The *Mycobacterium tuberculosis* Cell-surface Glycoprotein Apa as a Potential Adhesin to Colonize Target Cells via the Innate Immune System Pulmonary C-type Lectin Surfactant Protein A*  

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Tuberculosis is still a major health problem, and understanding the mechanism by which *Mycobacterium tuberculosis* (Mtb) invades and colonizes its host target cells remains an important issue for the control of infection. The innate immune system C-type lectins (C-TLs), including the human pulmonary surfactant protein A (PSP-A), have been recently identified as determinant players in the early recognition of the invading pathogen and in mounting the host defense response. Although the antigenic lipoglycan mannosylated lipoarabinomannan is currently considered to be the major C-TL target on the mycobacterial surface, the recognition by some C-TLs of the only mycobacterial species composing the “Mtb complex” indicates that mannosylated lipoarabinomannan cannot account alone for this specificity. Thus, we searched for the mycobacterial molecules targeted by human PSP-A, focusing our attention on the Mtb surface glycoproteins. We developed an original functional proteomic approach based on a lectin blot assay using crude human bronchoalveolar lavage fluid as a source of physiological PSP-A. Combined with selective cell-surface protein extraction and mass spectrometry peptide mapping, this strategy allowed us to identify the Apa (alanine- and proline-rich antigenic) glycoprotein as a new potential target for PSP-A. This result was supported by direct binding of PSP-A to purified Apa. Moreover, EDTA addition or deglycosylation of purified Apa samples completely abolished the interaction, demonstrating that the interaction is calcium- and mannose-dependent, as expected. Finally, we provide convincing evidence that Apa, formerly considered as mainly secreted, is associated with the cell wall for a sufficiently long time to aid in the attachment of PSP-A. Because, to date, Apa seems to be restricted to the Mtb complex strains, we propose that it may account for the selective recognition of those strains by PSP-A and other immune system C-TLs containing homologous functional domains.

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² The on-line version of this article (available at http://www.jbc.org) contains supplemental “Experimental Procedures.”

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With 2 millions deaths each year (2% increased incidence) and the sporadic resurgence of highly virulent strains, *Mycobacterium tuberculosis* (Mtb),³ the etiologic agent of human pulmonary tuberculosis, is considered to be a major public health threat, requiring a global mobilization of the international community (1). Indeed, the alarming emergence of multidrug-resistant forms of the bacillus in developed countries has unveiled the weakness of the existing therapeutic arsenal available to fight this important disease. One reason for this paradox is that, despite decades of effort, the molecular basis of the pathogenicity of Mtb is still poorly understood. Central to the Mtb infection lies the ability of the pathogenic bacillus to hide from the infected host defense mechanisms when entering and colonizing the host’s immune cells, principally alveolar macrophages, which paradoxically should be in charge of its clearance (2). Hence, the initial step of the interaction between the infectious bacillus and its target cell is likely to be decisive for the outcome of the infection because it must determine the mechanism by which facultative intracellular Mtb will escape from the primary immune response (3). Many intracellular pathogens have been found to produce species-specific adhesion molecules to recognize and bind specifically definite receptors of their target cells. On the contrary, current data strongly suggest that mycobacteria have developed a divergent strategy to cheat the immune system by taking advantage of different “pathogen recognition receptors” of the host immune system, hijacking them from their original defense role. In this context, the mannose-specific “C-type” lectins (C-TLs) of the host primary defense line have been the focus of considerable attention, as they have emerged as preferential Trojan horses used by mycobacteria to enter their target cells (4). Indeed, either the soluble pulmonary surfactant protein (PSP) A (5) and PSP-D (6) or the membrane-associated macrophage mannose receptor (7) and DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin) (8) have been shown to bind mycobacteria and facilitate their entry into phagocytes. Although extremely divergent in their structure, location, and organization, these C-TLs contain from 1 to 18 highly homol-

³ The abbreviations used are: Mtb, *M. tuberculosis*; C-TLS, C-type lectins; PSP, pulmonary surfactant protein; CRDs, carbohydrate recognition domains; ManLAMs, mannosylated lipoarabinomannans; BALF, bronchoalveolar lavage fluid; CSP, cell-surface protein; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; h, human; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
ogous carbohydrate recognition domains (CRDs), which recognize and bind mannose-containing glycoconjugates exposed on the surface of numerous infectious pathogens, including mycobacteria (9). The discovery of the mycobacterial glycoconjugates recognized by these immune system mannose-specific lectins therefore constitutes a major challenge to gain insight into the host-pathogen molecular interaction. Several previous studies (6, 8, 10–12) have identified the mannosylated lipoarabinomannans (ManLAMs), key antigenic lipoglycans of the pathogenic mycobacterial envelope, as the most likely target ligand of this class of pathogen-associated molecular pattern recognition receptors, which appear to play a fundamental role in the protection of the host against microbial infection.

Indeed, ManLAM has been proposed as being responsible for the development of new pharmacological competitors/inhibitors of the C-TL-mediated Mtb invasion (14), we reconsidered the presence in M. bovis BCG of still uncharactherized mycobacterial surface glycoproteins recognized by PSP-A. With this aim, we developed an original functional proteomic strategy based on the use of crude human bronchoalveolar lavage fluid (BALF) from alveolar proteinosis patients (as a source of physiological native PSP-A) to detect and identify the putative mycobacterial surface ligand for PSP-A. Using this innovative lectin blot analysis approach combined with a specific extraction procedure for surface proteins, two-dimensional electrophoresis, and mass spectrometry peptide fingerprinting, we definitively confirmed the presence of such protein ligands that have been identified as the Apa protein complex.

