25684 (named 129) produced antibodies that recognized a 36-kDa protein, corresponding to the Rv2707 protein's theoretical molecular mass (lane 3). Rabbit 249 (immunized with polymer 25680) recognized the same 36-kDa band (lane 4). Rabbit 129 preimmune serum did not display any type of recognition to membrane fraction proteins (lane 5), while antibodies induced after three immunizations did recognize several proteins from the *M. tuberculosis* membrane fraction, among which a signal corresponding to Rv2707 protein molecular weight was found (lane 6).

This suggested that the peptides used induced the production of antibodies which specifically recognized a protein corresponding to the Rv2707 protein's theoretical molecular mass (35.05 kDa), and that the translated protein was present not only in *M. tuberculosis* sonicate but in the membrane fraction as well.

Immunoelectron microscopy

IEM was used for determining Rv2707 location in the *M. tuberculosis* H37Rv structure. Rabbit 129's immune

serum was selected as a source for primary antibodies. Although some colloidal gold particles were located inside the mycobacteria (Fig. 3B), the majority were specifically attached to the mycobacterial surface (Fig. 3A,B). No labeling was observed using the preimmune 129 serum as negative control (data not shown).

Figure 2. Western blotting was performed with preimmune and post-third inoculation rabbit sera against *M. tuberculosis* sonicate (lanes 1–4) and a mycobacterial membrane fraction (lanes 5 and 6). Preimmune sera (lanes 1, 2, and 5). Immune sera after three immunizations with polymeric peptides 25,684 (129 in lane 3) or 25,680 (antisera 249 in lane 4). Antiserum 129 was also analyzed against the *M. tuberculosis* membrane fraction (immune serum in lane 6). MW markers are shown on the right-hand side.

Figure 3. Subcellular localization of Rv2707 protein in *M. tuberculosis* H37Rv. (A) IEM of *M. tuberculosis* H37Rv using a 1:10 dilution of serum 129 as primary antibody, followed by a 1:50 dilution of 10 nm gold-labeled anti-rabbit IgG as a secondary antibody. Panel B shows a similar experiment using a 1:50 dilution of 5-nm gold-labeled anti-rabbit IgG as a secondary antibody. The arrows indicate the protein's location on the surface of mycobacteria.

High specific binding peptide

Radio-labeled peptides were incubated with target cells in the presence (nonspecific binding) or absence (total binding) of the same non-radio-labeled peptide in A549 and U937 cell-binding assays. Total binding minus non-specific binding gave the specific binding (Forero et al. 2005; Garcia et al. 2005; Vera-Bravo et al. 2005). The specific-binding slope is the ratio between specifically bound radio-labeled peptide and added radio-labeled peptide, called specific binding activity. Any peptide showing specific binding activity equal to or higher than control peptide (1%) was called A549 or U937 cell HABP (Vera-Bravo et al. 2005).

Based on this methodology, previously used for recognizing HABPs in *M. tuberculosis* (Forero et al. 2005; Garcia et al. 2005; Vera-Bravo et al. 2005), three A549-

HABPs (16072, 16083, and 16084) and two U937-HABPs (16072 and 16084) were found in the Rv2707 protein. This indicated that these peptides were essential for A549 and U937 cell-binding activity, those underlined being common to both cell types (Fig. 4). These results support the transmembrane topology predicted by TMHMM and TMPRED for Rv2707, since none of the peptides predicted to be lying in the transmembrane portion displayed binding while all HABPs found were located in exposed regions. HABP 16,072 was predicted to be located inside pathogen cells while HABPs 16,083 and 16,084 were exposed on the bacillus surface.

Saturation assay

Figure 5 shows 16072, 16083, and 16084 saturation profiles from which A549 cell dissociation constants were determined ($K_d = 500, 900, \text{ and } 680 \text{ nM}$, respectively). The number of binding sites per cell was also determined; the greatest number of sites per cell (3,935,000 sites/cell) were found for peptide 16084 (3,300,000 sites/cell for 16072 and 795,000 sites/cell for 16083). The Hill coefficient was 1.7 for 16,072, 1.1 for 16083, and 1.1 for 16084, suggesting a degree of positive cooperativity due to the values being >1 .

