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Mycobacterium tuberculosis surface protein Rv0227c contains high activity binding peptides which inhibit cell invasion

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ABSTRACT

Mycobacterium tuberculosis surface proteins involved in target cell invasion may be identified as a strategy for developing subunit-based, chemically-synthesized vaccines. The Rv0227c protein was thus selected to assess its role in the invasion and infection of *Mycobacterium tuberculosis* target cells. Results revealed Rv0227c localization on mycobacterial surface by immunoelectron microscopy and Western blot. Receptor–ligand assays using 20-mer, non-overlapping peptides covering the complete Rv0227c protein sequence revealed three high activity binding peptides for U937 phagocytic cells and seven for A549 cells. Peptide 16944 significantly inhibited mycobacterial entry to both cell lines while 16943 and 16949 only managed to inhibit entrance to U937 cells and 16951 to A549 cells. The Jnet bioinformatics tool predicted secondary structure elements for the complete protein, agreeing with elements determined for such chemically-synthesized peptides. It was thus concluded that high activity binding peptides which were able to inhibit mycobacterial entry to target cells are of great importance when selecting peptide candidates for inclusion in an anti-tuberculosis vaccine.

1. Introduction

Tuberculosis represents one of the major causes of morbidity and mortality worldwide. This infectious disease caused by *M. tuberculosis* (*Mtb*) caused an estimated 9.4 million new cases, 1.3 million deaths and 0.5 million cases of multi-drug resistant tuberculosis (MDR-TB) in 2008 [63]. The search for new and improved alternatives for designing vaccines providing greater protection than the actual BCG vaccine is thus compelling and necessary.

Mycobacteria reach the alveolar space during invasion and become engulfed by the macrophages and other types of non-phagocytic cells, such as endothelial cells and fibroblasts, where they stay during the earlier stage of infection acting as potential reservoirs for the bacteria and thereby inducing a localized pro-inflammatory response leading to mononuclear cells being recruited from the nearest blood vessels [19,23]. *Mtb* ability to enter and multiply inside non-phagocytic cells explains this microorganism's physiopathological versatility. Although macrophages act as the host's first line of defense, these also become a mycobacterial replication center [7]. *In vitro* assays have shown that mycobacteria are capable of invading and surviving inside A549 epithelial cells (type II pneumocytes) as part of *Mtb* translocation through the alveolar wall [4]. *Mtb* H37Rv's ability to invade type II A549 alveolar epithelial cells and macrophages has shown the mycobacterium's specific strategies for surviving in different hosts [12]. *Mtb* expresses multiple ligands which bind to different host cell receptors involved in entry, such as the mannose receptor [14], surfactant protein A [20], complement receptor [6], scavenger receptors, Toll-like receptors TLR 2 and TLR4 [52] and Fc receptors [46]. These receptors are used by the bacteria in actin filament- and microtubule-dependant binding to and entering target cells through phagocytic or endocytic vesicles.

Both mycobacterial membrane proteins and host cell surface receptors must be taken into account in cell–mycobacteria interaction. The characteristic cell envelope of mycobacteria has not just one lipid layer similar to that of other gram-positive bacteria having a regular internal membrane but rather it has another mycolic acid-rich lipid layer interconnected to the internal layers of peptidoglycan and arabinomannan forming the cell wall. The complex cell surface is composed of proteins, sugars and lipids; the protein is mostly immersed in the cell wall; however, some proteins are exposed on the surface, playing roles in important processes for mycobacteria such as cell–cell interactions, ion and nutrient transport and cell signaling, and participate in key pathogenically-relevant cellular mechanisms. Many

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proteins required for mycobacteria pathogenicity are surface proteins, enzymes, receptors, transporters or signal transductors that are involved in lipid metabolism and transport across the cell envelope [10]. Proteins localized on the membrane have been involved in metabolic and biosynthetic biochemical reactions promoted by the cell membrane's lipid environment [54]. The cell membranes' structural environment promotes cell metabolism since it contains the multi-enzymatic complexes necessary for such metabolic processes [24,55]. Membrane proteins have been the object of growing interest as the target for designing vaccines and drugs based on such proteins' characteristics, acting as essential enzymes, receptors and transporters. Exhaustive bioinformatics screening has suggested some proteins from the PE, PPE and MCE families to be potentially-exposed membrane proteins, many of which have been studied today as vaccine candidates [41]. Other authors have focused on these important proteins in invasion, stimulating an immune response helping to define antigens having vaccine potential [56].

The *Mtb* Mce surface protein family has been implicated in invasion and its members have been presented as anti-tuberculosis vaccine candidates [25]. The Mce1A protein, together with antibiotic treatment, has been shown to be able to prevent the development of new cases of active tuberculosis from cases of latent tuberculosis [38]. Gioffre et al., have produced mutants in Mce-1, Mce-2 and Mce-3 protein operons which they used to infect mice by intra-tracheal route, observing that these mutants became hipovirulent strains leading to the total survival of the mice, having few colony-forming units and limited tissue damage [21]. Suppressing Mce-2 and Mce-3 protein expression led to live vaccines' increased immunogenicity and their reduced virulence [1].

Previous studies have demonstrated that mycobacterial invasion of macrophages and other non-phagocytic epithelial cells may be mediated by mycobacterial surface proteins (*i.e.* Mce family proteins) [16]. Experimental results obtained in our laboratory have led to some *Mtb* proteins' involvement in such invasion having been described, assessed and characterized in terms of their specific binding ability and role in mycobacterial entry to A549 cells and U937 monocyte-derived macrophages [5,18,40,42,50,59]. Identifying surface proteins (or their specific segments) in intracellular pathogens such as *Plasmodium falciparum* has been shown to counteract invasion, thereby becoming an important approach in the search for minimal subunit-based, multi-epitope, synthetic vaccine candidates [43].

The methodology proposed by our group has been based on the use of synthetic peptides for which advantages have been recognized regarding other molecules for identifying fragments for a subunit-based vaccine. When compared to recombinant proteins and/or other types of molecules (even though being less immunogenic than recombinant proteins) the peptides involve lower production costs, greater activity per unit of mass, high purity and stability thereby allowing longer storage periods at room temperature, great sequence diversity leading to finding highly efficient, selective and specific fragments, minimum risk of toxicity since their degradation products are aminoacids and have a low level of accumulation in tissue [61].

