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Peptidoglycan-Associated Polypeptides of *Mycobacterium tuberculosis*

GREGORY R. HIRSCHFIELD, MICHAEL MCNEIL, AND PATRICK J. BRENNAN*
Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523

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Important protein-based immunoreactivities have long been associated with the cell wall core of mycobacteria. In order to explore the molecular basis of such activities, purified cell walls of *Mycobacterium tuberculosis* were extracted with sodium dodecyl sulfate to produce an insoluble residue composed of the mycolylarabinogalactan-peptidoglycan complex and about 2% of unextractable protein. Treatment of the product from an avirulent strain of *M. tuberculosis* with trifluoromethanesulfonic acid released a single polypeptide with a molecular size of 23 kilodaltons, accounting for all of the insoluble cell wall protein. Extensive purification and then analysis of the 23-kilodalton protein demonstrated the absence of diaminopimelic acid, muramic acid, or other peptidoglycan components, pointing to either a novel linkage between protein and peptidoglycan or a noncovalent but tenacious association. The released 23-kilodalton protein showed amino acid homology and other similarities to the outer membrane protein OmpF of *Escherichia coli*. Although a similar product was released in small quantities from cell walls of the virulent *M. tuberculosis* Erdman and H37Rv by lysozyme treatment, the cell walls of virulent bacilli were dominated by the presence of poly-α-L-glutamine, accounting for as much as 10% of their weight. The poly-α-L-glutamine was successfully separated from the cell wall proper, demonstrating again the absence of a covalent association between peptidoglycan and the polymer. The antigenicity of these products is demonstrated, and their roles vis-a-vis analogous polypeptides from other bacteria in immunogenicity, pathogenicity, and bacterial physiology are discussed.

Tuberculosis remains a threatening disease, with an estimated 8 to 10 million cases and 3 to 4 million deaths per year worldwide (12); in the United States during 1985, 22,201 cases were reported, for an incidence rate of 9.3 cases per 100,000 population (Centers for Disease Control, Tuberculosis—United States, 1985; Morbidity and Mortality Weekly Report 35:699–703, 1986). Despite intensive study for over 50 years, the antigenic composition of *Mycobacterium tuberculosis* is not clearly defined and, consequently, candidate subcellular vaccines thought necessary for the eradication of tuberculosis have not been identified. Since T-cell intervention is the primary means for the acquisition of protective immunity in tuberculosis (17, 20), proteins are regarded as the key immunogens. Previous work had demonstrated that protein associated with mycobacterial cell walls can potentially induce protective immunity against challenge with *M. tuberculosis* (2) and *Mycobacterium leprae* (R. H. Gelber, P. J. Brennan, S. W. Hunter, M. W. Munn, J. M. Masson, L. P. Murray, P. Siu, M. Tsang, E. G. Engleman, and N. Mohagheghpour, submitted for publication), elicit a delayed type hypersensitivity response in sensitized humans and animals (25, 27), and activate appropriate T lymphocytes (3a, 15, 24). The intractable nature of this protein and the notorious resistance of mycobacterial cell walls to muramidase digestion (3) have made characterization of the cell wall protein difficult.

In this report, we describe the release, purification, and characterization of a prominent antigenic 23-kilodalton (kDa) protein tenaciously associated with but apparently not covalently attached to peptidoglycan from *M. tuberculosis* H37Ra and, to a lesser extent, the virulent Erdman and H37Rv strains. In addition, the isolation of an antigenic poly-α-L-glutamine from the cell walls of the virulent strains by nondegradative means is demonstrated, again revealing a noncovalent association with peptidoglycan. The implications of these associations in interpreting the molecular basis of the high immunogenicity of the mycobacterial cell wall core are considered.

* Corresponding author.

MATERIALS AND METHODS

Preparation of cell walls. *M. tuberculosis* H37Ra was obtained from Kuni Takayama (William S. Middleton Memorial Veterans Hospital, Madison, Wis.). The virulent *M. tuberculosis* H37Rv and *M. tuberculosis* Erdman were strains no. 102 and 107, respectively, from the Trudeau Mycobacterial Culture Collection. Cells were grown to mid-log phase in a glycerol-alanine-salts-containing medium (40) in Fernbach flasks with gentle shaking. The virulent *M. tuberculosis* strains were heat-killed at 80°C for 1 h. Cells were harvested by centrifugation at 5,000 × *g* and washed several times with distilled water. Cells (50 g wet weight) were suspended in 100 ml of breaking buffer consisting of 0.1% (vol/vol) Tween 80, 1 mM $MgCl_2$, and 1 mM benzamidine, in phosphate-buffered saline (PBS) (pH 7.4). The suspension was sonicated in an ice bath for 15 min with a W-385 Sonicator Ultrasonic Liquid Processor (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) operating at optimal cavitation intensity. The sonic extract was passed three times through a French pressure cell (model SA073; American Instruments Co., Urbana, Ill.) at 20,000 lb/in^2 and treated with 30 mg of ribonuclease (R4875; Sigma Chemical Co., St. Louis, Mo.) and 30 mg of deoxyribonuclease (D5025; Sigma) overnight at 4°C. The sonic extract-pressate was centrifuged at 27,000 × *g* for 20 min, and the resulting cell wall-containing pellet was subjected to 2% (wt/vol) sodium dodecyl sulfate (SDS) extraction for 2 h at 60°C to remove soluble protein and membrane. The insoluble cell walls were recovered by centrifugation at 27,000 × *g* for 20 min, and the SDS extraction was repeated twice. The extracted cell walls were washed extensively with PBS and

then with distilled water and 80% (vol/vol) aqueous acetone to remove SDS. Cell walls were suspended in a small volume of PBS containing 0.1% Tween 80 and placed on a discontinuous sucrose gradient composed of 15, 25, 30, 40, and 60% (wt/vol) sucrose in PBS–0.1% Tween 80. The gradient was centrifuged at 100,000 × *g* for 2 h, using an SW27 swing-out rotor (Beckman Instruments, Inc., Fullerton, Calif.). The major visible cell wall band was recovered and washed several times with PBS and then with distilled water.

