LipC (Rv0220) Is an Immunogenic Cell Surface Esterase of Mycobacterium tuberculosis

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We have reported previously the identification of novel proteins of Mycobacterium tuberculosis by the immunoscreening of an expression library of M. tuberculosis genomic DNA with sera obtained from M. tuberculosis-infected rabbits at 5 weeks postinfection. In this study, we report the further characterization of one of these antigens, LipC (Rv0220). LipC is annotated as a member of the Lip family based on the presence of the consensus motif “GXSXG” characteristic of esterases. Although predicted to be a cytoplasmic enzyme, we provide evidence that LipC is a cell surface protein that is present in both the cell wall and the capsule of M. tuberculosis. Consistent with this localization, LipC elicits strong humoral immune responses in both HIV-negative (HIV−) and HIV-positive (HIV+) tuberculosis (TB) patients. The absence of anti-LipC antibodies in sera from purified protein derivative-positive (PPD+) healthy subjects confirms its expression only during active M. tuberculosis infection. Epitope mapping of LipC identified 6 immunodominant epitopes, 5 of which map to the exposed surface of the modeled LipC protein. The recombinant LipC (rLipC) protein also elicits proinflammatory cytokine and chemokine responses from macrophages and pulmonary epithelial cells. rLipC can hydrolyze short-chain esters with the carbon chain containing 2 to 10 carbon atoms. Together, these studies demonstrate that LipC is a novel cell surface-associated esterase of M. tuberculosis that is highly immunogenic and elicits both antibodies and cytokines/chemokines.

There are an estimated 9 × 10^6 new cases of tuberculosis (TB) and 2 × 10^6 TB-related deaths every year (55). Infection with Mycobacterium tuberculosis is initiated with the inhalation of a droplet bearing bacteria, and it takes months or years to progress to clinical TB. During this progression from initial infection to clinical disease, the in vivo bacteria adapt to continuously changing environments by altering their gene expressions (31, 34, 48, 50). Previous studies to delineate culture filtrate (CF) proteins of M. tuberculosis that are recognized by antibodies during the natural course of disease progression demonstrated that the repertoire of antigens enlarges with the progression of infection (34–36, 41). Interestingly, several of the M. tuberculosis antigens (malate synthase, MPT51, and ESA16, for example) that elicit immune responses during the early stages of active infection have also been demonstrated to play important roles in the host-pathogen interaction (19–21, 57).

To identify additional antigenic proteins of M. tuberculosis that are expressed during the early stages of active infection, we used sera obtained from M. tuberculosis aerosol-infected rabbits that were bled at 4 to 5 weeks postinfection to screen an expression library of M. tuberculosis genomic DNA (44). Antibodies in these sera identified several proteins known to contribute to the infection and survival of M. tuberculosis (exported repetitive protein [ERP], KatG, and MtrA) as well as novel proteins (proline threonine repetitive protein [PTRP], PE-PGRS51, and LipC [Rv0220]) (3, 26, 44, 59). Interestingly, ERP, KatG, and PTRP are cell wall proteins of M. tuberculosis (3, 43, 58), and while the precise localization of MtrA in M. tuberculosis has not been reported, the Mycobacterium leprae homolog is also a cell wall protein (27). The current studies are focused on LipC.

LipC is annotated as a member of the Lip family based on the presence of the consensus motif “GXSXG” characteristic of esterases and members of the hydrolyase fold family (40). The Lip family is comprised of 24 putative carboxyl ester hydrolases. Of these, studies of 3 members, LipF (Rv3487c), LipH (Rv1399c), and LipY (Rv3097c), have been reported so far (5, 10, 60). The current studies demonstrate that LipC is a cell surface protein that is present in both the cell wall and the capsule of M. tuberculosis. Consistent with this localization, LipC is a strong inducer of antibodies in patients with active TB, and immunodominant epitopes of the protein have been mapped. LipC also elicits cytokine responses from both macrophage-like THP-1 and pulmonary epithelial A549 cells. Moreover, a recombinant and enzymatically active M. tuberculosis LipC was produced and purified from M. smegmatis, and the biochemical characterization revealed that LipC hydrolyzes short-chain esters. Together, these results suggest that LipC participates in the progression of active TB both by contributing to the utilization of lipid substrates for bacterial growth and replication and by modulating immune responses.