Some peptides' direct antimicrobial activity has been described as being immunomodulatory, meaning that some of them have been used as adjuvant [39]. β-Defensins are some of the few peptides related to *M. tuberculosis* infection; they are cationic natural antimicrobial peptides which are mainly produced by epithelial and phagocytic cells. High mycobacteria-associated β-defensin production has been reported during *Mtb* infection of pulmonary epithelial cells *in vitro*, suggesting its participation in eliminating *Mtb* by bacterial lysis [49] and it has been proposed, in the murine experimental model, that defensins could be involved in establishing a protection-inducing Th1 response against *Mtb* H37Rv, thereby suggesting their use as therapeutic agents or vaccine adjuvants [48].

Bioinformatics have been used for rationally selecting sequences which seem to be better immunomodulator candidates from amongst a microorganism's thousands of genes. Bioinformatics can thus be used for limiting the number of candidates to be tested and studies carried out in our laboratory have led to validating bioinformatics tools which are currently available for studying bacterial proteins [60]. The 421 amino-acid-long Rv0227c protein has been selected as part of such ongoing search for vaccine candidate proteins since it is a probable conserved membrane protein [24,35,64] and has been included in previous studies within a set of conserved genes from intracellular pathogenic mycobacterial species [34]. The present study was aimed at assessing Rv0227c localization on mycobacterial surface by immunoelectron microscopy (IEM) and its presence in *Mtb* sonicates and their fractions by Western blot. A tried-and-tested, robust, highly specific, sensitive receptor–ligand binding assay was used to assess 20-mer, synthetic, non-overlapping Rv0227c protein peptides' (covering the protein's complete sequence) specific binding to A549 and U937 cell receptors; these high activity binding peptides (HABPs) were used in invasion inhibition assays.

2. Materials and methods

2.1. Chromosomal DNA extraction and PCR assay

Mycobacteria were grown in 7H9 (Difco) supplemented with oleic acid, albumin, dextrose, NaCl (10% OADC) and incubated at their optimum temperature until cultures reached 0.5–1.0 OD_{600}. This should have provided a mid- to late-log phase culture; bacilli were harvested by spinning at 12,500 × *g* for 20 min at 4 °C, suspended in PBS and stored at −20 °C. The *rv0227c* gene's presence and transcription were assessed in *Mtb* strains (see Fig. 1).

Genomic DNA was isolated from mycobacterial species and strains using the commercial kit Ultra Clean® Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. The PCR assay was carried out in a thermal cycler (Gene Amp PCR system 9600, PerkinElmer) by incubating 100 ng genomic DNA with a PCR mixture containing: 1.25 units of BioTaqTM DNA Polymerase (Bioline, London, UK), 1× Taq polymerase reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs and 1 μM of each primer (sense: 5′-GCGACGGCACTGGAACG-3′ and antisense: 5′-GTGGGTCACGAGCGAAGT-3′) in a final 25-μL reaction volume. The reaction was carried under the following conditions: an initial denaturing step at 95 °C for 5 min followed by 35 cycles consisting of: 1 min at 58 °C, 1 min at 72 °C and 1 min at 95 °C. A final extension cycle at 72 °C for 5 min was performed. Sense and antisense oligonucleotides amplified a 711 bp fragment that was visualized in a 1% agarose gel stained with SYBR SafeTM (Invitrogen, Carlsbad, CA, USA). Extracted DNA quality was assessed by amplifying a 360 base pair fragment from the rpoB gene (encoding the B subunit of RNA polymerase) using the sense (5′-TCAAGGAGAAGCGCTACGA-3′) and antisense (5′-GGATGTTGATCAGGGTCTGC-3′) primers. DNase- and RNase-free water was used as a negative PCR control. Amplification products were electrophoretically separated on SYBR Safe-stained (Invitrogen) agarose gels. Extracted DNA quality was assessed by amplifying a 360 bp fragment from the *rpoB* gene. DNase- and RNase-free water was used as negative PCR control.

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

The bacterial pellet was suspended in an equal volume of guanidine thiocyanate, GTC (4 M guanidine isothiocyanate, 0.5%

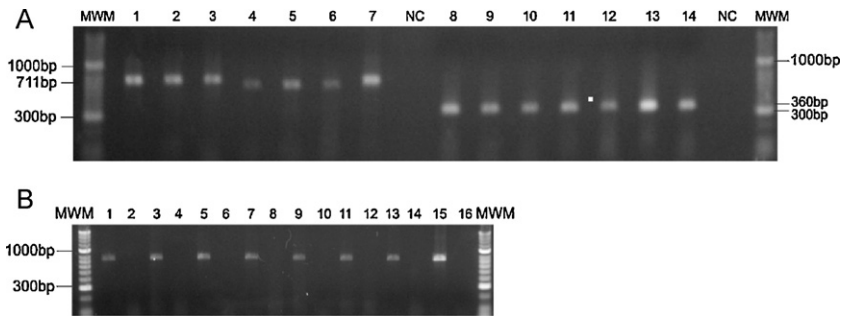

Fig. 1. Molecular assays. (A) PCR amplification of genomic DNA products isolated from mycobacterial species and strains. Lanes 1–7: *rv0227* gene amplification; lanes 8–14: *rpoB* gene amplification. Lanes 1 and 8: *M. tuberculosis* H37Rv (ATCC 27294). Lanes 2 and 9: *M. tuberculosis* H37Ra (ATCC 25177). Lanes 3 and 10: *M. bovis* (ATCC 19210). Lanes 4 and 11: *M. bovis* BCG (ATCC 35734, *pasteur* substrain). Lanes 5 and 12: *M. africanum* (ATCC 25420). Lanes 6 and 13: *M. microti* (*pasteur* substrain, kindly donated by Dr. F. Portaels, Institute of Tropical Medicine, Belgium). Lanes 7 and 14: *M. intracellulare* (ATCC 13950). MWM: 200 bp molecular weight marker. NC: negative PCR control. (B) 711-bp *rv0227* RT-PCR product amplified from different mycobacterial species and strains. MWM: molecular weight marker (200 line – Bioline, London, UK); 1 (positive) and 2 (negative) *M. tuberculosis* H37Rv synthesis, 3 and 4 *M. tuberculosis* H37Ra (as above), 5 and 6 *M. bovis* (as above), 7 and 8 *M. bovis* BCG (as above), 9 and 10 *M. africanum* (as above), 11 and 12 *M. microti* (as above), 13 and 14 *M. intracelullare* (as above). 15. PCR positive control (*M. tuberculosis* H37Rv DNA) and 16 PCR negative control.