EXPERIMENTAL PROCEDURES

Materials—BALFs from patients with alveolar proteinosis were a generous gift from Dr. M. C. Prévost (INSERM U326, Purpan Teaching Hospital, Toulouse, France). ManLAMs and lipomannan from M. bovis BCG 1173P2 and M. tuberculosis H37Rv were purified in our laboratory by J. Nigou (15, 16). 45–47-kDa purified protein and mouse monoclonal antibodies (clone AsA3) against M. tuberculosis 45–47-kDa protein were kindly provided by Drs. G. Marchal and F. Romain (Pasteur Institute, Paris, France). All chemicals were from Sigma unless stated otherwise.

Mycobacterial Cultures—M. tuberculosis H37Rv, M. bovis BCG 1173 (Pasteur vaccine strain), Mycobacterium smegmatis mc²155, and M. smegmatis mc²155:ApaMtb were grown as a surface pellicle at 37 °C in Middlebrook 7H9 medium supplemented with 5% albumin/dextrose/catalase (Difco). After 7 days (for the M. smegmatis strains) or 3 weeks (for Mtb and BCG) of culture, cells were harvested, and the surface components were extracted as described below. M. smegmatis expressing the 45-kDa glycoprotein was obtained by transforming M. smegmatis mc²155 with pSMT3-19.

Preparation of Mycobacterial Surface Material Extract—Harvested cells were submitted to gentle agitation for 1 min with 4-mm diameter glass beads (VWR International) and suspended in distilled water. Bacillus suspension was immediately filtered under vacuum through a 0.2-μm pore size 150-mL sterile filter unit (NALGENE Labware). The amount of protein in the cell-surface protein (CSP) extract was estimated using the Bradford method (Bio-Rad), and aliquots corresponding to 60 μg of proteins were stored at −20 °C.

Trichloroacetic Acid Precipitation—Trichloroacetic acid (20% w/v) final concentration was added to 60 μg of equivalent protein from CSP extract, and the proteins were allowed to precipitate on ice for 30 min. Precipitated proteins were recovered by centrifugation at 14,000 rpm for 15 min at 4 °C, and residual trichloroacetic acid was removed by two washes and centrifugations with cooled acetone.

Monosaccharide Analysis by Capillary Zone Electrophoresis/Laser-induced Fluorescence Detection—CSP extract samples corresponding to 0.25 μg of equivalent protein were hydrolyzed under acidic conditions (2 n trifluoroacetic acid, 120 min, 110 °C). The resulting monosaccharides were derivatized with 1-aminoxyrene-3,6,8-trisulfonate and analyzed by capillary zone electrophoresis/laser-induced fluorescence detection as described previously (17).

Gel Electrophoresis—One-dimensional electrophoresis was performed using protein (10 μg/lane) from M. bovis BCG CSP extract on 15% hand-cast analytical SDS-polyacrylamide gels (0.75 mm thick, 8.3 × 7.3 cm) with a Mini-PROTEAN 3 electrohoresis system (Bio-Rad). Samples were run at a constant 20 mA for 1 h (PowerPac 300, Bio-Rad) in 25 mM Tris, 192 mM glycine, and 0.1% SDS. The masses of the molecular standards (catalog no. 10748-010, Invitrogen) are those reported by the manufacturer. CSP extract/SDS-polyacrylamide gels were developed with the PlusOne™ protein silver staining kit from

Printing, we definitively confirmed the presence of such protein ligands that have been identified as the Apa protein complex.
Amersham Biosciences. For two-dimensional electrophoresis, 60 µg of precipitated proteins were solubilized in 125 µl of rehydration buffer (7 M urea (99.5% purity; Prolabo Chemicals & Reagents, Fontenay Souss Bois, France), 2 M thiourea (>99% purity; Fluka), 4% CHAPS (+98% purity), 30 mM dithiothreitol, 0.5% carrier ampholytes buffer (pH 3–10; Bio-Rad), and trace bromphenol blue). The sample was incubated with constant shaking for 4 h at room temperature before loading onto a 7-cm immobilized pH gradient strip (broad pH 3–10 range or narrow pH 3–6 range; Bio-Rad). Isoelectric focusing was performed with the IPGphor isoelectric focusing system (Amersham Biosciences). The following voltage profile was used: a constant 50 V, 12 h (active rehydration step); a constant 200 V, 30 min; a constant 500 V, 30 min; a constant 1000 V, 30 min; and linear increase to 8000 V to reach a total current value of 8000 V-h. The strips were then successively equilibrated for 15 min in 30 mM dithiothreitol, followed by 15 min in 135 mM iodoacetamide in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and trace bromphenol blue). After equilibration, the strips were applied to a 15% hand-cast precast Tris/glycine/methanol buffer (48 mM Tris base, 39 mM glycine, 0.1% (v/v) SDS, and 20% (v/v) methanol (pH 9.2)). Membranes were then blocked for 2 h with 5% (w/v) nonfat dry milk in Tris-buffered saline (25 mM Tris-HCl, 140 mM NaCl, and 3 mM KCl (pH 8)) with 0.2% Tween 20 (TBS-T) and finally washed three times with TBS-T. Specific immunological identification of the Api proteins was obtained by a 2-h incubation of the blocked membranes with rabbit anti-\( M.\) \textit{tuberculosis}\ 45–47-kDa polyclonal antibodies diluted 1:5000 in TBS-T, followed by exposure to horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Sigma). Membranes exposed to horseradish peroxidase conjugates were washed and overlaid with enhanced chemiluminescence reagent (Lumi-Light ECL substrate, Roche Diagnostics) and then exposed to Lumifilm chemiluminescent detection film (Roche Diagnostics) for 10 s to 5 min before development. Control membranes were stained with secondary antibodies only. Supplemental Western blot analyses were performed after membrane stripping by a 30-min incubation in 100 mM glycine buffer (pH 2.5).