Cross-linking assay

The cross-linking assay showed that peptide 16084 was able to bind to proteins located on U937 cell membrane (Fig. 6) having apparent 40-kDa and 50-kDa molecular masses (lane 1). This binding was inhibited when the assay was done in the presence of an excess of the same unlabeled peptide (lane 2). Greater recognition of the

Peptide number	Sequence		Specific Binding Activity to A549 cells			Specific Binding Activity to U937 cells		
			0%	1%	2%	0%	1%	2%
16069	¹ MSDQVPKPHRHIIWRTRTY	20						
16070	²¹ LSKSWDDSFSESQAQAFWSY	40						
16071	⁴¹ ALSPLPLLGLSLAYVAP	60						
16072	⁶¹ LFGPDTLPAIEKSALSTAHSY	80						
16073	⁸¹ FFSPSVNIEIPTIGDITNY	100						
16074	¹⁰¹ NARGEVASLGFUSLWAGSSY	120						
16075	¹²¹ AISAFVDVVEAHDQTPLRHY	140						
16076	¹⁴¹ PVRQRFFALFLYVMLVFLV	160						
16077	¹⁶¹ ATAPVMVVGPRKVSSEHPIESY	180						
16078	¹⁸¹ LANLLRYGYYPALIGLTVG	200						
16079	²⁰¹ VILLYRVALPVPLPTRLVL	220						
16080	²²¹ GAVLAIAVFLIATLGLRVYL	240						
16081	²⁴¹ AWITRTGYTYGALATPIAFL	260						
16082	²⁶¹ LFAFFGGFAIMLGAELNAAYY	280						
16083	²⁸¹ QEEWPAPATHAHLRGNWLKAY	300						
16084	³⁰¹ RIGVGTTTYSSTAQHSAAVA	320						
16085	³⁰⁵ GTTTYSSTAQHSAAVAEEPS	324						
11095	PEPTIDE CONTROL							

Figure 4. Peptide-specific A549 and U937 cell-binding activity. Protein and peptide numbers are shown. The bars represent binding activity.

Figure 5. Saturation assay. Saturation curves result from graphing free ^{125}I -peptide versus specifically bound ^{125}I -peptide concentration. The curves for A549-HABPs 16072, 16083, and 16084 can be observed.

50-kDa band was observed in the autoradiography. No signal was observed when performing the same peptide's cross-linking assay with HepG2 cells (lanes 3 and 4). These results indicated that peptide 16084 binding to U937 cell membrane was highly specific.

Invasion inhibition assay using HABPs

Peptides 16072, 16083, and 16084 were used during a highly sensitive and specific assay which has been recently developed at our institute for measuring the ability of *M. tuberculosis* to invade A549 cells. It was found that HABPs 16083 and 16084 were able to reduce invasion by $30.2 \pm 3.9\%$ to $52.3 \pm 7.7\%$ in a dose-response-dependent manner (Fig. 7), thereby showing these peptides' great ability to inhibit *M. tuberculosis* invasion of alveolar cells. It was determined that the invasion inhibition presented by HABP 16072 was not significant at any of the concentrations analyzed.

Another assay revealed $56.5 \pm 4.1\%$ and $45.5 \pm 1.7\%$ inhibition using a mixture of the 3 HABPs at 50 μM and 100 μM , respectively. These results were significantly different with HABP 16072 (at both concentrations) and 16083 at 50 μM . Likewise, HABPs 16083 and 16084 were mixed, showing $43.8 \pm 12.4\%$ to $48.8 \pm 10.9\%$ inhibition ability. The significant difference was determined regarding HABP 16083 at 50 μM and 100 μM . The foregoing showed that the mixture of HABPs did not lead to increasing invasion inhibition percentages since the differences found were mainly against the treatments which had not shown any effect when used alone.

It was determined that HABP 16083 presented greater inhibitory ability at greater concentrations. Using the peptide at 100 μM concentration increased its inhibition twofold compared to 50 μM ; however, when evaluating it at concentrations $>100 \mu\text{M}$ its inhibition ability remained almost stable, which could have been due to receptor saturation. Peptide 16084 presented greater inhibition ability than 16083; this effect was most notable at 50 μM ($48.6 \pm 0.6\%$) and 100 μM ($52.3 \pm 7.7\%$), since percentage invasion became reduced at greater concentration ($40.5 \pm 0.7\%$). These results showed that HABPs

identified in the Rv2707 protein play an important role in *M. tuberculosis*-target cell interaction, making them excellent candidates for being included in components of a multi-epitope, subunit-based, chemically synthesized, anti-tuberculosis vaccine.