sodium n-lauroyl sarcosine, 25 mM TRIS sodium citrate and 0.1 M 2-mercaptoethanol) to maintain RNA integrity. Total RNA was isolated from the pellet by adding 1 mL TRIZOL® reagent (Invitrogen), following manufacturer's instructions; it was then treated with DNAse I (Invitrogen) and used as template for RT-PCR assays. Complementary DNA (cDNA) synthesis was carried out using SuperScript III reverse transcriptase (Invitrogen) using random hexamers for first-strand cDNA synthesis, following the manufacturer's recommendations. A negative synthesis control (substituting SuperScript III enzyme for DEPC-treated water) was also included for each sample. 5 μL of cDNA was used as template for PCR amplification, following conditions previously described for DNA. *Mtb* H37Rv DNA and DNase- and RNase-free water were used as positive and negative PCR controls, respectively.

2.3. Rabbit immunization

The Rv0227c peptide sequences chosen for immunizing rabbits were obtained using BepiPred 1.0b Server epitope prediction software, available at http://www.cbs.dtu.dk/services/BepiPred/ [32]. Two New Zealand rabbits (previously determined as being non-reactive to *Mtb* sonicate by Western blot) were inoculated with 0.5 mg polymerized N-terminal peptide 25886 (CG^{81}ANADVVTLQVGSSLRRTDKQ^{100}GC) and polymerized C-terminal peptide 25888 (CG^{241}NEQITMTRYYAAQRTFWVDP^{260}GC). These polymers were emulsified with Freund's incomplete adjuvant (FIA) and administered on days 0, 20, and 40. Bleeding was carried out 20 days after the third inoculation, according to previously reported protocols [18,42,50,59].

2.4. Separating culture filtrate and subcellular fractions

According to already reported methodology [47,60], 25 mL *M tb* H37Rv culture aliquots were centrifuged at 10,000 × *g* for 20 min at 4 °C and the supernatant was filtered in sterile conditions using a 0.2-μm pore membrane to obtain the culture filtrate. Subcellular fractions were obtained by washing the pellet thrice with PBS, suspending it in protease inhibitor cocktail (100 μg/mL PMSF, 50 μg/mL pepstatin A, 50 μg/mL leupeptin), sonicating it for 15 min in an ice bath (work cycle: 80%, amplitude: 3), leaving it to settle for 15 min at 4 °C and sonicating it, the same as before. This procedure was carried in two stages to avoid samples overheating and thus maintain protein integrity. The sonicate was spun at 3000 × *g* for 5 min at 4 °C, the pellet (unbroken cells) was discarded and the supernatant was spun at 27,000 × *g* for 1 h at 4 °C. The pellet was suspended in lysis buffer containing lysozyme (without DNAse and RNAse) and spun once more for 1 h at 4 °C and 27,000 × *g*. The cell wall pellet from this centrifugation was suspended in ammonium bicarbonate, while the supernatants from the first and second centrifugations were pooled and spun at 100,000 × *g* for 4 h at 4 °C. The supernatant obtained from such spinning was labeled as cytosolic fraction and spun for 4 h at 100,000 × *g* and 4 °C to remove traces of membrane proteins (pellet). All pellets were pooled and labeled as membrane fraction.

The cell wall, membrane, cytosolic and culture filtrate were washed with DNAse-RNAse free lysis buffer, poured into 3500 MWCO dialysis tubing (Spectra/Por, CA, USA) and dialyzed against 10 mM ammonium bicarbonate for 24 h at 4 °C, changing the buffer thrice. Dialyzed fractions were concentrated with polyethylenglycol, quantified by BCA and stored at −70 °C.

2.5. Immunodetection assays

Mtb total sonicate and subcellular fraction proteins were separated in a discontinuous SDS-PAGE system using a 10–20% (W/V) acrylamide gradient; they were then transferred to a nitrocellulose membrane and incubated with rabbit sera obtained by inoculating the aforementioned polymer peptides, in 1:100 dilution. Samples were then incubated with alkaline phosphatase-160 conjugated antirabbit IgG in 1:3000 dilution; the reaction was developed with NBT/BCIP (Promega, Madison, USA).

Mycobacteria were embedded in LR-White resin (SPI supplies) [13] for IEM and analyzed by Hitachi Hu-12A electron transmission microscope to determine Rv0227c subcellular location using anti-goat IgG coupled to 10 nm colloidal gold particles and adding 6% uranyl acetate to enhance image contrast.

2.6. Peptide synthesis

Twenty-one peptides having 20 sequential non-overlapping amino acids, spanning the entire length of the Rv0227c sequence (NCBI Reference Sequence: NP_214741.1), were synthesized using t-Boc amino acids (Bachem) and MBHA resin (0.5 mequiv./g), following the solid-phase multiple peptide synthesis methodology [37]. The sequences of these chemically-synthesized Rv0227c peptides are shown in Fig. 3, indicating the name assigned to each peptide according to our institute's serial numbering system. Peptides were cleaved by the low-high hydrogen fluoride technique [58], purified by reversed-phase high-performance liquid chromatography and analyzed matrix assisted laser desorption/ionization time-of-flight mass spectrometry. A tyrosine residue was added to the carboxyl-terminus of those peptides

not containing this residue in their sequence to enable radiolabeling with $Na^{125}I$ [5,40,42,45,50]. Briefly, peptides were individually radio-labeled using 5 μL Na125I (100 mCi/mL; MP Biomedicals) and 15 μL chloramine-T (2.8 mg/mL) as oxidizing agent. The reaction was stopped after 15 min by adding 15 μL sodium metabisulfite (2.3 mg/mL), and radio-labeled peptides were separated by eluting the reaction mixture through a Sephadex G-10 column (Pharmacia, Uppsala, Sweden). Each eluted fraction was then analyzed in a gamma counter (Auto Gamma Cobra II Packard) [2,5,27,42,50,59]. Most peptides were soluble in PBS and those that were insoluble were dissolved in a minimal amount of DMSO prior to addition of PBS to bring the peptides to an initial 1 mg/mL concentration.