In the case of virulent strains, cell walls prepared in this way were dominated by the presence of poly-L-α-glutamine and had to be treated specially to attain its removal.

Release of peptidoglycan-bound protein. To facilitate the release of SDS-insoluble proteins, SDS-treated cell walls (50 mg) were suspended in 1.5 ml of trifluoromethanesulfonic acid (CF_3SO_3H)-anisole (2:1) (10) and held at room temperature for 30 min with constant stirring; cell walls were completely dissolved at this point. The solution was cooled in an ice bath, and diethyl ether (precooled to −20°C) (1.5 ml) and 50% aqueous pyridine (vol/vol) (cooled to 4°C) (3 ml) were added slowly with gentle mixing. The ether phase was discarded. The aqueous phase was further extracted with ether and dialyzed against large volumes of deionized water overnight. The resulting protein-pyridinium salts coprecipitate and the soluble contents within the dialysis sac were separated by centrifugation, dried on a Savant Speed Vac Concentrator (Savant Instruments, Farmingdale, N.Y.), and stored at −20°C for subsequent use.

Cell walls were also digested with lysozyme, after an initial Smith degradation (14), in order to release bound proteins, as follows. SDS-extracted cell walls (100 mg) were suspended in 35 ml of 0.03 M sodium acetate buffer (pH 4) containing 15% ethanol and 0.05 M sodium periodate and then constantly agitated for 48 h at 25°C. The treated cell walls were recovered by centrifugation at 20,000 × *g* for 20 min, and the process was repeated for 24 h. Ethylene glycol was added to destroy residual $NaIO_4$, and repeated washings and centrifugations were then done. The oxidized cell walls were reduced in 35 ml of a solution containing $NaBH_4$ (10 mg/ml) and 15% ethanol at pH 10.0 for 1 h at 25°C. $NaBH_4$ was destroyed with glacial CH_3COOH. Cell walls were washed and hydrolyzed with dilute HCl (0.05 M in 15% ethanol) for 18 h at room temperature. The freed mycolic acid was removed with ether, and ethanol and water washes were then done. The delipidated, deglycosylated cell walls were then treated for 24 h at 25°C with egg white lysozyme (Worthington Diagnostics, Freehold, N.J.) in 100 mM ammonium acetate buffer (pH 6.3) in 0.02% sodium azide at an enzyme-substrate ratio of 20%. Subsequent centrifugation at 20,000 × *g* resulted in a soluble lysozyme digest and an insoluble residue.

Purification of the 23-kDa peptidoglycan-associated protein. The protein released by trifluoromethanesulfonic acid was freed from the pyridinium trifluoromethanesulfonate coprecipitate by solubilization in 6 M guanidine hydrochloride buffered with 50 mM Tris hydrochloride (pH 7.8) and chromatography on a Sephadex G-75 column (90 by 1 cm) equilibrated with the same buffer. Further purification was carried out on a Sephacryl S-200 column (90 by 1 cm) in 6 M guanidine hydrochloride in 50 mM Tris hydrochloride (pH 7.8); anion exchange high-performance liquid chromatography (HPLC) on a Synchropak AX-300 column (250 by 4.1 mm; Synchrom, Inc., Lafayette, Ind.) in 7 M urea in 50 mM Tris hydrochloride (pH 7.2) was then done. Elution was achieved with a linear gradient of 0.0 to 0.3 M LiCl. Subsequent chromatography involved a hydroxylapatite Bio-Gel HPHT column (100 by 7.8 mm; Bio-Rad Laboratories, Richmond, Calif.) in 0.1% SDS–0.01 M sodium phosphate (pH 6.8) and a linear gradient of 0.01 to 0.35 M sodium phosphate at a constant pH. Removal of detergent was accomplished on an Extracti-Gel D column (2-ml bed volume; Pierce Chemical Co., Rockford, Ill.) equilibrated in 7 M urea–50 mM Tris hydrochloride (pH 7.8). In all cases, fractions were monitored for UV absorption at 280 nm and for protein colorimetrically, using the bicinchoninic acid protein assay reagent (Pierce) (38). The urea and buffer salts were removed from the final product by dialysis against 0.1 M ammonium bicarbonate (pH 8.0), and the protein was lyophilized, reconstituted with distilled water, lyophilized, and stored at −20°C for subsequent use.

Trypsinization of the 23-kDa protein and purification and sequencing of peptides. The 23-kDa protein (150 μg) was reduced for 3 h under nitrogen in 8 M urea–0.1 M Tris (pH 7.5) after the addition of 2 μg of dithiothreitol. Alkylation was then carried out by the addition of 5 μl of 4-vinylpyridine, allowing the mixture to react for 3 h at room temperature, after which the sample was exhaustively dialyzed against 0.1 M ammonium carbonate. The pyridylethylated protein was then treated with 7 μg of TPCK-trypsin (T-8642, Sigma Chemical Co., St. Louis, Mo.) for 4 h at 37°C. An additional 7 μg was added and allowed to react overnight at 37°C. The sample was lyophilized to dryness. Peptides were purified by reverse-phase high-pressure liquid chromatography on an octadecyl-4PW column (150 by 4.6 mm; Toso Haas, Philadelphia, Pa.) in 200 mM ammonia (pH 10.8), eluting with a 50-min linear gradient of acetonitrile from 0 to 60%. Peptides were detected by absorbance at 214 mm. An estimated 100 pmol of each peptide was analyzed by a model 470A gas-phase sequencer (Applied Biosystems Inc., Foster City, Calif.).