\textbf{Lectin Blotting}—Lectin blotting was performed according to a procedure very similar to the immunoblot procedure described above, except that, after blocking, the membranes were incubated with human BALF diluted 1:10 in TBS-T plus 5 mM CaCl\textsubscript{2} for 12 h at 4 °C. After careful washing with the same buffer, binding of human (h) PSP-A was revealed using rabbit anti-hPSP-A polyclonal antibodies (Ab3420, Chemicon; working dilution of 1:2500) using the same procedure as described above, except that 5 mM CaCl\textsubscript{2} was added to all buffers.

\textbf{Glycoprotein Detection}—Glycoproteins of the \textit{M. bovis} BCG CSP extract were specifically identified after transfer to nitrocellulose membrane using the digoxigenin glycan detection kit (Roche Diagnostics) according to the manufacturer’s recommended procedure.

\textbf{In-gel Protein Digestion}—Protein spots excised from Coomassie Blue-stained two-dimensional gels were cut into smaller gel pieces and submitted to the standard protocol available on the University of California, San Francisco, web site (donatello.ucsf.edu/ingel.html).

\textbf{Mass Spectrometry and Protein Data Bank Mining}—The samples were analyzed using a 4700 proteomics analyzer MALDI-TOF/TOF system (Applied Biosystems) equipped with a \(/ \) laser operating at 200 Hz. All mass spectra were recorded in reflector mode using positive ion detection and were obtained by accumulating 2500 subspectra generated by 250 laser pulses on 10 distinct areas of the dried droplet. Mass spectra were recorded for all sample spots on the plate and calibrated using the instrument external calibration procedure. Each sample mass spectrum was further internally calibrated with Data Explorer Version 4.4 software (Applied Biosystems) using signals from autoprotoelytic fragments of trypsin (peptides 58–77 and 98–107) present in the spectrum. Data Explorer peak detection parameters were adapted on each mass spectrum to generate monoisotopic peptide mass lists corresponding to the mass values of the 30 highest intensity ions peaks found between 1000 and 3500 Da. These lists were then used to mine the two major public protein data banks NCBI/TrEMBL and Swiss-Prot/TrEMBL using the Protein Prospector MS-Fit search engine of the University of California, San Francisco. Standard search parameters were set as follows: trypsin digest with one missed cleavage; fixed carbamidomethylation of cysteines and alternative oxidation of methionines; species, all/microorganisms; and peptide mass tolerance, 50 ppm.

\textbf{Solid-phase Cell Binding Assays}—Solid-phase binding assays were carried out on microtiter plates (Nunc-Immuno, Nalge Nunc International, Rochester, NY). Coating of mycobacterial cells was achieved by incubating cells (5 or 2.5 µg/100 µl) in suspension in coating buffer for 1 h at 37 °C. The remaining steps of the protocol were performed at room temperature unless stated otherwise. Wells were blocked for 2 h in blocking buffer (25 mM Tris-HCl, 140 mM NaCl, 3 mM KCl, and 30% low fat dry milk (pH 7.4)). The wells were extensively rinsed with wash buffer (10 mM Tris-HCl, 140 mM NaCl, 1 mM CaCl\textsubscript{2}, 0.05% Tween 20, and 0.4% low fat dry milk (pH 7.4)). 100 µl of diluted (1:10 in wash buffer) alveolar proteinosis patient BALF containing hPSP-A were added and allowed to react overnight with coated mycobacterial cells. After extensive washing, 100 µl of rabbit anti-hPSP-A polyclonal antibodies (working dilution of 1:4000 in wash buffer) were loaded onto the wells and incubated for 3 h. After extensive washing, 100 µl of alkaline phosphatase-conjugated mouse anti-rabbit monoclonal immunoglobulins (clone RG16; 1:2000 dilution in wash buffer) were added and allowed to react for 1 h. Microtiter wells were then extensively washed with substrate buffer (15 mM Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 35 mM NaHCO\textsubscript{3}, and 0.1 mM MgCl\textsubscript{2} (pH 9.5)), and alkaline phosphatase activity was detected by incubation of 100 µl of p-nitrophenyl phosphate (1 mg/ml in substrate buffer) at 37 °C. Absorbance at 405 nm was measured on a microtiter plate reader (µQuant, BioTek Instruments, Inc). Each value reported is the mean of triplicate measures ± S.D. from four independent experiments.
RESULTS