2.7. Rv0227c peptide interaction with target cells

U937 cells (2×10^5/mL, ATCC CRL-1593.2) were differentiated using 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich) for 3 days [11], to become adherent and A549 (ATCC CLL-185) cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum and kept at 37 °C in 5% CO_2.The two adherent cell lines were dislodged by suspension in 1× non-enzymatic cell dissociation solution (NECDS) (Sigma) for 3 min at 37 °C and collected by spinning at 500 × *g* for 5 min. The pellet was suspended in non-supplemented RPMI 1640 medium until further use [50].

Peptides were tested for their ability to bind to A549 and U937 cells. Briefly, cells (1.5×10^6) were incubated with increasing radio-labeled peptide concentrations (0–950 nM) for 90 min at 4 °C in the presence (non-specific binding) or absence (total binding) of the same unlabelled peptide (30 μM). An aliquot of this reaction mixture was passed through a 60:40 dioctylphthalate–dibutylphthalate cushion ($d = 1.015$ g/mL) and spun at 4500 × *g* for 3 min. Cell-associated radioactivity was quantified in an automatic gamma counter (Gamma Counter Cobra II). The assay was carried out in triplicate.

Specific binding values were obtained by subtracting non-specific binding from total binding values and specific binding for each cell line was calculated from the specific binding curve slope. Any peptide having ≥1% specific binding activity was called a high activity binding peptide (HABP) [59], suggesting a relevant biological interaction, since these peptides recognized more than 40,000 specific binding sites per cell at the ^{125}I-radio-labeled peptide concentrations used.

Saturation assays were carried out for characterizing HABP target cell binding. 1.5×10^6 A549 cells and 1.5×10^6 U937 cells were incubated with increasing (0–7000 nM) radio-labeled HABP concentrations in the presence or absence of 30 μM unlabeled HABP. Following incubation, unbound peptide was removed from cells by sedimentation through a dioctylphthalate–dibutylphthalate cushion ($d = 1.015$ g/mL). The curves so obtained were analyzed by saturation and Hill analysis [5,18,28,59,62].

2.8. Mycobacterial invasion and cytotoxicity assays

M. tuberculosis was sonicated once for 20 s, at 3.0 amplitude and 80% working cycle in an ice-bath using a Branson Sonifier Ultrasonic Cell Disruptor 450, the bacteria was labeled with SYBR c (Applied Biosystems), 20× final concentration at 37 °C for 20 min in the dark, then washed twice and spun at 12,000 rpm for 20 min; excess dye was removed. Bacteria were then suspended in RPMI 1640 and labeling was further verified by fluorescence microscopy.

Peptide ability to inhibit mycobacterial invasion was assessed following Bermudez and Goodman's technique [3], modified by our group [9]. Briefly, A549 and U937 cells (1×10^6), after having been seeded overnight on 6-well plates, were incubated with three logarithmic peptide concentrations (2 μM, 20 μM and 200 μM) for

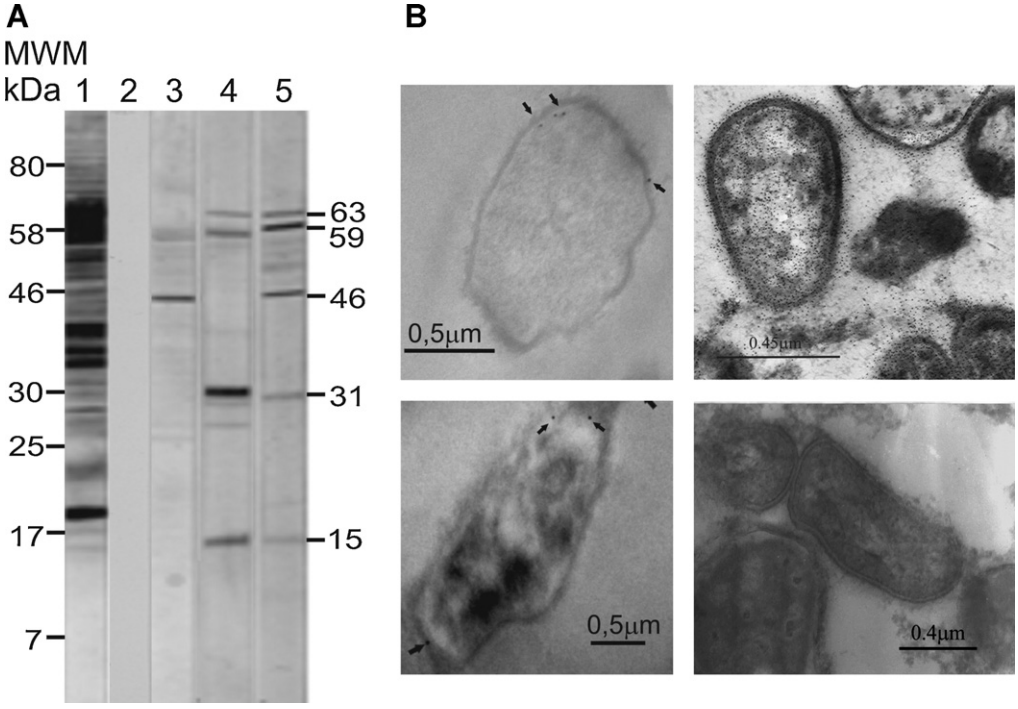

Fig. 2. Immunodetection. (A) Western blot. Positive control, total sonicate recognition with *Mtb* hyper-immune anti serum (lane 1). Negative control (pre-immune serum) (lane 2). Rabbit serum obtained after the third immunization, recognized a 46 kDa protein (close to the Rv0227c protein's theoretical molecular weight: 45.5 kDa) in whole mycobacterial sonicate (lane 3); 31 and 15 kDa molecular weight bands were recognized in the cell membrane fraction (lane 4) and five bands (63, 59, 46, 31 and 15 kDa) were recognized in the cytosol fraction (lane 5). (B) Immunoelectron microscopy. The presence of the Rv0227c protein can be observed on *Mtb* cell surface. The upper and lower panels on the left show the different slices for mycobacteria labeled with immune sera against Rv0227c peptides, arrows indicate anti-rabbit antibody-associated colloidal gold particles (10 nm). The result of hyperimmune serum is shown on the right upper and preimmune serum is on right down.