The cell walls of *M. tuberculosis* Erdman and H37Rv: purification of poly-α-L-glutamine. The SDS-extracted, 27,000 × *g* pellet obtained from the virulent strains of *M. tuberculosis* Erdman and H37Rv were subjected to sucrose density gradient centrifugation as described above. The cell walls proper settled at the 30 to 40% interface, whereas the associated poly-α-L-glutamine pelleted to the bottom of the tube in conjunction with small amounts of peptidoglycan. Repeated sucrose density centrifugation yielded cell walls virtually devoid of poly-α-L-glutamine.

The combined poly-α-L-glutamine pelleted material was applied to a tube containing 80% Percoll (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) in PBS–0.1% Tween 80 which, when centrifuged at 100,000 × *g* for 20 min, allowed in situ formation of a gradient and distinct banding of the insoluble, pure poly-α-L-glutamine.

Electron microscopy. Electron microscopy was conducted on cell wall specimens which had been prefixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h at room temperature, washed with sodium phosphate buffer, and postfixed in 1% OsO_4 in sodium phosphate buffer for 1 h at 4°C; washings with water (8) were then done. Specimens were dehydrated in a graded ethanol series (up to 100% ethanol) and embedded in Spurr resin (39), and sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and examined on a Philips 400T transmission electron microscope.

Chemical analysis. Samples of cell wall, with added 2-deoxyglucitol as the internal standard, were hydrolyzed with 2 M CF_3COOH at 121°C for 1 h, and the component neutral sugars were analyzed by gas chromatography (GC) of the alditol acetates on an SP 2340 fused silica column (23).

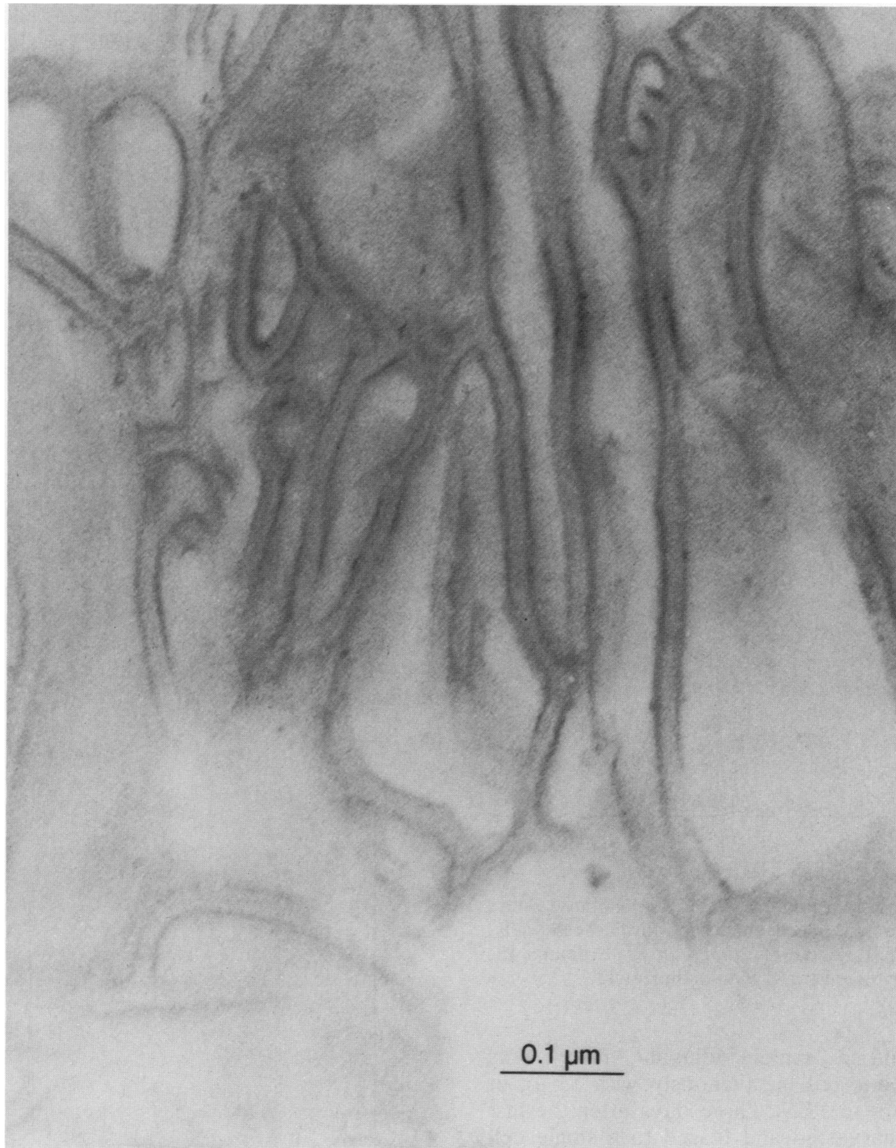

FIG. 1. Electron micrograph of a stained thin section of SDS-extracted, sucrose gradient-purified cell walls of *M. tuberculosis* H37Ra.

Analysis of amino acids and amino sugars was performed after hydrolysis of samples at 110°C for 20 h with 6 N HCl by GC or GC-mass spectrometry of the *N*(O)-heptafluorobutyryl isobutyl esters (21), using D,L-α-aminoadipic acid as the internal standard (15). To distinguish between D- and L-amino acids, the optically active S-(+)-2-butyl derivatives rather than the *iso*-butyl esters were chromatographed. The mycolic acids of cell walls were analyzed as the 4-bromophenacyl esters by HPLC as described elsewhere (46); hydrolyzed samples contained tricosanoic acid as the internal standard to allow quantitation. Simpler fatty acids were obtained by methanolysis in CH_3OH-toluene-H_2SO_4 (30:15:1) at 75°C overnight of samples of cell wall to which nonadecanoic acid was added as the internal standard. The fatty acid methyl esters were resolved by GC on a Durabond-1 fused silica column operating at an initial temperature of 150°C for 4 min, increasing by 4°C per min to 270°C. In all cases, the detector responses of appropriate standards were compared with those of internal standards, and a correction factor was applied to all quantitations.