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MATERIALS AND METHODS

Bioinformatics analysis. Nucleic and protein BLAST searches with the current databases were performed at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Predictions of theoretical molecular weights (ProtParam), transmembrane helices (TMHMM), and signal peptides (SignalP) were carried out with the respective software at the ExPASy Proteomics server (http://ca.expasy.org/). The NetO Glyc program was used to predict potential glycosylation sites, and MeMo (http://www.bioinfo.tsinghua.edu.cn/~tigerchen/memo/links.html) was used to predict potential arginine or lysine sites that may be methylated. A model of the three-dimensional (3-D) structure of LipC was generated from the automatic protein structure homology modeling server using the I-TASSER software (33). The Rv0220 sequence was submitted against the nonredundant sequence database structural classification of proteins and the Protein Data Bank (PDB). The molecular graphics software program PyMol (http://www.pymol.org/) was used to display and draw the structure from the PDB files generated by the I-TASSER server.

Bacterial strains, plasmids, and growth media. Mycobacterial strains were grown in Middlebrook 7H9 broth (Becton Dickinson and Company [BD], Sparks, MD) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-saline (ADS) (0.5% bovine serum albumin, fraction V [Sigma, St. Louis, MO]; 0.2% dextrose; and 0.085% NaCl) at 37°C under constant shaking at 110 rpm. Escherichia coli strains were grown in LB broth (Difco Laboratories) at 37°C with shaking (220 rpm) in the presence of either hygromycin (200 μg/ml), kanamycin (50 μg/ml), amphicillin (100 μg/ml), or chloramphenicol (34 μg/ml), as required.

Immunoscreening of the Agt11 library. The Agt11 expression library of M. tuberculosis H37Rv DNA from the World Health Organization (WHO) was screened with pooled sera obtained from M. tuberculosis aerosol-infected rabbits at 5 weeks after aerosol infection as described previously (44). The reactive Agt11 clones were purified, and the inserts were sequenced to identify the gene encoding the immunoreactive protein (44).

Expression and purification of rLipC. For the expression of recombinant LipC (rLipC) in E. coli, a 1,235-bp DNA fragment containing the entire lipC open reading frame was amplified from M. tuberculosis H37Rv genomic DNA using primers F1 5'-CCCATATGAAACACGGCAGCGG-3' and R1 5'-GCTTCTGATGTCGCGGCTGTCG-3' (underlined sequences indicate NdeI and XhoI sites, respectively) and cloned into the pCR-Blunt cloning vector (Invitrogen, Carlsbad, CA). This intermediate plasmid (pCR-Blunt-lipC) was digested with NdeI and XhoI, and the resulting lipC gene was cloned into the PET14b expression vector (Novagen, EMD Biosciences, Inc., San Diego, CA) at NdeI and XhoI sites to produce an in-frame fusion with the His tag at the N-terminal position. The open reading frame of the recombinant plasmid (pET14b-lipC) was verified by DNA sequencing. The recombinant plasmid (pET14b-lipC) was transformed into E. coli BL21(DE3)(pLysS) cells (Invitrogen). After isopropyl-β-D-thiogalactopyranoside (IPTG) induction, the recombinant LipC protein was expressed in inclusion bodies, and standard procedures with urea were used to obtain the purified His-tagged rLipC protein by affinity chromatography on a Ni-nitrilotriacetic acid (NTA) agarose column (Qiagen, Chatsworth, CA). Endotoxins were removed by washing the protein-loaded affinity column with 10 mM Tris-HCl in 6 M urea, followed by 0.5% amidosulfofobetaine 14 (ASB-14) in 6 M urea. rLipC was eluted (20 mM Tris-HCl [pH 7.9], 1 M imidazole, 6 M urea), and fractions containing purified rLipC were pooled and dialyzed against 10 mM ammonium bicarbonate (pH 8.0) with stepwise-decreased concentrations of urea. The purified rLipC protein formed aggregates that were readily solubilized in 0.1% SDS. The Limulus amoebocyte lysate (LAL) assay was used to determine the endotoxin content in the purified LipC preparation according to the manufacturer’s instructions (Bio Whittaker, Walkersville, MD). Protopic analysis of rLipC was performed by quadrupole time-of-flight (Q-TOF) mass spectrometry at the New York University (NYU) protein analysis facility. All studies except for the assessment of enzymatic activity were performed with this rLipC protein.