Discussion

BCG has presented contradictory results without inducing protection against pulmonary tuberculosis in adults. Developing a new vaccine has thus become absolutely indispensable for controlling tuberculosis. There are two potential anti-tuberculosis vaccination strategies: prophylactic (preexposure) and therapeutic (once an individual has been exposed). The former have been most studied, including recombinant BCG (rBCG), *M. tuberculosis* attenuated strains, subunit vaccine approaches and live, nonreplicating viral vector-based delivery systems used alone or in prime-boost regimens (Skeiky and Sadoff 2006).

To date, no vaccine has been found showing a better level of protection than the existing one; designing and implementing new methodologies for selecting vaccine candidates has thus become necessary.

Initial host cell interaction with *M. tuberculosis* is mediated by many cell receptors such as mannose, glucan, Toll-like, surfactant protein, scavenger, and complement receptors 1, 3, and 4 (Ernst 1998; Rooyackers and Stokes 2005). In vitro studies have shown that there are two main recognition mechanisms. The first involves the direct interaction of host cell receptors with the mycobacteria's constituent components (Schlesinger et al. 1996), the second involves specific recognition of seric elements which function as opsonins (collectins and their receptors, complement and its receptors, immunoglobulin and their receptors) (Hirsch et al. 1994).

Figure 6. Sixteen thousand eighty-four radiolabeled peptide cross-linking assay. U937 cells were incubated with ^{125}I -labeled peptide 16084 in the absence (total binding, lane 1) or presence (inhibited binding, lane 2) of unlabeled peptide. Peptide 16084 bound to a 40-kDa and a 50-kDa band. The same assay was carried out on normal HepG2 cells (total binding lane 3 and inhibited binding lane 4).

Figure 7. Invasion inhibition assay. Inhibition percentages using different concentrations of peptides 16072, 16083, and 16084, using colchicine as control. The results correspond to average inhibition percentage calculated for each treatment $\pm$ SD. **P* value ≤ 0.05 , ***P* value ≤ 0.01 .

Many studies have been carried out with the *M. tuberculosis* H37Rv proteome to identify proteins which will facilitate designing novel measures for preventing tuberculosis (Jungblut et al. 1999; Sinha et al. 2002, 2005). Likewise, some membrane proteins have been determined; however, it is still not known which of these play a role in *M. tuberculosis* invasion (Gu et al. 2003). Understanding *M. tuberculosis*–host cell interaction and identifying the bacteria's surface molecules serving as ligands with the many cell receptors will lead to characterizing the various proteins directly involved in invasion.

Since *M. tuberculosis* infection of target cells begins with initial recognition and adherence (being essential for mycobacterial survival), then studies carried out at our institute with different *M. tuberculosis* H37Rv genes (Rv1510, Rv2004c, and Rv2536) (Forero et al. 2005; Garcia et al. 2005; Vera-Bravo et al. 2005) have evaluated their presence in *M. tuberculosis* complex strains and their subsequent transcription and translation. Synthetic peptides have been used for identifying sequences found to be directly implicated in pathogen–host interaction and thereby determining HABPs for each protein.

In spite of great complexity in *M. tuberculosis* composition, results obtained in this work led to us confirming that the gene encoding the hypothetical Rv2707 membrane protein (Cole et al. 1998) was exclusively present in all *M. tuberculosis* complex strains, but was not found in MOTT. RT-PCR also revealed that this gene was preferentially transcribed in *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. africanum*, but not in *M. bovis* or *M. microti*. A study (Mahairas et al. 1996) aimed at evaluating genetic differences between *M. bovis* and *M. bovis* BCG determined the effect of deletion regions on gene expression. The BCG Brazil (deficient in RD1 and RD3), *M. bovis* and *M. tuberculosis* (containing RD1 and

RD3 regions) strains were evaluated, finding that BCG expressed higher levels of some proteins and at least 10 additional proteins. Another study (Mostowy et al. 2004) produced an H37Rv-deficient *M. tuberculosis* strain from the RD1 region (H37Rv: RD1), finding that nine open reading frames in six genomic regions were induced in H37Rv:RD1 by comparison with H37Rv. These results showing mycobacterial strains having deletions expressing some additional proteins regarding nondeleted ones are similar to our Rv2707 results; however, the physiological implications of this behavior are still to be determined.