SDS-PAGE and two-dimensional PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed under reducing conditions by the method of Laemmli (16). Samples were prepared in SDS-mercaptoethanol buffer by boiling for 2 min. Electrophoresis was performed in slabs 0.75 mm thick and 16 cm long with a 6% stack over a 12.5% resolving gel. Each gel was run at a constant current of 10 mA until the tracking dye entered the resolving gel; the current was then increased to 15 mA. Two-dimensional analysis was performed by separation in the first dimension by nonequilibrium pH gradient electrophoresis (30) at 400 V for 4 h and then by SDS-PAGE in the second dimension. Proteins were visualized by staining with Coomassie brilliant blue R250 or by silver staining (29).

Monoclonal antibody production to the 23-kDa peptidoglycan-associated protein. BALB/c mice were immunized intraperitoneally with 150 μg of purified cell walls from *M. tuberculosis* H37Ra in Freund incomplete adjuvant, followed 2 weeks later by a subcutaneous injection with 50 μg of the CF_3SO_3H-released, chromatographically purified 23-

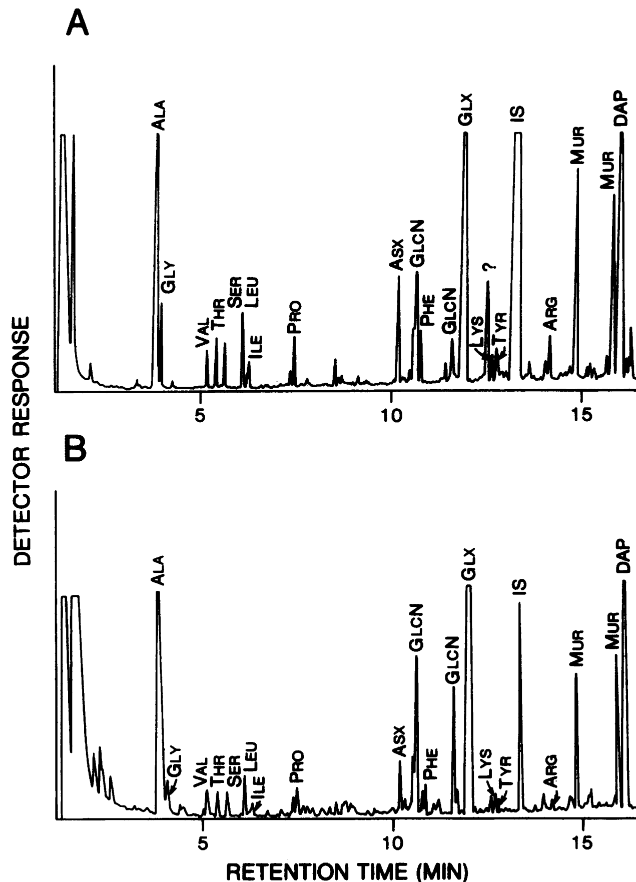

FIG. 2. GC of the heptafluorobutyryl amino acid isobutyl esters prepared from SDS-extracted, sucrose gradient-purified cell walls. (A) *M. tuberculosis* H37Ra; (B) *M. tuberculosis* Erdman. IS, Internal standard of derivatized D,L-α aminoadipic acid.

kDa protein in Freund incomplete adjuvant. Two weeks later, the mice were boosted intravenously with 15 μg of purified 23-kDa protein in PBS. Three days after the last boost, spleens were harvested and teased to a single cell suspension, which was fused to SP2/0-AG14 myeloma cells with polyethylene glycol 4000; successful fusions were selected, using standard procedures (13). Clones were screened and selected by plate enzyme-linked immunosorbent assay (ELISA). After limited dilution cloning, hybridoma cells were injected into Pristane-primed BALB/c mice for ascites fluid production.

Immunological techniques. Polyclonal antisera were obtained by immunizing rabbits with heat-killed (80°C; 1 h) *M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv, or *M. tuberculosis* Erdman emulsified in incomplete Freund adjuvant. Plate ELISA was conducted as described elsewhere (6), with minor modifications. Briefly, the coating of antigen was performed in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Nonspecific binding sites were blocked with 1% bovine serum albumin in PBS (pH 7.4)–0.05% Tween 80 at 37°C for 1 h. Rabbit antiserum appropriately diluted in PBS–0.05% Tween 80 containing 10% normal goat serum was added and incubated at 37°C for 1 h. After the rabbit antiserum was discarded and the plate was washed with PBS, peroxidase-conjugated goat anti-rabbit immunoglobulin G, immunoglobulin M, and immunoglobulin A (Organon Teknika Corp., Cappell Div., Malvern, Pa.) diluted 1:2,000 in PBS–10% normal goat serum were added for 1 h at 37°C. Substrate was added after the PBS washes, and the reaction was stopped after 15 min with 2.5 N H_2SO_4. Absorbances were read at 490 nm in a multiscan microdilution plate reader.

Western blot (immunoblot) analysis was accomplished by electrophoretic transfer of protein onto nitrocellulose paper (Bio-Rad) (42) followed by ELISA (15), using a 1:1,000 dilution of monoclonal antibody Tb1C7 ascites fluid.