Mycobacterial Cell Wall Protein Extraction—hPSP-A is a highly multimeric protein produced by type II alveolar cells as lipoprotein complexes. The quantitatively major physiological form of hPSP-A is composed of six heterotrimers (18 subunits) of the SPA1 and SPA2 gene products. Hence, with regard to the substantial size (>100 kDa) of the octadecameric native protein, which may limit its free diffusion within the bacterial envelope, we hypothesized that, to be recognized, the ligands must be easily accessible to such high molecular mass receptors and thus localized in the outermost layer of the envelope. Thus, we chose to limit our investigation and to probe PSP-A binding only to the surface components of the mycobacterial cell wall. With this aim, we tentatively applied an approved specific mechanical treatment with glass beads to strip off and extract selectively the outermost components (18). In brief, freshly filtered M. bovis BCG cells were gently shaken with glass beads to limit bacterial cell disruption and subsequent excessive contamination by intracellular compounds. The intact cell bodies were removed by centrifugation to obtain the crude cell wall extract (supernatant). According to the current structural and architectural model of the mycobacterial envelope, this extract was found to contain large amounts of the outermost exposed soluble neutral polysaccharides, viz. glucan and the arabinomannan (almost 40%; see “Experimental Procedures” for details) (18). The proteins released from the surface were then further concentrated by trichloroacetic acid precipitation to remove selectively the unwanted trichloroacetic acid-soluble saccharide material. As expected, the resulting pellet was found to contain up to 85% of the protein (in mass). When analyzed by SDS-PAGE analysis and silver nitrate staining, this so-called CSP extract showed no discernible qualitative protein composition with the crude cell-surface extract. As a corollary to this, the quasi-absence of protein bands detectable in the non-precipitated trichloroacetic acid-soluble fraction supports the idea that the surface proteins were wholly recovered in the pellet. Carbohydrate analysis by capillary zone electrophoresis/laser-induced fluorescence detection of the 1-aminopyrene-3,6,8-trisulfonate derivatives of the CSP hydrolysis products revealed almost exclusively the presence of glucose, mannose, and arabinose, as expected from the current knowledge of the carbohydrate contents of the mycobacterial cell surface. In contrast, no galactose was detected in the CSP extract. Because this compound is a specific marker restricted to the peptidoglycan-arabinogalactan-mycolyl complex, this absence strikingly demonstrates that the integrity of the innermost envelope layer was unaffected by the mild extraction procedure used.

The protein content of the CSP extract was further analyzed by SDS-PAGE, and the complex pattern revealed by silver nitrate staining (Fig. 1A, lane 1) was very similar to that reported for Mtb H37Rv by Ortalo-Magné et al. (19). Although some of these bands have been identified in previous studies, the exhaustive protein content of these CSP extracts has been poorly investigated to date and remains quite elusive despite the considerable knowledge these molecules may provide in gaining a better understanding of the interplay between the pathogen and the host.

![Image](https://example.com/image.png)

FIGURE 1. Evidence for mycobacterial cell-surface glycoprotein ligands of hPSP-A by lectin blot proteomic analysis. 10 or 60 μg of equivalent protein of M. bovis BCG cell-surface proteome were resolved by one-dimensional (A) or two-dimensional (B and C) electrophoresis, respectively, and revealed by silver nitrate staining (A, lane 1; and B) for whole protein content detection. Replicas of the stained one- and two-dimensional gels transferred onto nitrocellulose membrane were then probed by lectin blotting with whole human BALF to detect potential hPSP-A ligands (A, lane 2; and C). Lectin blot nonspecific detection was controlled by incubating the membrane in sham buffer without BALF (A, lane 3), whereas putative mycobacterial glycoproteins (lane 4) were specifically detected (see “Experimental Procedures” for details). Apparent molecular masses were estimated according to molecular mass marker standards (A, lane 5).

Evidence of Potential Mycobacterial Glycoprotein Ligands of hPSP-A—To determine whether some proteins contained in the extract were recognized by hPSP-A, the precipitated CSPs of M. bovis BCG were resolved under denaturing conditions by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was then incubated overnight with diluted human BALF. BCG CSPs recognized by hPSP-A were selectively detected through specific exposure of the bound hPSP-A to rabbit anti-hPSP-A antibodies. Under these conditions, four main bands at ~50, 47, 45, and 30 kDa were clearly detected (Fig. 1A, lane 2). The results from incubation of a control membrane in sham buffer without BALF (Fig. 1A, lane 3) strongly suggest that the highest apparent molecular mass band (~50 kDa), as well as probably the lowest apparent molecular mass band (~30 kDa), can be attributed readily to nonspecific cross-reaction of the anti-hPSP-A polyclonal antibodies. With the aim of reducing this unwanted non-specific binding, the anti-hPSP-A polyclonal antibodies were tentatively pre-adsorbed on BCG cells before use. However, this procedure did not signifi-
Binding of Mycobacterial Glycoprotein Apa to hPSP-A

cantly reduce the adverse cross-reactions (data not shown), strongly suggesting that they may result from the recognition of some non-conformational epitopes naturally masked in the native "on-cell" presentation, but unveiled by the denaturing blot conditions.

Finally, the two remaining bands at 47 and 45 kDa specifically labeled by hPSP-A were then considered as candidate ligands. To support this assumption, we first tried to demonstrate the presence of carbohydrate motives that were associated with these bands and that could account for the binding of hPSP-A at the molecular level. With this aim, the β-diol functions, solely predictable on carbohydrate rings (at least on the nonreducing terminal monosaccharides), were selectively oxidized by periodic acid, and the resulting carbonyls were chemically derivatized with digoxigenin hydrazide. The so-labeled glycosylated molecules were then revealed through detection of the digoxigenin nucleus by anti-digoxigenin antibodies.

As shown in Fig. 1A (lane 4), this strategy clearly revealed the presence of carbohydrate motives associated with two bands with strikingly similar apparent molecular masses to those recognized by hPSP-A. Thus, we logically hypothesized that these two bands may correspond to the two hPSP-A candidate ligands evidenced by lectin blotting.