One interesting feature that the Rv2707 protein displays is its high content of alanine and leucine. A previous study describes a group of simple sequence proteins (SSPs) in prokaryote genomes, where repeated amino acids are found (Subramanyam et al. 2006). Although amino acids such as glycine, proline, alanine, and leucine (the last two highly represented in Rv2707) were frequently found to be repeated both in SSPs and non-SSPs, these amino acids have as a common feature a low biosynthetic cost.

Immunoblotting studies revealed the protein in *M. tuberculosis* total sonicate, since antibodies induced in two of the rabbits immunized with polymeric peptides derived from Rv2707 predicted amino acid sequence specifically recognized a 36-kDa band, this being the protein's approximate molecular weight (35.05 kDa). This technique also led to determining that the Rv2707 protein was found in the membrane fraction (Fig. 2, lane 6), supporting what had been predicted with PSORT. IEM confirmed this protein's localization on *M. tuberculosis* surface.

Bearing in mind the importance of studying the biological activity of membrane proteins involved in *M. tuberculosis*–host cell interaction, it was determined that membrane protein Rv2707 presenting HABPs 16072

(⁶¹LFGPDTLPAIEKSALSTAHSY⁸⁰) and 16084 (⁶¹LFGPDTLPAIEKSALSTAHSY⁸⁰) bound to both A549 and U937 cells, while HABP 16083 (²⁸¹QEEWPAPATHAHLGNWLKAY³⁰⁰) selectively bound to A549 cells. HABP 16084 specifically bound to possible 40-kDa and 50-kDa receptor molecules on U937 cell membrane in cross-linking assays.

HABPs 16083 and 16084 were located near the C terminus of the Rv2707 protein, suggesting the presence of an extreme C-terminal binding region. The region consisting of residues lying between Q²⁸¹ and A³²⁰ was involved in *M. tuberculosis*–host cell interaction. Studies regarding the effect of identified HABPs on the A549 cell line led to finding that they presented high invasion inhibition ability.

The receptor for HABP 16083 possibly became saturated, since it inhibited invasion by around $16.5 \pm 8.1\%$ at 50- μ M concentration. However, inhibition became $30.2 \pm 3.9\%$ when this concentration was doubled. Inhibition was around $33.4 \pm 3.0\%$ at the greatest concentration evaluated, suggesting that this HABP had dose-dependent behavior, clearly seen by increased peptide concentration and subsequent inhibition of *M. tuberculosis* invasion of A549 cells.

HABP 16084 exhibited $48.6 \pm 0.6\%$ and $52.3 \pm 7.7\%$ inhibition at 50 μ M and 100 μ M, respectively, making this HABP most able to inhibit *M. tuberculosis* invasion of A549 cells.

It could be concluded that HABP 16084 presented equal inhibition regarding *M. tuberculosis* ability to invade A549 cells, 16,083 inhibited it a little less and 16072 definitively did not present inhibitory activity when comparing each HABP against colchicine. These results could be explained in line with that predicted by bioinformatics, since 16072 (although solvent-exposed) seemed to be intracellularly located and its inhibition ability was thus nil or greatly reduced.

This work has led to identifying the presence of the Rv2707 gene, as well as its transcription and expression in *M. tuberculosis* and proposes using some of these HABPs derived from *M. tuberculosis* membrane as components of multiepitope, subunit-based, anti-tuberculosis synthetic vaccines.

Materials and Methods

Bioinformatics analysis

Protein alignments were obtained by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Altschul et al. 1997) and CLUSTALW (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) (Thompson et al. 1994) multiple alignments. Different parameters were evaluated for the Rv2707 protein such as hydrophathy, transmembrane regions, cell local-

ization, and signal sequence. The grand mean of hydrophathy (GRAVY) scores were calculated using the PROTPARAM tool (<http://us.expasy.org/tools/protparam.html>) (Gasteiger E. 2005) in which a score ≥ 0.4 (mean score for the cytosolic proteins) suggests probability for membrane association; the higher the score, the greater the probability (Kyte and Doolittle 1982). Transmembrane regions were predicted using TMHMM (<http://www.cbs.dtu.dk/services/TM>) (Moller et al. 2001) and TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) (Hoffman and Stoffel 1993). Cell localization, lipid attachment sites, and signal sequences were predicted from PSORT (<http://psort.nibb.ac.jp>) (Nakai and Kanehisa 1991).