RESULTS

Isolation and properties of cell walls of *M. tuberculosis*. Traditional cell wall isolation procedures (8) were applied to harvests of *M. tuberculosis* H37Ra, H37Rv, and Erdman. Sonication and then multiple passes through a French pressure cell were required to produce an extract virtually devoid of whole bacteria, as determined by acid-fast staining. Sucrose gradient centrifigation of the 27,000 × *g*, SDS-extracted pellet yielded one major visible band at the 25 to 30% interface; incidentally, heat-killed bacteria, regardless of strain, yielded a corresponding band at the 30 to 40% interphase. Cell walls from the H37Rv and Erdman strains required repeated sucrose density centrifugation to yield a product devoid of poly-α-L-glutamine. Stained ultrathin sections of the embedded purified cell walls showed fragments distinguished by an inner electron-dense layer overlaid by a

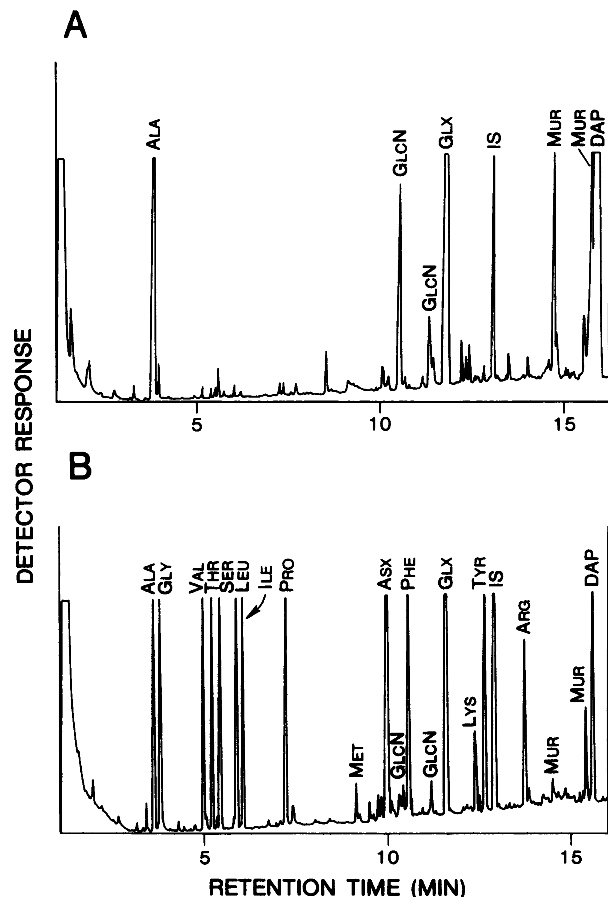

FIG. 3. GC of the heptafluorobutyryl amino acid isobutyl esters prepared from CF_3SO_3H-hydrolyzed, purified cell walls. (A) Nondialyzable, water-soluble products; (B) nondialyzable, insoluble protein-pyridinium salts coprecipitate.

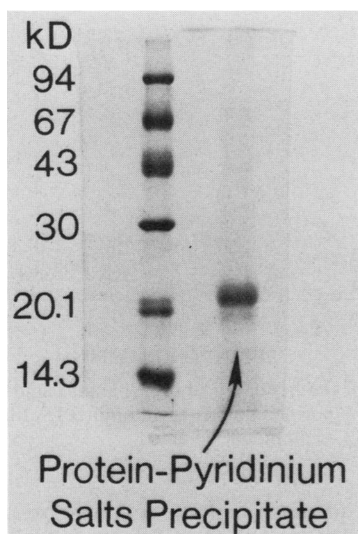

FIG. 4. SDS-PAGE of the protein-pyridinium salts coprecipitate. Conditions are described in Materials and Methods. Approximately 2 μg of protein was applied. Phosphorylase b (molecular size, 94 kDa), bovine serum albumin (molecular size, 67 kDa), ovalbumin (molecular size, 43 kDa), carbonic anhydrase (molecular size, 30 kDa), soybean trypsin inhibitor (molecular size, 20.1 kDa), and α-lactalbumin (molecular size, 14.3 kDa) (Pharmacia LKB Biotechnology) served as molecular markers. Protein bands were visualized by Coomassie brilliant blue staining. kD, Kilodaltons.

TABLE 1. Amino acid and amino sugar composition of the released 23-kDa protein after HPLC

Amino acid or amino sugar	Molar ratio	
	After initial resolution by HPLC[a]	After full HPLC purification[b]
Gly	29	28
L-Ala	13	14
D-Ala	2	0
Cys	ND[c]	ND
Ser	18	18
Thr	13	13
Pro	11	11
Tyr	4	4
Trp	ND	ND
Asx	22	22
L-Glx	16	16
D-Glx	2	0
Lys	2	2
Arg	7	7
His	2	2
Phe	9	9
Leu	14	14
Ile	8	8
Met	1	1
Val	11	11
DAP	1	0
GlcN	2	0
Mur	1	0

[a] Using weak anion exchange chromatography.
[b] After hydroxylapatite chromatography.
[c] ND, Not determined accurately by the GC method employed.

diffuse electron-transparent zone (Fig. 1) typical of mycobacterial cell walls (8, 9). Wall fragments also showed trilamellar staining in the form of two electron-dense layers overlaying an electron-transparent zone; this has been interpreted as adjacent cell walls associating through interaction of their outer diffuse electron-transparent layers (9).