Since the rLipC protein obtained from E. coli was enzymatically inactive, lipC was also expressed in M. smegmatis to determine enzymatic activity and substrate specificity. Briefly, the lipC gene was amplified from M. tuberculosis H37Rv genomic DNA with primers F2 (5'-GCTATCATG GTACAGGACGGCCGCGGTTGACTC-3') and R2 (5'-TACTGGAGCT TAGTACCCTTTGTGGCGACCTG-3'), containing NcoI and HindIII restriction sites (underlined), respectively. The amplified lipC fragment was double digested (with NcoI and HindIII), and after purification, the fragment was cloned into vector pMgNt (a shuttle vector containing a tobacco etch virus (TEV)-cleavable N-terminal 6His tag, an acetamide-inducible promoter, and a hygromycin resistance gene; provided by EMBL-Hamburg). The resulting recombinant plasmid (pMyNt-lipC) was verified by DNA sequencing and electroporated into M. smegmatis mc^155. The bacteria expressing rLipC were cultured until an optical density at 600 nm (OD600) value of 3 was reached, induced for 16 h by the addition of 0.2% (wt/vol) acetamide, and harvested by centrifugation. The bacterial pellet was suspended in 10 mM Tris buffer (pH 8.0) containing 300 mM NaCl and 1% N-laurylsarcosine (Sarkosyl) and broken with a French press. The His-tagged rLipC protein was purified by Ni-NTA agarose column-based affinity chromatography and eluted with 10 mM Tris buffer (pH 8.0) containing 300 mM NaCl and 100 mM imidazole. The fractions containing rLipC were pooled and further purified with a Superdex 200 column equilibrated with 10 mM Tris buffer (pH 8.0) containing 300 mM NaCl.

Localization of LipC. Purified rLipC from E. coli suspended in incomplete Freund’s adjuvant (IFA; Sigma) was used to immunize a New Zealand White rabbit to obtain polyclonal antibodies (16). Immunoglobulin G (IgG) from normal rabbit serum or serum from the immunized rabbit was purified by protein A-Sepharose 4B columns (Amersham Biosciences). M. tuberculosis H37Rv subcellular protein fractions (cytoplasm [Cyt], total cell wall [TCW], SDS-extracted cell wall [SDS-CW], and culture filtrate [CF]) (NIH/NIAID TB Research Materials contract, CSU) and M. tuberculosis capsular material (kindly provided by Mary Jackson, Colorado State University) were separated by SDS-PAGE (5.0 μg/lane), and the Western blots were probed with rabbit anti-LipC IgG (1:1,000) or normal rabbit IgG, followed by alkaline phosphatase (AP)-conjugated anti-rabbit IgG (1:2,000) and BCIP (5-bromo-4-chloro-3-indolylphosphate)-Nitro Blue Tetrizolium (NBT) substrate (KPL, Inc., Gaithersburg, MD). Previous studies have demonstrated that M. tuberculosis malate synthase is present in all the subcellular fractions of M. tuberculosis (19). Identical Western blots of all subcellular fractions were probed with anti-malate synthase IgG (1:1,000) to ensure the integrity of the preparations used. The localization of LipC on intact bacterial cells was confirmed by immunoelectron microscopy. Briefly, M. tuberculosis clinical isolate CDC1551 and a transposon mutant with a lipC mutation in CDC1551 (CDC1551 lipC::Tn) were obtained from TARGET (Tuberculosis Animal Research and Gene Evaluation Taskforce, John Hopkins University [http://webhost.nshu.edu/target/]). Bacteria from mid-log-phase cultures of M. tuberculosis H37Rv, M. tuberculosis CDC1551, and M. tuberculosis CDC1551 lipC::Tn were fixed and processed as described previously (43). The sections were examined under a Philips CM 10 TEM electron microscope at the NYU Image Core Facility.