Mycobacterial species and strains

The ATCC and Trudeau Mycobacterial Collection (TMC) were the sources for the following mycobacterial species and strains used: *M. tuberculosis* H37Rv (ATCC 27294), *M. tuberculosis* H37Ra (ATCC 25177), *M. bovis* (ATCC 19210), *M. bovis* BCG (ATCC 27291, Pasteur substrain), *M. africanum* (ATCC 25420), *M. microti* (Pasteur strain, kindly donated by Dr. F. Portaels, Institute of Tropical Medicine, Belgium), *M. flavescens* (ATCC 14474), *M. fortuitum* (ATCC 6841), *M. szulgai* (ATCC 65799), *M. peregrinum* (ATCC 14467), *M. phlei* (ATCC 11758), *M. scrofulaceum* (ATCC 19981), *M. avium* (ATCC 25291), *M. smegmatis* (ATCC 14468), *M. nonchromogenicum* (ATCC 19530), *M. simiae* (TMC 1595), *M. intracellulare* (ATCC 13950), *M. gastri* (TMC 115754), *M. kansasii* (ATCC 12478), *M. diernhoferi* (ATCC 19340), *M. gordonae* (ATCC 14470), *M. marinum* (ATCC 927), *M. terrae* (ATCC 15755), *M. chelonae chelonae* (ATCC 35752), *M. xenopi* (ATCC 35841), *M. vaccae* (ATCC 4978), and *M. triviale* (ATCC 23292). All mycobacterial species and strains were grown for 5–15 d in Middlebrook 7H9 (Difco Lab) and Middlebrook 7H10 (Difco).

Chromosomal DNA extraction and PCR assay

DNA was isolated from mycobacterial species and strains by the previously described phenol-chloroform method (Del Portillo et al. 1991; Parra et al. 1991; Mahairas et al. 1996). DNA was precipitated with 2-propanol, washed with 70% ethanol, and suspended in 1 $\times$ TE. The PCR assay was carried out in a thermal cycler (Gene Amp PCR system 9600, Perkin-Elmer) by incubating 100 ng genomic DNA with 40 μ L PCR master mix, 1.5 mM MgCl₂, 0.1 mM of each deoxynucleoside triphosphate, 0.4 μ M each oligonucleotide primer (sense 5'-ATGCTCGGCGCTGAAGCTC-3' and antisense 5'-ATTGCAAGCGGATTTACGAC-3'), and 1.5 U *Taq* polymerase (Promega) in a final 50- μ L reaction volume. Sense primer anneals between nucleotides 811 and 828 and antisense primer anneals 19–38 nucleotides downstream the gene's stop codon. Primer design was difficult due to the high Rv2707 GC content (62%); therefore, antisense primer had to be designed downstream this gene. Then 30 cycles of the following thermal profile were carried out after 5 min DNA denaturing at 94°C: 58°C for 15 sec, 72°C for 15 sec, and 94°C for 30 sec. A final 5-min extension cycle performed at 72°C was used for PCR amplification.

RNA isolation and reverse transcription-PCR (RT-PCR)

Sodium azide 10 mM was added to the culture just before harvesting. The cell pellet was suspended in cold lysis buffer (Katoch and Cox 1986) and sonicated twice for 15 min.

The pellet was suspended in 100 μ L of Trizol (Gibco) and stored in aliquots at -80°C . Total RNA was precipitated with isopropanol, washed with 70% ethanol, and suspended in distilled water.

cDNA synthesis involved target RNA (500 μ g) and 10 μ g/mL random primers (Gibco) being incubated for 10 min at 70°C . Next, $1\times$ RT buffer (0.14 M KCl, 8 mM MgCl_2 , 50 mM Tris-HCl, pH 8.1), 10 mM dithiothreitol (DTT), 0.5 mM dNTPs, 40 U Human Placenta Ribonuclease Inhibitor (Promega), 200 U M-MLV reverse transcriptase (Gibco) were added in a 30- μ L final volume. This mixture was kept at 37°C for 1 h. PCR was carried out as described above.