Chemical analysis of the purified cell walls of all strains of *M. tuberculosis* showed that neutral sugars composed about 23% of the total weight; arabinose (molar ratio, 44) and galactose (molar ratio, 32) predominated; small amounts of mannose, rhamnose, and glucose (molar ratio for each, ca. 1) were also present. Residual bound SDS interfered with the quantitation of mycolic acids, which represented about 45% of the weight of cell walls prior to SDS extraction. There were no appreciable simpler fatty acids present. The amino acid and amino sugar content of the isolated SDS-extracted cell walls, as determined by GC of *N*(*O*)-heptafluorobutyryl isobutyl esters, is shown in Fig. 2. On the basis of the content of glucosamine (GlcN), muramic acid (Mur), and diaminopimelic acid (DAP), it was estimated that about 20% of the SDS-extracted cell walls consisted of peptidoglycan. Nonpeptidoglycan amino acids represented about 1 and 2% of the mass of purified cell walls of *M. tuberculosis* Erdman and H37Ra, respectively. This value remained constant throughout repeated extractions with 2% SDS at 60°C and 100°C; 2% SDS containing 0.5 M NaCl and 1.5 mM EDTA at 37°C, 60°C, or 100°C; or 7 M urea overnight at room temperature.

Trifluoromethanesulfonic acid facilitates the release of a 23-kDa peptidoglycan-associated protein from *M. tuberculosis* H37Ra. The purified cell walls from *M. tuberculosis* H37Ra were exposed to a mixture of CF_3SO_3H and anisole, a treatment expected to cleave glycosidic (10, 22) and ester (41) linkages within cell walls (9), while leaving putative peptide bonds intact. The treatment was effective in completely solubilizing cell walls. After neutralization with pyridine and extraction with ether to remove freed mycolic acids, the aqueous products were dialyzed, a process which served to remove the hydrolyzed neutral sugars. Amino acid-amino sugar analysis of the nondialyzable soluble materials showed almost exclusively GlcN, Mur, Ala, Glx, and DAP (Fig. 3A), suggesting large, water-soluble peptidoglycan fragments. The protein amino acids were primarily associated with the protein-pyridinium salts precipitate which arose during dialysis (Fig. 3B). SDS-PAGE of this precipitate showed only one major polypeptide with a molecular size of 23 kDa (Fig. 4). The faint band observed below the 23-kDa protein is probably a degradative product of the 23-kDa protein, as it reacts with the 23-kDa monoclonal antibody (results not shown). Gel filtration on Sephadex G-75 followed by Sephacryl S-200 and subsequent weak

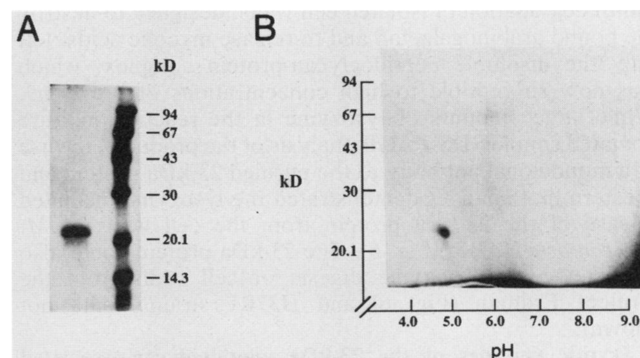

FIG. 5. SDS-PAGE (A) and two-dimensional PAGE (B) of the purified 23-kDa peptidoglycan-associated protein. In each case, 2 μg of protein was applied. Protein was visualized by silver staining.

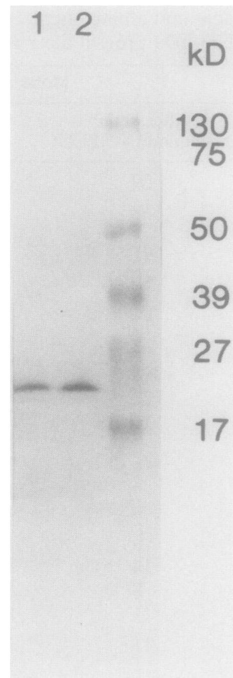

FIG. 6. Western blot analysis of the lysozyme-digested cell walls from *M. tuberculosis* Erdman (lane 1) and *M. tuberculosis* H37Ra (lane 2), using the monoclonal antibody (TblC7) generated against the CF_3SO_3H-released, purified 23-kDa protein. kD, Kilodaltons.

anion exchange HPLC yielded a product homogeneous by SDS-PAGE (data not shown); however, amino acid-amino sugar analysis (Table 1) showed the continuing presence of GlcN, Mur, DAP, D-Ala, and D-Glx, suggesting for a time that these might represent a terminal element, perhaps the remnants of an original link between polypeptide and peptidoglycan. However, further purification by HPLC on a hydroxylapatite column equilibrated with SDS yielded a product (Fig. 5) devoid of peptidoglycan components as analyzed by GC and GC-mass spectrometry (Table 1). Seemingly, then, the 23-kDa protein was not covalently linked to peptidoglycan by an amide linkage (5).

Release of the 23-kDa peptidoglycan-associated protein with lysozyme. Isolated cell walls of *Mycobacterium* spp. are notoriously resistant to lysozyme (3), perhaps because of the presence of *N*-glycolyl rather than *N*-acetyl functions on the Mur*N*, or perhaps because of steric hindrance from the massive, attached mycolyl-arabinogalactan. However, Smith degradation of isolated cell walls, designed to destroy the bound arabinogalactan and to release mycolic acids, left only the insoluble peptidoglycan-protein complex, which was now susceptible to high concentrations of lysozyme. While large amounts of lysozyme in the reaction mixture obviated simple SDS-PAGE analysis of the products, the use of a monoclonal antibody to the purified 23-kDa protein and Western blot analysis demonstrated the lysozyme-mediated release of the 23-kDa protein from the cell walls of *M. tuberculosis* H37Ra (Fig. 6). The 23-kDa protein could also be detected in lysozyme digests of cell walls from the virulent Erdman (Fig. 6) and H37Rv strains (data not shown).