Patients and control subjects. Sera from 19 purified protein derivative-negative (PPD−) and 29 PPD-positive (PPD+) healthy subjects; 70 HIV-negative (HIV−), acid-fast-bacillus (AFB) sputum smear-positive TB patients (HIV−TB+); and 45 HIV-positive (HIV+) AFB smear-positive TB patients (HIV+TB+) from India, a country where TB is endemic, were included in these studies. As per the DOTS (directly observed therapy, short term) programs guided by the WHO, the diagnosis of TB in countries where the disease is endemic is based on the microscopic examination of sputum smears for AFB, since this test is highly specific (54). Cultures are too expensive and complex for TB diagnosis in high-burden settings; for this reason, the bacteriological confirmation of
The LipC stock (400 μg/ml) was diluted to 1 μg/ml in medium (final concentration of SDS of 0.00025% [~0.01 mM]), and 200 μl of solution was added to ~4 × 10⁴ A549 cells/well or 2 × 10⁵ phorbol myristate acetate (PMA)–differentiated THP-1 cells in triplicate for 24 h. At the end of the incubation, the viability of cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s instructions (Invitrogen).

**Confirmation of the lack of endotoxin contamination.** The LAL assay showed that the concentration of endotoxin in the purified rLipC preparation was <10 ng/ml. For the biological confirmation of the absence of contamination with endotoxin, 2 × 10⁶ PMA-differentiated THP-1 cells were exposed to 200 μl medium containing 1 ng/ml lipopolysaccharide (LPS), 1 ng/ml LPS that was preincubated with 10 ng/ml polymyxin B for 1 h, 1 μg/ml LipC, and 1 μg/ml LipC that was preincubated with 10 ng/ml polymyxin B in triplicate. Culture supernatants were collected 24 h later and used for cytokine assessments with commercially procured ELISA kits according to the manufacturer’s instructions (BD Biosciences Pharmingen, San Diego, CA).

**Cytokine responses to rLipC.** A total of 2 × 10⁶ THP-1 or 4 × 10⁴ A549 cells/well (in 200 μl of medium) were exposed to different concentrations of the rLipC protein for 24 h, and the supernatants were harvested and assayed for interleukin-12 (IL-12), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and IL-8 using commercial sandwich ELISA kits (BD Biosciences Pharmingen), as described above.

**Enzymatic characterization of rLipC.** The activity of rLipC expressed in *M. smegmatis* was measured by using p-nitrophenyl (pNP) esters (Sigma) with carbon chain lengths ranging from C₂ to C₁₆. The release of pNP was monitored at 410 nm by using a 96-well plate spectrophotometer and quantified by using a calibration curve of pNP ([pNP]ₐ = 410 nm = 30 mM⁻¹). Enzymatic reactions were performed with 2.5 mM Tris buffer (pH 8.0) containing 300 mM NaCl (with 0.1, and 4 mM sodium taurodeoxycholate [NaTDC]) at 37°C over a period of 15 min in a final volume of 300 μl containing various amounts of enzyme and 1 mM substrate. Results are expressed as specific activities in international units/mg, corresponding to 1 μmol pNP released per minute and per mg of enzyme. rLipC activity was also investigated by using pH-stat (Metrohm AG, Switzerland), fluorescent, and spectrophotometric assays for various lipids as well as phospholipids to detect lipase or phospholipase activity, respectively.

**Statistical analysis.** Comparisons between the reactivities with sera from PPD⁻ and PPD⁺ healthy controls as well as TB patients were performed by calculating P values with a nonparametric Mann-Whitney test using GraphPad Prism, version 5, software (GraphPad Software, Inc., San Diego, CA). A P value of <0.01 was considered statistically significant.