1 h, then washed twice with Hank's balanced salt solution (HBSS) to remove peptide excess. SYBR-safe stained mycobacteria were added (1:100 MOI) and incubated overnight at 37 °C. 20 μg/mL amikacin was added to each well and left to incubate for 2 h at 37 °C. Extracellular bacilli were removed by washing wells thrice with HBSS; the cells were dislodged using 3% Trypsin (Sigma) and analyzed in FACScan flow cytometer (Becton Dickinson) [9,60]. Student's *t*-test was used for analyzing treatment differences. Peptides' cytotoxic effect was evaluated using rezarsurine (*in vitro* toxicology assay kit, Sigma), following the manufacturer's recommendations. Briefly, cell cultures were removed from incubator, resazurin dye solution was added in an amount equal to 10% of the culture medium volume, then cultures were returned to incubator for 2 h. Samples were measured fluorometrically by monitoring the increase in fluorescence at a wavelength of 590 nm using an excitation wavelength of 560 nm.

2.9. CD spectroscopy

Rv0227c peptides' conformational elements were determined by CD spectroscopy [44]. Spectra were obtained in TFE or aqueous peptide solution by averaging three scans (Jasco J-810 spectropolarimeter) at 20 °C using a 1 cm cuvette, corrected for baseline deviation (260–190 nm wavelength range, 20 nm/min scan rate, 1 nm bandwidth) [57]. The protein's secondary structure was predicted using a Jnet algorithm [8].

3. Results

3.1. Molecular assays

PCR experiments carried out with mycobacterial genomic DNA showed the presence of the *rv0227* gene in all *M. tuberculosis* complex strains, as well as in *M. intracellulare* (Fig. 1A). The good quality of the extracted DNA was determined by amplifying *rpoB* control gene (Fig. 1A).

RT-PCR was carried out to determine whether the *rv0227* gene was being transcribed in 7H9 culture medium conditions. RT-PCR revealed a 711-bp fragment in the cDNA plus synthesis assay for all *Mtb* complex strains (*M. tuberculosis* H37Rv, H37Ra, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti*) and in the *M. intracellulare* specie. Lack of amplification in negative synthesis controls indicated contamination-free gDNA (Fig. 1B).

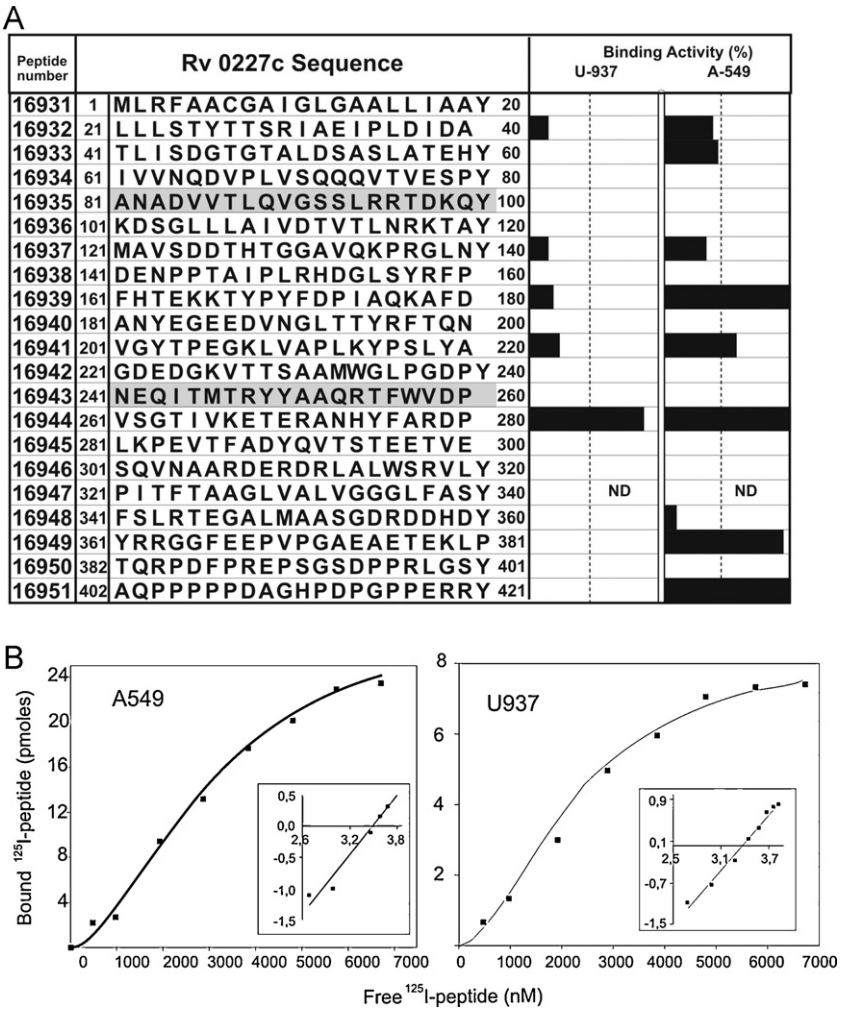

Fig. 3. Binding and saturation assays. (A). A549 and U937 cell binding profiles for Rv0227c synthetic peptides. The institute's synthetic peptide serial numbers and amino acid sequences are shown on the left of the figure. Horizontal black bars represent specific binding capacity, expressed as percentages for each peptide in both cell lines tested. Vertical dotted lines show the 1% specific binding cut-off above which a peptide was considered to be a HABP. Amino acid sequences used for rabbit immunization are shown in gray. (B) Saturation curves. These were obtained by plotting free ^{125}I-peptide concentration against ^{125}I-peptide specifically bound to HABP 16944 in U937 and A549 cells. The larger graph shows the saturation curve and the smaller one the Hill plot. Hill plot abscissa is Log *F* and ordinate is Log $[B/(B_{max} - B)]$, where *B* is bound peptide concentration, B_{max} maximum bound peptide concentration and *F* free peptide concentration.