In conclusion, this preliminary observation strongly supports the previous assumption of the presence, in the mycobacterial envelope, of glycosylated proteins that may constitute potential targets for the fixation of hPSP-A. It is worth mentioning that the target proteins characterized herein in M. bovis BCG are likely to differ by their apparent molecular masses from the previous putative 60-kDa candidate characterized in Mtb by Pasula et al. (5).

Two-dimensional SDS-PAGE Separation of the M. bovis BCG Protein Ligand of PSP-A—With regard to the above observations, the in-gel trypsin digestion of the protein band, followed by mass fingerprint analysis of the proteolytic peptides, appears to be the more appropriate strategy to identify these proteins. However, the weak intrinsic resolution of one-dimensional SDS-PAGE (Fig. 1A, lane 1) prevents any possibility of direct unambiguous identification by such an approach because of the high level of overlap between the protein bands. Thus, to overcome this limit, the M. bovis BCG CSPs were tentatively resolved by two-dimensional gel electrophoresis. The best separation was obtained using a narrow pH 3–6 range gradient for the electric focalization in the first dimension. Fig. 1B shows a standard silver-stained two-dimensional electrophoresis map obtained for the M. bovis BCG CSP extract. The two putative PSP-A ligands were then localized by lectin blotting as described above. In brief, a replica of the silver-stained two-dimensional electrophoresis gel was electrotransferred to a nitrocellulose membrane and successively incubated overnight in BALF and probed with anti-hPSP-A antibodies to detect bound collectin. Under these conditions, two series of three spots were observed (Fig. 1C). The pattern of the spot was reasonably interpreted as the imprint of two protein species isoforms differing in their apparent size (vertical distribution) as well as in their pl (horizontal resolution). These spots were then localized with accuracy on the silver-stained gel by superimposition of the two images. Their apparent molecular masses between 40 and 45 kDa and between 45 and 50 kDa (from external molecular mass calibration), respectively, fit fairly well with the estimated molecular masses of the two putative hPSP-A target glycosylated proteins characterized on the one-dimensional electrophoresis gel between 40 and 50 kDa. Finally, it can be roughly estimated that these proteins have pl values between 4.5 and 5 assuming that the pH gradient is linear.

Identification of the M. bovis BCG Protein Ligand of PSP-A by Peptide Mass Fingerprinting—Further identification of these proteins was performed by MALDI-TOF mass spectrometry analysis. The major spots of the two proteins observed on the silver nitrate-stained gel and assumed to correspond to those revealed by hPSP-A binding were individually excised from the two-dimensional electrophoresis gel and submitted to in-gel trypsin digestion. The resulting proteolytic peptides were recovered from the gel and analyzed by MALDI-TOF mass spectrometry using α-hydroxycinnamic acid solution as the matrix. Fig. 2 (upper panel) shows the characteristic peptide mass fingerprint obtained with the trypsin digest mixture of the 45–50-kDa spot by MALDI-TOF mass spectrometry between m/z = 1100 and 3200. The accurate mass value adjustment was performed by internal calibration using the autodigested peptides of the added trypsin or the generally observed contaminant corresponding to keratin proteolytic fragments (Fig. 2, middle panel). Peptide mass fingerprint analysis of the second spot (40–45 kDa) surprisingly offered a very similar mass spectrum containing the same major ions (data not shown), suggesting that these two spots may correspond to different isoforms of the same protein. Analysis of these peptide mass fingerprints readily identified the Mtb modD (or apa) gene product (also named the Apa, MPT32, or 45–47-kDa antigenic complex (20)). Indeed, among the 17 monocharged ions that could not be attributed to known contaminants and then selected to mine the data bases, seven ions, including the most intense at m/z 1155, 1820, 1836, 1852, 2431, and 3132, were found to fit with a remarkably high accuracy (ΔM < 10 ppm) with the predicted Apa protein tryptic peptides (35% sequence coverage, 77% (7/9) of the theoretically expected peptides (with 1000 Da < molecular mass < 4000 Da) from trypsin cleavage of Apa) (Fig. 2, lower panel). The theoretic pl of 4.6 calculated from the Apa sequence is in a good agreement with the pl estimated from the electrophoretic mobility of the plots observed in the two-dimensional electrophoresis map. In contrast, the apparent molecular mass of the protein estimated by SDS-PAGE (~45–50 kDa) is likely to be much higher than expected for Apa (28,780 Da). However, it has been established previously that Apa has an aberrant migratory behavior upon SDS-PAGE probably because of its high content of proline, but also because of the presence of carbohydrate appendages (21, 22), which slow down its electrophoretic displacement to a higher apparent molecular mass. Moreover, as pointed out by one of the Apa synonym names, “45–47-kDa antigenic complex,” this protein appears as a doublet on one-dimensional electrophoresis gels with an apparent molecular mass of 45–47 kDa and as a characteristic six-spot pattern (doublet of three horizontally dispersed closed spots) on two-dimensional electrophoresis gels,
very similar to the spot pattern revealed by lectin blotting. To date, no definitive structural explanation has been given for this unforeseen behavior. From our previous investigations by mass spectrometry, we hypothesized that the difference between the two observed isoforms results from alternate post-translational processing and proteolysis affecting both extremities of the protein sequence. However, the ion peaks observed in the MALDI-TOF mass spectra were found to signal only internal protein sequence peptides and unfortunately could not discriminate between the two isoforms because of the lack of the terminal peptide ions. Nevertheless, altogether, these data strongly converge and suggest that these spots correspond to the Apa antigen.