## RESULTS

**Identification and characteristics of LipC.** The Agt11 clone identified by the serum pool from *M. tuberculosis*-infected rabbits expressed the C-terminal half (aa 231 to 403) of the lipC (Rv0220) gene product (data not shown). Bioinformatics analysis predicts LipC to be a 44-kDa (403-aa) protein with a pI of 10.4. Three amino acids, Ala, Arg, and Val, are overrepresented (12.3, 11.1, and 10.5%, respectively) in the LipC amino acid sequence. SignalP analysis predicted no signal sequence in the LipC amino acid sequence. Bioinformatics analysis revealed the LipC family. BLAST-P identified homologous proteins (100% identity) in the LipC protein sequence. The LipC topology (GXXXG) and the hydrolase fold that is characteristic of lipases/esterases. The amino acid sequence of LipC shows <40% identity with the other 23 members of the Lip family. BLAST-P identified homologous proteins (~100% identity) in *M. tuberculosis* H37Rv, *M. bovis* AF2122/97, *M. bovis* BCG strain Pasteur 1173P2, *M. bovis* BCG strain Tokyo 172, and all *M. tuberculosis*.
M. ulcerans clinical isolates sequenced so far. The protein is absent in M. leprae TN and M. leprae Br4923. Proteins showing 70 to 80% identity are present in the nontuberculous mycobacteria (NTM) M. avium paratuberculosis K-10, M. avium ATCC 2529, M. ulcerans Agy99, M. kansasii ATCC 12478, M. parascrofulaceum ATCC BAA-614, M. intracellulare ATCC 13950, M. marinum, and M. smegmatis mc²155. In M. abscessus, a protein showing only 23% identity was found. No glycosylation sites but 3 potential Arg residues that may be methylated were predicted.

The LipC protein model was generated from 10 templates available in the PDB that are structurally closest to proteins like carboxylesterase from the brefeldin A esterase (PDB accession number 1JKM) or the available in the PDB (from E. coli) probed with anti-His (lane 3), rabbit anti-LipC IgG (lane 4), and normal rabbit IgG (lane 5); lane 6, rLipC purified from M. smegmatis separated on a 10% SDS-PAGE gel and stained with Coomassie blue; lanes 7 to 9, Western blots of rLipC (from M. smegmatis) probed with anti-His (lane 7), rabbit anti-LipC IgG (lane 8), and normal rabbit IgG (lane 9).

**FIG 2** Expression and purification of rLipC. Lane 1, molecular mass markers; lane 2, rLipC purified from E. coli separated on 10% SDS-PAGE gels and stained with Coomassie blue; lanes 3 to 5, Western blots of rLipC (from E. coli) probed with anti-His (lane 3), rabbit anti-LipC IgG (lane 4), and normal rabbit IgG (lane 5); lane 6, rLipC purified from M. smegmatis separated on a 10% SDS-PAGE gel and stained with Coomassie blue; lanes 7 to 9, Western blots of rLipC (from M. smegmatis) probed with anti-His (lane 7), rabbit anti-LipC IgG (lane 8), and normal rabbit IgG (lane 9).

**TABLE 2** Sequencing of rLipC by Q-TOF mass spectrometry

<table>
<thead>
<tr>
<th>LipC peptide fragment mapped</th>
<th>No. of identified peptides</th>
<th>LipC aa positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAGSTGVAYIR</td>
<td>1</td>
<td>6–16</td>
</tr>
<tr>
<td>HASDFSATK</td>
<td>2</td>
<td>60–70</td>
</tr>
<tr>
<td>DLLTPGINEVR</td>
<td>3</td>
<td>71–81</td>
</tr>
<tr>
<td>GIVSPDLAVEWPPAPER</td>
<td>12</td>
<td>98–114</td>
</tr>
<tr>
<td>VLYGDDPAQQLDVWR</td>
<td>22</td>
<td>123–147</td>
</tr>
<tr>
<td>AIOQGYAVLSR</td>
<td>1</td>
<td>173–182</td>
</tr>
<tr>
<td>FVDFLER</td>
<td>2</td>
<td>290–296</td>
</tr>
<tr>
<td>TGPTAHAILFLNQVHR</td>
<td>1</td>
<td>377–393</td>
</tr>
</tbody>
</table>
LipC Is an Immunogenic Cell Surface Esterase