3.2. Localization of Rv0227 protein on mycobacterial surface

The recognition of a 46 kDa band showed that the inoculated polymers could induce antibodies production; these antibodies are able to recognize the whole Rv0227c protein. Proteins in *M. tuberculosis* total lysate having different band heights were recognized in the positive control, whereas the negative control (pre-immune rabbit sera) showed no recognition of Rv0227c protein (Fig. 2A). A clearly defined band having a molecular weight around 46 kDa was observed in lane 3 corresponding to the total sonicate, which agrees with Rv0227c theoretical molecular weight (45.5 kDa). An additional weak band was observed at 59 kDa (Fig. 2A, lane 3).

Regarding *M. tuberculosis* H37Rv subcellular fractions, the recognition of bands having around 31 and 15 kDa molecular weights was observed; thus the possibility of proteolytic processing of this protein once it is located on the envelope could not be ignored (Fig. 2A, lane 4). Bands corresponding to the protein with and without processing were recognized in the mycobacterial cytosolic fraction, along with 46, 31 and 15 kDa bands (Fig. 2A, lane 5). In Addition, mycobacteria protein immune-localization using anti-rabbit secondary antibodies coupled to colloidal gold particles showed the Rv0227c protein on mycobacterial surface (Fig. 2B, panels on left). The results of hyperimmune and preimmune sera are shown.

3.3. Rv0227c peptides showing high specific binding ability

Protein segments which are specifically able to interact with A549 and U937 cell receptors were assessed using 21 synthetic peptides, containing 20 non-overlapping amino acids covering the protein complete sequence. Three HABPs for U937 (16943, 16944 and 16949) and seven for A549 cells (16934, 16939, 16941, 16944, 16945, 16949 and 16951) were found from the total number of peptides assessed for each cell line. Fig. 3A shows the position and amino acid sequence for each peptide. The black bars represent the specific binding percentages for all peptides assessed in both cell lines; the dotted line indicates 1% specific binding.

A saturation assay was performed to determine the dissociation constant (K_d) as an affinity measure, the Hill coefficient was calculated (n_H) and the number of receptors on U937 and A549 cells surface was determined to make a first approach to assess the nature of such HABP-receptor interaction. It was thus decided to work with HABP 16944, which had high specific binding to both A549 and U937 cell lines.

Fig. 3B shows the results of these saturation assays, where K_d = 3 500 nM was obtained for A549 cells and K_d = 2 400 nM for U937 cells, leading to assume that the peptide bound to the receptor due a high affinity and specificity interaction with similar characteristics for both cell lines. The Hill coefficient obtained in saturation assayed for both cell lines was greater than one ($n_H > 1$), thereby inferring that it presented a positive cooperativity interaction. Despite the aforementioned characteristics, the number of binding sites per cell varied from 6×10^4 in U937 cells to 16×10^6 in A549 cells; further studies will lead to the characterization of these receptor sites.

3.4. Rv0227 HABPS inhibited invasion

HABPs ability to inhibit invasion was assessed for each cell line using a tried-and-tested methodology [18,42,50,59], once all HABPs presenting specific binding to each cell line were established. A 37% of invasion was found for A549 cell line treated with cytochalasin D control, which inhibits actin polymerization, compared to invasion of untreated cells (taken as being 100%).

Fig. 4B shows that HABPs 16944, 16949 and 16951 provided the greatest inhibition, being able to inhibit mycobacteria entry by more than a 50% at the highest concentration (200 μM); however, such activity became increased as peptide concentration rose.