Anti-Apa Antibodies Bind to the Spots Indicated by the PSP-A Lectin Blot Assay—To unequivocally confirm this finding, we performed an immunodetection of these protein spots using specific antibodies. For this, the nitrocellulose membrane probed by lectin blotting was stripped and further submitted to immunodetection using rabbit polyclonal antibodies against the Apa protein. As shown in Fig. 3 (lanes d and e), the protein spots revealed perfectly matched in both their position and pattern those recognized by PSP-A (lanes b and c). This result thus supports the above identification based on the peptide mass fingerprint analysis and strongly suggests that the M. bovis BCG Apa protein could serve as a target for PSP-A. However, these binding experiments were performed on a complex mixture of protein resolved by two-dimensional SDS-PAGE. Although considered as the most efficient and suitable approach to conveniently resolve complex mixtures of protein, this separation strategy is not exhaustive, and some proteins may still overlap under these conditions. Thus, we cannot absolutely exclude the possibility that the spots in question may correspond to two distinct proteins, one of which is recognized by PSP-A, whereas the other corresponds to Apa.

Recognition of Purified Apa by PSP-A from Alveolar Proteinosis Patient BALF—Definitive evidence came from the direct observation of PSP-A binding to purified Apa by lectin blotting. As shown in Fig. 4a, Western blot analysis of the Apa antigen purified from M. bovis BCG revealed, as described above, two protein bands attributed to the two isoforms of 47 and 45 kDa, with a higher relative intensity of the quantitatively major 47-kDa isoform in this purified sample. Overnight incubation of the transferred proteins with human BALF led to strong binding of PSP-A to the quantitatively major 47-kDa isoforms.

**FIGURE 2.** Identification of the M. bovis BCG protein ligand of hPSP-A by MALDI-TOF mass spectrometry peptide fingerprinting. Upper panel, MALDI-TOF mass spectrum of peptides obtained after in-gel trypsin treatment of the highest apparent molecular mass spot evidenced by PSP-A two-dimensional lectin blotting. The ion peaks attributed to Apa peptides are indicated. Middle panel, mass ion attribution (see “Experimental Procedures” for details). Sequences corresponding to putative Apa peptide ions are detailed. MetOx, oxidized methionine. Lower panel, complete sequence of the Apa protein showing the trypsin cleavage site (K and R in boldface) and the peptides (underlined) identified in the MALDI-TOF mass spectrum.
of purified Apa as evidenced by the specific immunodetection of bound PSP-A (Fig. 4c). The low detection level of PSP-A binding to the 45-kDa isoform can be attributed at least in part to its minor amount in this preparation. Alternatively, in previous studies, the 45-kDa isoform has been tentatively assigned to a proteolytic truncated product of the 47-kDa isofrom lacking the glycosylated C-terminal undecapeptide (22, 23). Although this assumption has never been definitively demonstrated, the putative reduced number of glycan chains on the 45-kDa isoform may drastically decrease the binding affinity for PSP-A. Thus, the combination of these two features can reasonably explain the low binding of PSP-A to the lower apparent molecular mass Apa isoforms contained in this purified sample.

As further confirmation, similar patterns were also obtained for Mtb purified Apa and recombinant Apa expressed in M. smegmatis (naturally devoid of the Apa antigen). These antigens are likely to vary only slightly from their carbohydrate moiety (22) and, as expected, are undoubtedly recognized by PSP-A whatever their source (Fig. 4, a and c).

Recognition Is Dependent on Carbohydrate-Lectin Interactions—The human immune system C-TLs, including PSP-A, are known to bind specifically mannconjugates through well described calcium-dependent mechanisms (24–26). On the other hand, as stated above, Apa is the only M. bovis BCG glycoprotein for which the structure of the saccharide moiety has been fully characterized. It contains up to nine hexoses (seven for the quantitatively major glycoform) (22, 23), consisting of α-mannoses, α-1,2-mannobioses, and α-1,2-mannotrioses (21) linked to Thr10, Thr18, Thr27, and Thr277. Thus, to verify whether the binding of PSP-A depends on the recognition of the Apa mann-o-igosaccharide appendages, the purified Apa proteins of different origins were deglycosylated by jack bean α-exomannosidase. As shown in Fig. 4b, removal of the carbohydrate chains led to a higher electrophoretic mobility of the two isoforms because of the loss of the mass associated with the carbohydrates. Moreover, this treatment completely abolished the interaction with PSP-A, as expected (Fig. 4c). This result therefore unambiguously confirms that this interaction is attributable to the recognition and binding of the Apa mann-o-igosaccharides by the hPSP-A CRDs.

Evidence of the Role of Apa as an Adhesin on the M. bovis BCG Surface—When taken together, these observations strongly suggest that Apa may contribute to the binding of the immune system C-TLs on the mycobacterial cell surface via its oligomannosyl moiety. However, this assumption obviously relies on binding experiments performed with membrane-blotted protein, which may have a particular conformation distinct from the native one. To bypass any irrelevant solid-phase molecular presentation, we chose to directly monitored the binding of hPSP-A to cell-surface Apa to definitely confirm that native Apa is a potential physiological ligand of hPSP-A.