Expression of LipC in vivo. To determine if LipC is expressed during M. tuberculosis infection in humans, the reactivity of rLipC with sera from PPD−, HIV+ TB−, as well as PPD+ healthy subjects and TB patients was examined by Western blotting. Visual examination showed that while sera from non-TB subjects showed background reactivity with the rLipC protein, there was no difference between the reactivities of sera from PPD− and HIV+ TB− subjects and those from the PPD+ subjects. In contrast, sera from most HIV− TB+ and HIV+ TB+ patients showed stronger reactivity (Fig. 4A). To confirm these results, sera from additional randomly selected PPD+ subjects and TB patients were tested, and similar results were obtained (Fig. 4B). When the intensity of the rLipC protein probed with anti-His antibodies was considered 100 units, the relative intensity obtained with individual sera from PPD−, HIV− TB−, and PPD+ subjects ranged from 1 to 14 units, and there was no difference among sera from the 3 subgroups of subjects (P > 0.01). In contrast, reactivity with the 6 HIV− TB+ sera ranged from 7 to 60 units (median reactivity = 28.5 units), and for the 6 HIV+ TB+ sera, it ranged from 33 to 75 units (median reactivity = 62.5 units). The difference in intensities between the non-TB subjects and the TB patients was statistically significant (P < 0.01). Using mean intensities of PPD−, PPD+, and HIV+ TB− sera plus 3 SD as a cutoff, sera from 11/12 TB patients in the first experiment (Fig. 4A and C) and from 8/10 TB patients in the second experiment (Fig. 4B and D) were positive for the presence of anti-LipC antibodies. Thus, sera from ~85% of the TB patients had anti-LipC antibodies. The reactivity of a subset of the control and TB sera was evaluated with rLipC expressed in S. aureus (data not shown).

Epitope mapping of LipC. When the reactivities of the 40 overlapping peptides of LipC were tested with sera from 13 PPD− and 23 PPD+ subjects, there was no difference in the reactivities of sera from these two types of subjects with any of the peptides. In contrast, sera from a vast majority (57/60; 95%) of the HIV− TB+ patients had antibodies against at least one LipC peptide (Fig. 5A); most sera (56/60; 93%) had antibodies to >1 peptide. As expected, there was a wide variation in the recognition of individual peptides with the HIV− TB+ sera, in which different peptides were recognized by 0 to 70% of the patients (Fig. 5A). Twelve peptides were recognized by >50% of the HIV− TB+ sera (52 to 70%). These peptides were retested twice for reactivity with the same sera. For 6 peptides (LipC3, LipC6, LipC24, LipC26, LipC34, and LipC39) (Fig. 5B and Table 3), the reactivity was maintained (52 to 65%). In contrast, the level of reactivity with the remaining 6 peptides was lower during the subsequent assays (32 to 43%). For the 6 peptides that were consistently recognized by >50% of the HIV− TB+ sera, there was no difference between the reactivities with sera from PPD+ and PPD− subjects (P = 0.29 to 0.74), while the level of reactivity of all 6 peptides was significantly higher (P < 0.001) with sera from patients. The combined level of reactivity with the 6 peptides was ~83% for HIV− TB+ patients. These 6 peptides were also reproducibly recognized individually with >50% of the HIV− TB+ sera (Fig. 5C), and the combined level of reactivity with the peptides was ~82%, while the vast majority of HIV− TB+ patients lacked antibodies to any of these peptides (Fig. 5C). Mapping of the 6 immunodominant peptides onto the model of the 3-D structure of LipC showed that 5 peptides (LipC3, LipC6, LipC24, LipC26, and LipC39) were totally exposed to the solvent, while LipC24 was buried in the folded conformation of the protein (Fig. 1).