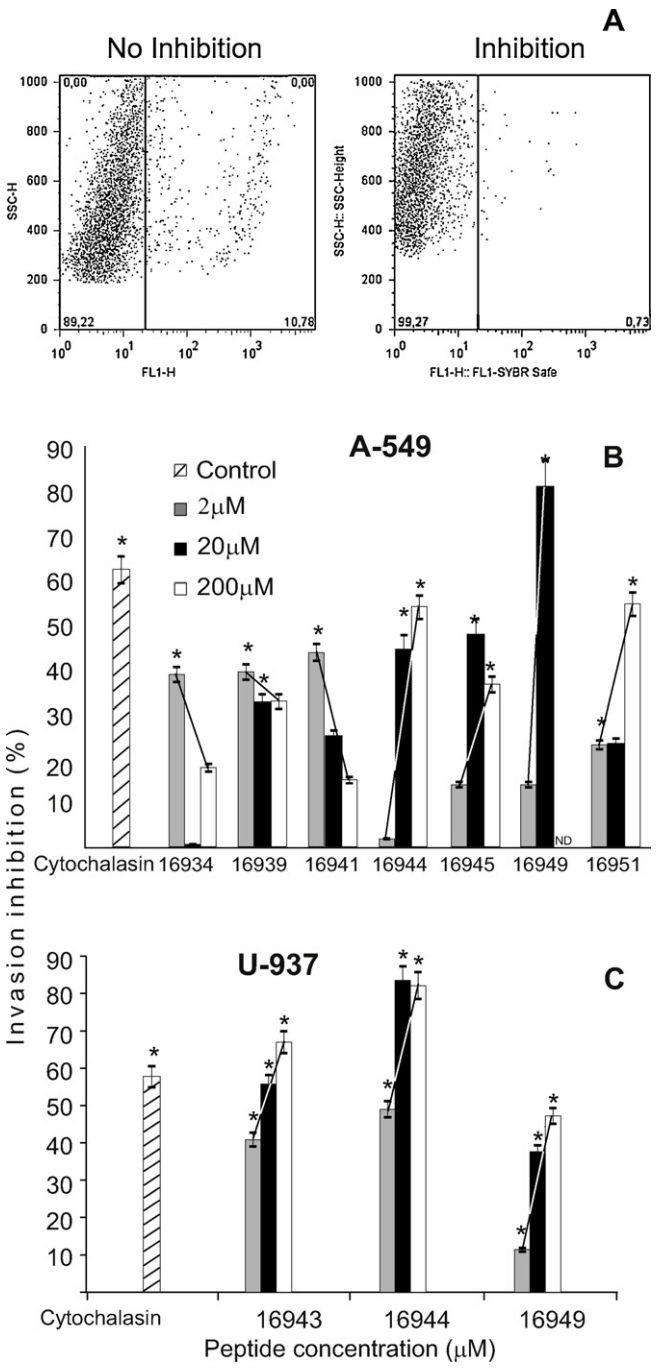

Fig. 4. Invasion inhibition percentages. (A) Density plots for invasion assay in HABP presence (inhibition) or absence (non-inhibition). (B) Percentages regarding *M. tuberculosis* H37Rv invasion of A549 cells. (C) Percentages regarding *M. tuberculosis* H37Rv invasion of U937 cells. Standard errors are indicated by black bars. $^*p = \leq 0.01$.

HABP 16951 gave no significant inhibition at 2 and 20 μM, although invasion decreased to 42% as concentration increased. HABP 16945 prevented a 48% of invasion at 20 μM, even though this percentage fell up to 37% when concentration was increased. HABPs 16934 and 16939 did not reduce mycobacterial entry by more than a 40% and there was no evidence showing that the inhibition trend was peptide concentration-dependent. Regarding HABP 16941, a trend showing an increase in the invasion percentage along with peptide concentration appeared.

U937 cells invasion decreased by more than 50% for all the concentrations of peptides 16943 and 16944. HABP 16943 reduced invasion to nearly 40% for all concentrations; HABP 16944 only

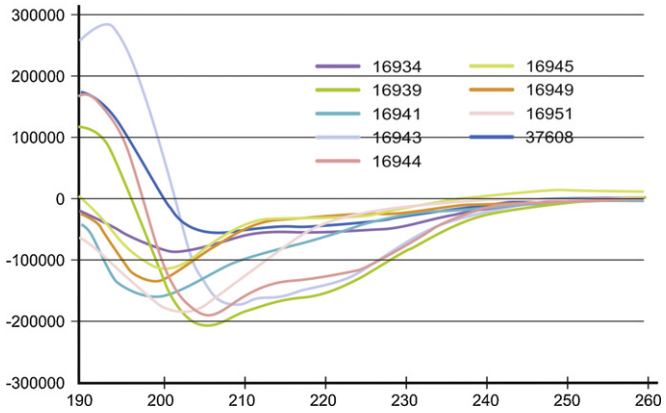

Fig. 5. Rv0227c peptide structure. The figure shows circular dichroism for Rv0227c HABPs and non-HABP 37608. The results are expressed in terms of mean residue ellipticity [θ], units being degrees $\times$ cm^2 $\times$ dmol according to $[\theta] = \theta \cdot \lambda / (100\,\mathrm{L\,cn})$.

allowed a 50% of invasion at a 2 μM concentration, and a 15% and 18% of invasion when working with 20 and 200 μM, respectively. There was a small decrease in the invasion percentage using peptide 16949, although this peptide had its best effect at 200 μM, preventing a 48% of invasion. Invasion rates tended to decrease as peptide concentration rose (Fig. 4C).

The results obtained from cytotoxicity assays demonstrated that any of the assessed peptides at different concentrations had cytotoxic effects over cells, keeping viability levels comparable to those obtained for control cells (cells which were incubated only with RPMI). None of the assessed treatments showed significant differences regarding this control.

3.5. CD spectroscopy

CD spectroscopy analysis was carried out to determine whether the chemically-synthesized peptides presented secondary structure elements that might be related to the predictions for the complete protein and whether these elements had any relationship with their activity. Fig. 5 shows the conformation for some of the HABPs found. HABPs 16939 and 16944 had a β-sheet conformation, having a maximum at 192 nm and a minimum toward 218 nm. It was also noted that HABPs 16941 and 16949 tended to form a random coil having a minimum toward 196 nm and that HABP 16951 tended to form a poly-proline form (Fig. 5). It was also observed that peptides 16943 and 37608 (which was not HABPs) showed a distinct α-helix conformation, having two clearly defined minima. The conformation observed after dichroism corroborated the results obtained by bioinformatic predictions.

4. Discussion

A completely effective vaccine against TB has thus still not yet been developed, since the molecular interactions happening between mycobacterial proteins and specific receptors on host cell surface must first be ascertained. Such interactions could be blocked by the immune system being activated by these molecules.

The search for specific antigens has been stepped up since the complete *Mtb* H37Rv genome has been reported. Our studies have been based on proteins reported within the 3924 open reading frames (ORF) for this genome, bearing in mind a series of principles for obtaining a logical and rational methodology aimed at developing minimum subunit-based synthetic vaccines which have been initially described for malaria. The principles underlying this methodology have been based on identifying high activity binding peptides (HABPs) from *Mtb* proteins involved in mycobacterial entry to the host cell for inducing an immune response which may be able to block their binding to host cells.

Molecular biology assays gave a positive result in all assessed species and strains by amplifying a specific segment for the *rv0227* gene, thereby confirming that this protein is highly conserved among *Mtb* complex strains [34]. It is possible that, amongst the many functions performed amongst *Mtb* complex strains by highly conserved proteins, they could be performing an essential role in virulence and invasion. The *rv0227c* gene was also assessed in *M. intracellulare* (pathogenic specie) as the designed primers annealed with the protein's orthologous sequence in this species.

The rabbit antisera produced against specific amino acid sequences corresponding to Rv0227 protein predicted B-cell epitopes recognized a protein in *Mtb* total sonicate by Western blot. The antisera clearly recognized a 46 kDa molecular weight protein, agreeing with Rv0227c's theoretical molecular (45.5 kDa), confirming that the inoculated polymers were able to induce rabbit antibodies which could recognize a protein (probably the Rv0227c molecule) having similar molecular weight in the *Mtb* sonicate.