The *rpoB* gene was used as positive transcription control (Lee et al. 2000). DNase-Q-treated *M. tuberculosis* H37Rv DNA was used as a negative cDNA synthesis control. Distilled water and *M. tuberculosis* H37Rv DNA were used as PCR negative and positive controls, respectively.

Cell culture

The lines A549 (human lung carcinoma) and U937 (monocyte cell line derived from human histiocytic lymphoma) were obtained from ATCC and kept in culture using RPMI 1640 (Gibco) and 10% fetal bovine serum (HyClone, Inc.) at 37°C and 5% CO_2 . The cells were dislodged with 0.1% EDTA-PBS. Both cell lines were collected and washed with PBS.

Peptides

Peptides were synthesized by multiple solid phase methodology (Houghten 1985). The peptides were 20 amino acids long, nonoverlapped, and spanned the entire Rv2707 sequence. One Tyr-residue was added at the C terminus of peptides lacking a Tyr-residue in their sequence to allow ^{125}I -radiolabeling. These peptides were characterized by HPLC and MALDI-TOF mass spectrometry.

Radiolabeling

^{125}I -radiolabeling was performed according to previously described techniques (Yamamura et al. 1978; Urquiza et al. 1996) in which chloramine T (2.25 mg/mL) and 3.2 μ L ^{125}I -Na (100 mCi/mL) were added to 5- μ L peptide solution (1 μ g/ μ L). Fifteen microliters of sodium bisulphite (2.75 mg/mL) were added after a 5-min reaction at 18°C . The radiolabeled peptide was then separated from reaction subproducts on a Sephadex G-10 column (Pharmacia).

Binding assay

A549 or U937 cells (2×10^6) were incubated with different radiolabeled peptide concentrations (10–200 nM), in the presence or absence of unlabeled peptide (40 μ M), for 2 h at 4°C (Forero et al. 2005). An aliquot of this reaction mixture was passed through a 60:40 dioctylphthalate-dibutylphthalate cushion (1.015 g/mL). A gamma counter was used for quantifying cell-associated radioactivity. Binding assays were performed in triplicate. Peptide 11095 of the Rv1510c protein (Vera-Bravo et al. 2005), which has been reported as specifically binding to A549 and U937 cells, was used as positive control of cell-binding assays.

Saturation assay

A549 cells (1×10^6) were incubated with increasing radio-labeled HAPB concentrations between 0 and 2400 nM, in the presence or absence of 80- μ M unlabeled HAPB. After incubation, unbound peptide was removed from cells by passing the reaction mixture through a dibutylphthalate-dioctylphthalate cushion (1.015 g/mL). The curves obtained were analyzed by saturation and Hill analysis.

Cross-linking assay

U937 cells (2×10^6) were subjected to a conventional binding assay with HAPB 16,084. Cells were washed with PBS following incubation and cross-linked with 25 μ M BS³, Bis (sulfo-succinimidyl suberate) (Pierce), for 15 min at 18°C . The reaction was stopped with 20 mM Tris-HCl pH 7.4 and washed again with PBS. The cells were then treated with a lysis buffer (5% SDS, 10 mM iodoacetamide, 1% Triton X-100, 100 mM EDTA, 10 mM PMSF). The obtained membrane proteins were separated in 12% SDS-PAGE. Those proteins cross-linked with radiolabeled peptide were exposed on BioRad Imaging Screen K for 7 d. The same cross-linking assay was performed simultaneously on HepG2 cells.

Rabbit immunization

Two New Zealand rabbits (having previously been determined to be nonreactive to *M. tuberculosis* sonicate by Western blot) were inoculated with 0.5 mg polymerized peptide 25680 (CG²¹LSKSWDDSIFFESAQAFAWSY⁴⁰GC) or polymerized peptide 25684 (CG³⁰⁵GTTTYSSTAQHSAAVEEPS³²⁴GC), corresponding to the amino acid sequences of monomer peptides 16070 and 16085, located in N-terminal and C-terminal protein regions, respectively. These polymers were emulsified with Freund's Incomplete Adjuvant (FIA) and administered on days 0, 20, and 40. Bleeding was carried out 20 d after the third inoculation. The Rv2707 protein peptide sequences chosen for immunizing rabbits were obtained by using SYFPEITHI epitope prediction software available at <http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm> (Rammensee et al. 1999).