Tryptic peptides of the 23-kDa peptidoglycan-associated protein. The released protein was not amenable to N-terminal sequencing. Accordingly, in order to obtain at least some structural information, three tryptic fragments were purified and sequenced, with the following results: fragment 1, Leu-Thr-Val-(Gln)-Gln-Cys (with ambiguity at residue 4); fragment 2, Leu-Ile-Ala-His-Thr-Gly-Asp-Asn-Val-Thr-Tyr-Gly-Glu-Pro-(Arg) (with ambiguity at residue 15); fragment 3, Try-Ile-Val-Ala-Gly-Pro-Gly-Ala-Asp-Phe-Glu-Gly-Thr-(Asn)-Glu-Leu-Gly-Try-Glu (with ambiguity at residue 14).

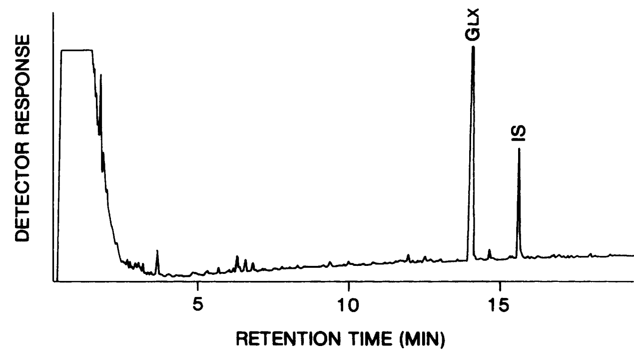

FIG. 7. GC of the heptafluorobutyryl amino acid isobutyl esters prepared from the purified poly-α-L-glutamine. IS, Internal standard of derivatized D,L-α aminoadipic acid.

In a search of the PIR and SWISSPROT data bases using a mutation data matrix scoring system (35a), the third tryptic fragment showed a relationship to residues 44 to 63 of the outer membrane protein F (OmpF) precursor of *E. coli* (15a); the OmpF sequence scored the highest of 3,497,762 and 2,763,453 residue segments searched in the PIR and SWISSPROT data bases, respectively, with a score of 49 out of a possible 99 maximum and −123 minimum (35a).

Poly-α-L-glutamine from virulent *M. tuberculosis* cell walls. The 27,000 × *g*, cell wall-containing pellet from the virulent *M. tuberculosis* Erdman and H37Rv strains contained considerable amounts of poly-α-L-glutamine, accounting for approximately 10% of the cell wall mass; the polymer remained associated with cell walls throughout repeated SDS extraction. Upon sucrose gradient purification of the pelleted SDS-extracted material, it was found that while the cell walls proper were predominantly at the 30 to 40% interface, the insoluble poly-α-L-glutamine pelleted to the bottom of the tube in conjunction with minor amounts of peptidoglycan (as revealed by amino acid-amino sugar analysis). Repeated sucrose and Percoll gradient purification of the pellet material yielded a pure insoluble product with no detectable peptidoglycan components as determined by amino acid-amino sugar analysis (Fig. 7). The poly-α-L-glutamine isolated by these conditions, which do not endanger the integrity of amide linkages, was at least 90% amidated (full structural analysis of the polymer will be reported separately).

Antigenicity of the polypeptides. The purified 23-kDa and poly-α-L-glutamine polypeptides reacted readily against rabbit anti-serum raised against the homologous whole bacterium (Fig. 8), indicating that both evoke a humoral immune response. The 23-kDa peptidoglycan-associated protein was not recognized by monoclonal antibodies which identify a free 23-kDa protein of *M. tuberculosis* (H. D. Engers and V. Houba, Letter, Infect. Immun. **51:**718–720, 1986) as analyzed by Western blot and plate ELISA. Similar analysis revealed the absence of the free 23-kDa protein in early culture filtrate derived from the bacillus Calmette-Guérin Russian strain of *M. bovis*, in which copious amounts of a secreted 23-kDa protein are found (1). In addition, it was not