Cytokine responses to LipC. The cytokine responses elicited from THP-1 and A549 cells by rLipC were also evaluated. The viabilities of both cell types were unaffected by the low concentration of SDS (0.00025% SDS) present in the antigen preparation used to stimulate them (Fig. 6A). THP-1 cells exposed to 1 ng/ml LPS produced ~2,400 pg/ml of IL-12 and ~2,500 pg/ml of TNF-α. The preincubation of LPS with 10 ng/ml of polymyxin B completely abrogated the production of both cytokines (Fig. 6B). Cells exposed to 1 µg/ml rLipC expressed ~2,800 pg/ml and ~2,700 pg/ml IL-12 and TNF-α, respectively. The preincubation of rLipC with polymyxin B had no effect on cytokine production, confirming that the observed cytokine responses were not due to a contaminating endotoxin (Fig. 6B).

THP-1 cells exposed to different concentrations of rLipC showed a dose-dependent production of IL-12, TNF-α, IL-8, and MCP-1, and in most cases, the peak response was attained at 0.1 µg/ml (Fig. 6C). MCP-1 was the most highly induced cytokine (~7,000 pg/ml), followed by IL-8 (~3,500 pg/ml), IL-12 (~3,000 pg/ml), and TNF-α (~2,500 pg/ml) (Fig. 6C). Minimal or no

FIG 3 Localization of LipC protein. (A) M. tuberculosis H37Rv subcellular protein fractions (5 µg/lane) and LipC (75 ng/lane) were separated on 10% SDS-PAGE gels and transferred onto a nitrocellulose membrane. The blots were probed with anti-maltose synthase (MS) IgG (top), anti-LipC IgG (middle), and normal rabbit IgG (bottom). Lanes: 1, rLipC; 2, cytoplasm; 3, total cell wall; 4, SDS-extracted cell wall; 5, capsule; 6, culture filtrate. (B) Immunoelectron microscopy of ultrathin sections of M. tuberculosis H37Rv (a and b), the M. tuberculosis CDC1551 lipC::Tn mutant (c), and M. tuberculosis CDC1551 (d). The bacterial sections were probed with either rabbit normal IgG (a) or rabbit anti-LipC IgG (b, c, and d).
cytokine levels (<400 pg/ml) were detected in supernatants from unstimulated cells (Fig. 6C).

A549 cells stimulated with rLipC expressed IL-8 and MCP-1 in a dose-dependent manner, and as was observed with THP-1 cells, the peak level of MCP-1 induction was higher (~5,500 pg/ml) than the peak level of induction of IL-8 (~1,600 pg/ml) (Fig. 6D).

**Enzymatic characterization of LipC.** To evaluate the substrate specificity of LipC, the enzymatic activity of proteins expressed and purified from *M. smegmatis* against p-nitrophenyl esters was investigated by using substrates with acyl chain lengths ranging from C₂ to C₁₆ in presence or absence of NaTDC (1 or 4 mM) (Fig. 7). The presence of NaTDC enhanced slightly the specific activity for substrates ranging from C₂ to C₁₀ compared to the buffer alone. The activity of rLipC increased with the chain length of the substrate, reaching maximum specific activities of 160 mU/mg (Kₘ = 2.89 mM; Vₘₐₓ = 0.48 μM/min) and 110 mU/mg (Kₘ = 2 mM; Vₘ = 0.69 μM/min), with and without NaTDC, respectively.

No activity was detected for substrates with carbon chain lengths greater than C₁₀ under any of the conditions used. Using the pH-stat method (12), fluorescent spectrometric assays (R. Verger, C. Serveau-Avesque, and H. Chahinian, 17 August 2006, PCT patent application WO2006/85009), and radioactive assays (37), no activity was detected on mono-, di-, or triacylglycerol whatever the carbon chain length, indicating that this enzyme is a strict esterase and does not have any lipase activity (data not shown). Also, all attempts to detect phospholipase activity failed (data not shown).