This rabbit's antisera also recognized a 59 kDa band, possibly corresponding to this protein's post-translational modifications such as protein glycosylation; this possibility was explored using the NetOGlyc 3.1 Server O-glycosylation predictor [30] as it can screen glycosylation in mycobacterial proteins [26]. Potential O-glycosylation sites were found in Thr^{377}, Thr^{382}, Ser^{392}, Ser^{394} and Ser^{401} amino acids; glycosylated bacterial proteins are often exposed on cell surface (as predicted for Rv0227c) so their biological function could involve binding and invasion.

Regarding *Mtb* H37Rv subcellular fractions, 31 and 15 kDa molecular weight bands were recognized, suggesting this protein's proteolytic processing once it had become located on the membrane. This hypothesis has been based on the knowledge that many secreted and transmembrane hormones and proteins often undergo proteolytic cleavage of precursor products. Proteolytic cleavage of recently synthesized polypeptide chains controls the folding and function of proteins which might be synthesized with extra amino acids to allow proteins to be cleaved by proteases as they pass or become anchored to the membrane [15,33].

It is interesting to observe that HABPs having specific binding to target cells are localized (after amino acid 160) meaning that the fraction hypothetically corresponding to the 31 kDa band may be the protein's active fragment. It was also observed that this protein fraction could be anchored to the membrane by the transmembrane region (predicted to lie between amino acids 319–340) by the transmembrane prediction using Hidden Markov Models (TMHMM) bioinformatics tool. No HABPS have currently been found in this region. It should be stressed that band intensity was greater in the membrane fraction where it was expected to find a greater amount of processed protein. Such hypothesis, however, requires further study.

Identifying *M. tuberculosis* proteins involved in target cells invasion as a method of choice when searching for vaccine candidates, implies that these proteins should be located on the mycobacterial surface, where they could interact with receptors to enter a target cell. The presence of the protein on the bacterial surface was confirmed here by TEM, supporting the notion that Rv0227c might be an important protein for mycobacterial entry. Previous studies demonstrate the presence of the protein in the mycobacterial envelope, using proteomics and computational approaches [24,35,64].

Once it had been confirmed that Rv0227c was a surface protein, binding assays using individual peptides spanning the entire protein sequence were carried out; seven of them bound with high specificity to A549 cells while only three showed high affinity for U937 cell receptors.

Mycobacterial entry into specialized macrophages involves previously described cellular receptors, such as complement receptors, mannose receptors, surfactant A protein receptors, and others such as scavenger and Fc-γ receptors. Such diversity indicates that mycobacteria use different internalization routes, ultimately determining whether these are destroyed by macrophages [17,36,53].

In turn, as type II pneumocytes are non-phagocytic cells, mycobacterial entry would have to rely on other routes and receptors different to those used by professional phagocytic cells [36]. In spite of this, peptides 16944 and 16949 might have been sharing a common receptor in both cell lines. Interestingly, peptide 16944 not only showed high specific binding to A549 and U937 cells having similar affinity constants, but it was also able to significantly inhibit mycobacterial entry to U937 cells and moderately to A549 cells. These characteristics make this peptide a promising candidate to be further assessed in designing a synthetic, peptide-based multiepitopic vaccine against tuberculosis.

HABPs capacity to bind to cellular receptors and block mycobacterial entry was assessed by flow cytometry, using fluorescently-labeled bacteria. HABP 16944 ability to reduce mycobacterial entry was evident for both cell lines, as percentages of target cell invasion were reduced to less than 50%. In general, HABPs 16943, 16944, 16949 and 16951 showed a clear inhibitory effect on mycobacterial entry, at least in one cell line; therefore, these HABPs will be studied in more detail in future work. It should be stressed that the last HABP mentioned contains many prolines, (this residue is repeated five times between positions 404–408) which has been recognized to be one of the most common apolar amino acids in protein–protein interaction regions [29]. Although several Rv0227 protein HABPs were able to inhibit mycobacterial entry to some degree for both cell lines, previous studies carried out by us suggested that this is not a generalized feature for all peptides, even those presenting high specific binding capacity [50].

Peptide 16943 in a polymeric form was inoculated; it proved to be a U937 cell HABP in binding assays. Anti-16943 serum was used as inhibitor in mycobacterial invasion assays. The serum (1:100) was able to inhibit mycobacterial entry to host cells by around 70%. Anti-16935 serum was used as control and did not inhibit mycobacterial entry. The HABP-antiserum's inhibitory effect on invasion has been associated with Rv0227c location on membrane surface and thus being involved in target cell invasion, thereby confirming the results of protein immune-localization. On the other hand, treating *Mtb* H37Rv with proteinase K (non-specific serin protease), removing surface proteins, has completely inhibited mycobacterial entry to epithelial cells and macrophages in *in vitro* assays, thereby showing the importance of mycobacterial surface protein components in the interaction with (infection) target cells. This is why HABPs which are able to inhibit mycobacterial entry to macrophages and epithelial cells *in vitro* have been of such special interest as they will be tested in assays using animal models for evaluating their protection-inducing potential against *Mtb* infection. There is general consensus that a Th1-type cell-mediated immune response involving both CD4+ and CD8+ lymphocytes is required in acquired immunity against TB [51]. It has long been held that antibodies were not involved in protection-inducing immunity against TB [31], even though serum therapy was extensively used for treating TB before the antibiotic era [22].

The CD spectroscopy data and information regarding other *Mtb* membrane-associated proteins has clearly suggested that HABPs involved in host cell binding and invasion have mainly displayed β-sheet or random structure configurations characteristic of proteins involved in receptor–ligand, protein–protein interactions, *etc.*, such data having a tremendous impact on designing a tailor-made anti-tuberculosis vaccine as we have thoroughly shown for some other infectious diseases like *P. falciparum* malaria.

5. Conclusion

Ascertaining that HABPs 16943, 16944, 16949 and 16951 were able to bind with high specificity to receptors on A549 and U937 cells in this study, and inhibit *Mtb* internalization in a dose-dependent manner, represents an important contribution to characterizing *Mtb* surface proteins, suggesting that these peptides (when properly modified) are excellent candidates for inclusion in a minimal subunit-based, chemically-synthesized, highly-effective anti-tuberculosis vaccine.

Conflict of interest

The authors declare that they have no competing interest.

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