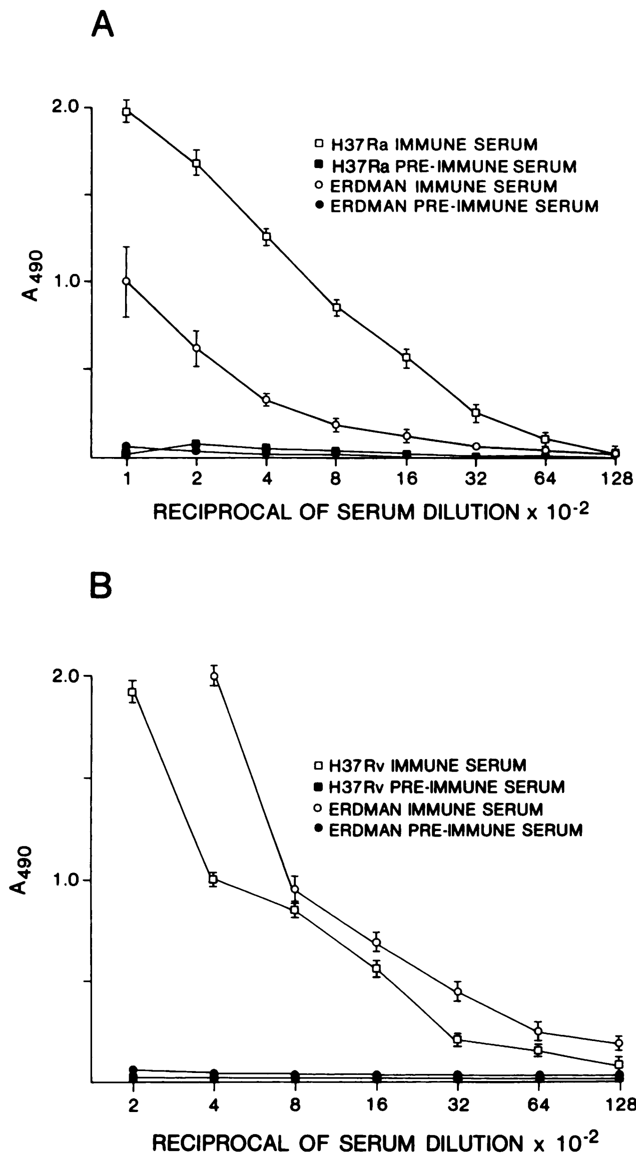

FIG. 8. Serological activity of the 23-kDa protein (A) and poly-α-L-glutamine (B) in ELISA against anti-*M. tuberculosis* rabbit anti-serum. Conditions for the assay have been described previously (6). The absorption values obtained for serum dilutions of rabbit serum pre- and postimmunization with *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv and *M. tuberculosis* Trudeau Mycobacterial Culture No. 107 Erdman are represented. Each dilution was assayed in triplicate wells containing 50 ng of the 23-kDa protein or 500 μg of the poly-α-L-glutamine. Reported values were corrected by subtracting background values for corresponding serum dilutions tested in wells with no antigen. Each point represents the corrected mean plus or minus the standard error of the mean.

detected in the culture filtrate or the cytosolic or membrane fractions of *M. tuberculosis* H37Ra from which it was isolated.

DISCUSSION

Others have previously commented on the presence of nonextractable, persistent polypeptides within mycobacteria (9, 32), and the accumulated evidence indicates an important role in mycobacterial immunity (3a, 24, 25, 27, 34; Gelber et al., submitted). For instance, in the latest of this body of work (25; Gelber et al., submitted), cell wall-associated proteins were shown to evoke a vigorous delayed type hypersensitivity response in sensitized animals and humans, to activate T cells from appropriate subjects, and to protect mice against challenge with live *M. leprae* (25; Gelber et al., submitted). However, the task of dissociating such putative immunogenic polypeptides from the mycobacterial cell wall proper without compromising peptide bonds was formidable largely because mycobacterial peptidoglycan is notoriously resistant to muramidase (3). Indeed, the fact that the nature of these tenaciously associated proteins had not previously been pursued may have been due to the lack of appropriate hydrolytic tools. Accordingly, innovative approaches to allow the release of such intact polypeptides were sought. Under appropriate conditions, trifluoromethanesulfonic acid cleaves glycosidic and ester linkages while leaving amide linkages intact (10, 11, 41). Its effects were decisive in solubilizing mycobacterial cell walls and in effecting the complete release of an intact 23-kDa protein (Fig. 3 and 4). In addition, Smith degradation of the cell walls designed to remove the attached mycolyl-arabinogalactan left the peptidoglycan susceptible to conventional lysozyme in high concentrations, liberating the 23-kDa protein and thereby establishing its association with the basal peptidoglycan.

The tenacity with which the 23-kDa protein adheres to the peptidoglycan was exemplified by the difficulty in resolving the two through several purification steps, initially suggesting an amide linkage. However, the ultimate removal of all traces of peptidoglycan by hydroxylapatite HPLC in detergent has convinced us that the relationship between peptidoglycan and protein does not involve a peptide bond; obviously, the presence of a novel linkage susceptible to CF_3SO_3H, such as an ester bond, cannot be excluded. In the case of gram-negative bacteria, extraction of cell walls with SDS in the presence of salt or at a high temperature generally solubilizes peptidoglycan-associated proteins (4, 18, 19); the exception is the lipoprotein of Braun and Sieglin (5). In its purified form, the 23-kDa protein is not amenable to N-group analysis. Yet, there is no evidence for the presence of acylglycerol-modified cysteine, a common feature of cell wall-associated lipoproteins of other procaryotes (47); nevertheless, the 23-kDa protein is extremely hydrophobic, on the basis of its behavior in reverse-phase chromatography (unpublished results).

Peptidoglycan-associated proteins have been described in gram-positive bacteria which resist exhaustive extractions with detergents and chaotropic agents, being released only by muramidases (7, 28, 35, 37). Perhaps the 23-kDa protein is intercalated within the highly cross-linked peptidoglycan, analogous to the postulated interaction of the staphylococcal protein A and the streptococcal M6 and G proteins with their respective peptidoglycans (31). Regardless of the form of the cell wall association, it is clear from present and past studies (25, 27) that peptidoglycan-associated proteins are concrete, immunologically important entities rather than contaminating remnants of other somatic proteins. The apparent absence of the 23-kDa polypeptide from culture filtrate and cytosolic or membrane fractions, combined with its apparent hydrophobicity, may presage a role in solute transport through the lipid barrier of mycobacteria. Indeed, the evidence for a relationship to the OmpF protein of *E. coli* gives some credence to this suggestion.

The poly-L-glutamate of virulent strains of *M. tuberculosis* has long been considered truly attached to the underlying peptidoglycan (26, 33, 43–45). The present work establishes

that the polymer is, in fact, primarily poly-α-L-glutamine (detailed results not shown) and that it can be resolved from the inherent peptidoglycan by nondegradative means. The physical properties of the polymer, particularly its insolubility, at least in detergents, chaotropes, organic acids bases, and a large variety of solvents (results not shown), could account for its copurification with cell walls and the view that it is attached to peptidoglycan; it now seems more likely that it provides an intracellular storage reserve of carbon and nitrogen, analogous to many of the inclusion bodies described in other procaryotes (36). The poly-α-L-glutamine has long been speculated to play a role in virulence because of its prevalence in cell walls from virulent strains of *M. tuberculosis* (for a review, see reference 32). The ability to isolate this polymer and peptidoglycan-associated polypeptides such as the 23-kDa protein as intact antigens will allow further investigations concerning their roles in the immunology, physiology, and pathogenesis of mycobacteria.

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