Apa was originally described (20) as a major immunodominant antigen specifically secreted in the culture filtrate of “Mtbc complex” mycobacterial species. Although the transient presence of Apa in the mycobacterial envelope during

![FIGURE 3. Immunoblot identification of M. bovis BCG Apa glycoprotein. Lane a, two-dimensional (2D) electrophoresis total CSP detection by silver staining; lanes b and c, two- and one-dimensional (1D) electrophoresis lectin blotting (Lectin-B), respectively, of the total BCG CSP; lanes d and e, two- and one-dimensional electrophoresis Western blotting (Western-B), respectively, with the anti-Apa polyclonal antibodies. (Western blotting was performed on the same membrane as the lectin blot after dehybridization.) NiAg, silver-nitrate.](image)

![FIGURE 4. Binding of PSP-A to purified Apa is carbohydrate-dependent. Shown are Western blots (a and b) and an hPSP-A lectin blot of purified native (a) and deglycosylated (b) Apa from Mtb, BCG, and Apa-transfected M. smegmatis (M. smegmatis::Apa). Carbohydrate removal by exomannosidase treatment induced a downshift of the band toward a reduced apparent molecular mass (b) and abolished recognition by the hPSP-A CRDs (c).](image)
the exportation step is evident, to date, this antigenic complex has never been described as associated with any external cell structure even temporarily. To support the proposal that Apa may serve as a mycobacterial surface ligand for the recognition and binding of Mtb by immune system C-TLs and more particularly by hPSP-A, we first verified its presence and accessibility on the mycobacterial cell surface. This was achieved by probing the M. bovis BCG cell surface with specific anti-Apa monoclonal antibodies. As demonstrated by the dose-dependent response observed (Fig. 5), the Apa antigen can be considered as being associated with the mycobacterial cell surface. Although it is not possible to distinguish between a permanent presence and a transient residence sufficiently long to allow its detection, this observation makes apparent that Apa is at least accessible to antibodies binding on the mycobacterial surface. On the other hand, as expected, Apa could not be detected on the surface (or in a CSP extract) of wild-type M. smegmatis (although these monoclonal antibodies are likely to react with an unknown M. smegmatis-specific antigen as reported previously (20)). This is consistent with the fact that M. smegmatis is known to be devoid of the modD gene (negative control experiment). On the contrary, M. smegmatis cells transfected with the Mtb modD gene readily express the Apa antigen on their surface as evidenced in Fig. 5. This observation thus validates the specificity of the antibodies and allows us to confirm the presence and accessibility of Apa on the M. bovis BCG surface as well as on Apa-transfected M. smegmatis cells.

Finally, to definitively confirm that M. bovis BCG surface Apa is a potential physiological ligand of hPSP-A, we performed competitive binding inhibition experiments with anti-Apa antibodies. As shown in Fig. 6 (upper panel), preincubation of the M. bovis BCG cells with anti-Apa antibodies significantly reduced the yield of hPSP-A surface binding by almost 30%. Moreover, this reduction was shown to be specific to anti-Apa antibodies because preincubation with unrelated mouse anti-IgG isotype antibodies had no inhibitory effect on hPSP-A binding. The fact that inhibition was only partial is consistent with the statement that Apa is not an exclusive ligand. Indeed, other mannose-containing mycobacterial cell-surface molecules, including the mycobacterial ManLAMs and lipomannan proposed previously, are most likely recognized and bound by hPSP-A. To further confirm that Apa is partly responsible for the binding of hPSP-A to mycobacteria, we compared the antibody-induced inhibition of hPSP-A binding to M. smegmatis. As expected, a similar preincubation of wild-type M. smegmatis with anti-Apa antibodies had a very limited inhibitory effect on the binding of PSP-A, suggesting that the anti-Apa antibodies
do not alter the recognition and interaction of PSP-A with other cell-surface antigens. In contrast, an almost 20% decrease in PSP-A binding was observed in the M. smegmatis strain expressing the Apa antigen, indicating that, in this strain, Apa accounts for at least 15% of the PSP-A binding. In conclusion, these data unambiguously demonstrate that the specific Mtb complex antigen Apa must be considered as a new ligand for PSP-A and most probably for other related immune system C-TLs sharing homologous CRDs with PSP-A.

DISCUSSION

Glycoconjugate structures exposed on the surface of invading pathogens are of prime importance in the first contact with the host innate immune system. Their rapid recognition and binding by host immunity lectins have a decisive role in the establishment of the initial immune response and in the outcome of the infection (27).

However, it is now well established that Mtb takes advantage of such carbohydrate-lectin interaction-based defense mechanisms to invade and colonize its target cells (2). Thus, the exhaustive identification of Mtb surface glycoconjugates involved in these processes is crucial for a better understanding of Mtb infectiousness. To date, the numerous studies devoted to the search for potential mycobacterial adhesins able to bind to immune system lectins have led to the identification of surface lipoglycans (ManLAM and lipomannan), a gluca and a mannos-containing glycolipid (phosphatidylinositol mannoside). However, besides these, several clues indicate the presence of still undefined highly glycosylated protein material potentially involved in these processes. Indeed, Pasula et al. (5) reported that the carbohydrate-mediated binding of PSP-A to Mtb is trypsin-sensitive. Independently, the Mtb Apa and LpqH proteins were reported to compete in some way, as ManLAM constitutes a discriminating structural determinant highly implicated in the adhesion function of the protein.

In this study, we have offered new clues to these observations by demonstrating definitively that the glycosylated Mtb Apa antigen must be considered as a probable physiological ligand of the host prime defense line PSP-A. Moreover, because the manno-oligosaccharides (i.e. α-mannoses, α-1,2-mannobioses, and α-1,2-mannotrioses) found on Apa are identical to those found to be responsible for the interactions of ManLAM with hPSP-A (11), the macrophage mannose receptor (28), and DC-SIGN (8), it is possible that Apa would be similarly recognized by these receptors.