SDS-PAGE and immunoblotting

Proteins from *M. tuberculosis* subcellular fractions were separated in a discontinuous SDS-PAGE system, using a 10% to 20% (W/V) acrylamide gradient. A total of 500 μ g/mL of each sample were loaded per gel, transferred to nitrocellulose membrane (Hybond, Pharmacia) (Kyhse-Andersen 1984), and then incubated with a 1:100 sera dilution for 1 h. The samples were then incubated with conjugated antirabbit IgG antibody alkaline phosphatase 1:3000 (ICN) for 1 h; the reaction was developed with NBT/BCIP (Promega).

Immunoelectron microscopy

IEM studies were carried out on a Philips CM 10 TEM. Fifty microliters of *M. tuberculosis* H37Rv wet pellet were fixed with a 4% paraformaldehyde–0.5% glutaraldehyde solution for 2 h at 4°C . The pellet was dehydrated in graded ethanol and then embedded in LR-white hard-grade acrylic resin (Sigma-Aldrich).

for 4 d at 4°C. Thin sections (400 nm) were cut and mounted on 300 mesh nickel grids. Grids were incubated in 1:10 dilution rabbit polyclonal serum for 1 h at 37°C and then immersed in a 1:50 dilution of either 5 or 10 nm gold-labeled antirabbit IgG (Sigma) for 1 h at room temperature. Grids were then washed with TBS-Tween and fixed in 2.5% glutaraldehyde and incubated for 15 min in 1% uranyl acetate. Grids were washed with distilled water and dried at room temperature before observation (Stirling and Graff 1995).

Mycobacterium for invasion assay and bacterial staining

The bacilli were collected at the logarithmic phase, washed, and suspended in 1× PBS. Due to the mycobacteria's tendency to form clumps, the suspension was briefly sonicated (30 W for 5 sec) in a Branson sonifier (VWR International). *M. tuberculosis* was labeled with SYBR Safe (Applied Biosystems) at 20× final concentration at 37°C for 20 min in the dark. Mycobacteria were washed twice, centrifuged at 12,000 rpm for 20 min, and excess dye was removed. Bacteria were then suspended in RPMI 1640 and labeling was further verified by fluorescence microscopy (FM) and flow cytometry (FC).

Inhibition invasion assay using HABPs

A549 cells (1×10^6) in suspension in complete RPMI 1640 medium without antibiotics were preincubated for 1 h at 37°C in the presence of HABPs (50 μM, 100 μM, and 200 μM) identified in Rv2707. *M. tuberculosis* H37Rv was then added at 1:10 multiplicity of infection (MOI) (Bermudez and Goodman 1996) to a final 200 μL volume. They were incubated at 37°C in 5% CO₂ for 2 h and then placed in medium supplemented with 20 μg/mL amikacin (ICN), for 30 min. Two RPMI 1640 washes were then carried out by centrifuging at 2500 rpm for 5 min. The cells were fixed in 1% paraformaldehyde (Sigma) in RPMI 1640 at 4°C for 1 h, washed, and suspended in RPMI 1640. The cells were counterstained with 3% methylene blue in 30% ethanol and incubated at room temperature for 5 min before being read for FC and FM. Cell viability was determined with trypan blue by counting in a Neubauer chamber.

Invasion inhibition controls were carried out in the presence of a tubulin-polymerization inhibitor such as 30 μM colchicine (Sigma). The inhibitor was added to A549 cells in suspension 1 h prior to adding bacteria for inhibiting mammalian cell internalization and incubated at 37°C for 1 h (Bermudez and Goodman 1996).

Flow cytometry quantification

Samples were analyzed on a FACScan (Becton Dickinson), equipped with 488-nm argon lasers. Cellquest software (Becton Dickinson) was used for FC capture and analysis. Uninfected A549 cells were discriminated from infected A549 based on light FL1 characteristics. Samples were run at 2500 events/sec; 40,000 events were collected.

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