It is noteworthy that, to date, the Apa glycoprotein seems to be restricted to the Mtb complex and has not been found in any other mycobacterial species investigated (i.e. Mycobacterium avium, Mycobacterium marinum, and M. smegmatis). Thus, this particular species specificity may account for the relative selective recognition by the C-TLs of the Mtb complex strains. This hypothesis is strengthened by the fact that hPSP-A binding to transfected M. smegmatis expressing the Mtb Apa glycoprotein is readily increased compared with the wild-type strain. However, it is obvious that Apa is not the unique compound of the mycobacterial surface targeted by PSP-A. This is evidenced by the only partial level of inhibition obtained with the blocking antibodies and further supported by the fact that no significant difference could be observed in the level of binding of PSP-A to 45-kDa isofrom-null mutants of M. tuberculosis compared with its wild-type counterpart (data not shown).

From a biological point of view, the current findings offer, for the first time, new evidence for the previously suspected (but never confirmed) adhesion function of Apa in the tuberculosis infection process, in addition to all the previously described biological activities of this unique Mtb glycoprotein (i.e. CD4+ and CD8+ T cell proliferation (29), in vivo induction of the delayed type hypersensitivity reaction (23), and protection against Mtb challenge in the guinea pig animal model (30)).

On the other hand, it is noteworthy that Apa has been found mainly as a secreted antigen of the Mtb complex species. Hence, it can be logically envisaged that this protein may be produced by the infecting bacilli to serve as a bait to trap and inactivate those major pathogen recognition receptors involved in the uptake of invading pathogens. Indeed, recent studies (32) have shown that purified ManLAM binding to the macrophage mannose receptor or DC-SIGN readily inhibits the TLR4-mediated pro-inflammatory immune response induced by lipopolysaccharide. Thus, by analogy, it can be reasonably speculated that the binding of secreted Apa to these same receptors may also modulate the response of the host adaptive immune system cells by down-regulating the Th1 cytokine pattern and more particularly the pro-inflammatory interleukin-12/interferon-γ loop (31–33) identified as a key element in immune resistance to mycobacterial infection (34). Such an impaired T lymphocyte response may contribute to the persistence of the pathogenic mycobacteria and the development of chronic tuberculosis (35). Interestingly, as demonstrated previously for the already elucidated biological activities of Apa (22, 23), we have also shown here that the manno-oligosaccharide moiety constitutes a discriminating structural determinant highly implicated in the adhesion function of the protein.

To date, Apa and the M. bovis MPB83 antigen (36) still remain the only fully characterized glycosylated proteins produced by the human Mtb complex pathogen species. However, it is remarkable that, whereas Apa contains α-1,2-manno-oligosaccharides, MPB83 has been shown to be substituted by α-1,3-linked manno-oligosaccharides. Although apparently minor, such a difference may have dramatic consequences on the selectivity of binding to C-TL and more particularly to DC-SIGN. Indeed, as opposed to the majority of the known manno-specific C-TLs that have been shown to recognize terminal sugar residues, crystallographic data have revealed that DC-SIGN CRD binding requires that the 3- and 4-hydroxys of the penultimate residue (of a manno-oligosaccharide) be free. We can obviously expect that DC-SIGN binds to Apa but not to the MPB83 α-1,3-linked manno-oligosaccharides, which do not fulfill the minimum stereochemical requirements for molecular interaction. With regard to the metabolic pathway, glycosylation of these proteins implies the coexistence in Mtb of at least two different mannosyltransferases with α-1,2 and α-1,3 specificity, respectively. Although not surprising, this feature raises, however, questions about the determinism and molecular mechanisms of the substrate selectivity of these enzymes, i.e. why does the α-1,2-mannosyltransferase affect only Apa, whereas the α-1,3-mannosyltransferase modifies...
MPB83? A second fundamental question is whether these different glycosylation patterns have 1) sense and consequences on the biological activities of these two molecules and 2) a significant involvement in the host selectivity of the respective producing strains (i.e. M. tuberculosis and M. bovis). Further studies are still necessary to find the answer to this fundamental open question.

Besides Apa and MPB83, few other major antigenic proteins produced by mycobacteria are suspected to be modified by saccharide appendages, although for most of them, the precise nature and structure of these motives have not been sufficiently defined to definitively confirm these assumptions. However, it is worth mentioning that most of these putatively mannosylated proteins (including Apa) are secreted antigens. Moreover most of them have been found to be involved in the alteration of the host’s adaptive immune response and thus potentially associated with the pathophysiological disorders characteristic of the infection by Mtb. Recently, VanderVen et al. (37) offered some clues to this observed potential link by elegantly evidencing the association between the O-mannosylation of a recombinant chimeric protein (based on a Sec-dependent secreted protein fused with a glycosylation cassette of Apa) and its exportation by the Sec protein translocation system of Mtb. Moreover, these results have revealed unsuspected analogies between the protein glycosylation pathway in Mtb and the essential post-translational protein O-mannosylation processes highly conserved among eukaryotes. However, although the O-mannosylation of proteins is likely to be a ubiquitous and greatly represented post-translational modification in eukaryotic organisms, to date, the extent of this process in Mtb remains largely unknown. These observations thus raise the question of the utility and of the possible roles that the post-translational glycosylation of antigenic protein may represent for the mycobacteria in terms of evolutionary advantages regarding infectiousness, host specificity, or virulence. Answering these questions should not only offer new insights into a better understanding of the host-pathogen relationship but also may assess whether this post-translational glycosylation process can constitute a valid strategic target to develop new therapeutic approaches against tuberculosis as suggested recently (14).

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