**DISCUSSION**

The sequencing of the *M. tuberculosis* genome revealed that *M. tuberculosis* possesses ~150 genes that code for enzymes involved in fatty acid degradation (6), suggesting that *M. tuberculosis* utilizes host lipids during growth and replication in vivo. The analysis of the genes by bioinformatics methods identified a family of 24 putative carboxyl hydrolases with the characteristic GXSXG motif. Twenty of the Lip family members are predicted to be present in the cytoplasm of the bacterium, and four of these family members are predicted to be present in the periplasmic space; LipC is predicted to be a cytoplasmic enzyme (40). However, the experimental approaches used in this study clearly demonstrate that native LipC is present primarily in the cell wall and the capsule of *M. tuberculosis*. This is reminiscent of the findings with 2 other Lip family members, LipF and LipY, both of which are also associated with the cell wall (28, 45). In confirmation of this localization in vivo, like LipF and LipY, LipC also participates in immune responses by eliciting antibodies. It is interesting that anti-LipC antibodies raised by immunization with rLipC (~45 kDa) recognize the appropriate protein band as well as an additional ~47-kDa protein in the native cell wall subcellular fractions but only the ~47-kDa band in the capsular fraction. This finding suggests that the native LipC present on the bacterial surface in the capsule may have posttranslational modifications, while the cytoplasm and cell wall have both the modified and the nonmodified LipC proteins. The nature of these posttranslational modifications remains to be determined.

Previous studies suggested that there may be interspecies differences in the profiles of antigens recognized by antibodies (17, 18). Since LipC was originally identified by antibodies in sera from *M. tuberculosis*-infected rabbits, it was important to confirm its expression during TB in humans. Thus, sera from subjects with latent or active *M. tuberculosis* infection were evaluated for the presence of anti-LipC antibodies. Previous studies also reported differential expressions of some *M. tuberculosis* antigens in HIV⁻ TB⁻ and HIV⁺ TB⁺ patients (34, 36). For this reason, sera from both HIV⁻ TB⁻ and HIV⁺ TB⁺ patients were included. Sera from healthy subjects with no evidence of *M. tuberculosis* infection...
(PPD−) as well as HIV+ TB− subjects (from the United States) were included as negative controls. Antibodies to LipC were present in sera from 90% of the TB patients tested, whether or not coinfected with HIV, indicating the in vivo expression of the proteins during active infection in both types of patients. In contrast, sera from PPD+ subjects showed reactivity similar to that of sera from PPD− subjects, demonstrating the absence of an in vivo expression of LipC during latent TB.

We have demonstrated that immunodominant epitopes of antigenic proteins can replace and improve upon the detection of antibodies to the parent protein (39, 43). Antibodies to at least one epitope of LipC were present in 95% of the HIV+ TB− patients and absent in the vast majority of PPD− subjects, confirming the results of the Western blot studies. Moreover, reactivity with just the 6 immunodominant peptides was sufficient to identify a vast majority (80%) of the HIV+ TB− patients. This level of reactivity is similar to that observed with immunodominant peptides of PTRP, which is also a cell wall protein that was identified by antibodies in rabbit sera obtained at 5 weeks postinfection and whose immunodominant epitopes were mapped with sera from the same cohorts as those that were used in this study (43). Moreover, as was observed for PTRP and its immunodominant peptides, there was no difference in the recognition of LipC and its peptides between HIV− TB− and HIV+ TB+ patients. Our earlier studies with culture filtrate proteins (CFP) of M. tuberculosis showed that some antigens (malate synthase and MPT51, for example) are well recognized by antibodies from both cavitary and noncavitary TB patients, demonstrating that these antigens are expressed in vivo in both types of patients (34, 53). In contrast, other antigens (38-kDa protein and MPT32, for example) appear to be expressed primarily during cavitary TB, since noncavitary TB patients lack antibodies to them (34). The similar immunogenicities of LipC and its peptides in sera from smear-positive HIV− TB− patients, most of

\[
\begin{array}{|c|c|}
\hline
\text{Peptide} & \text{Sequence} \\
\hline
\text{LipC3} & \text{ARPADYMLALSVAGGSLPVVA}^a \\
\text{LipC6} & \text{TAIGVWGBASHFDLSATAK}^b \\
\text{LipC24} & \text{IAVAGCSAGHLSALAGLTA}^c \\
\text{LipC26} & \text{NDPQYQAELEPSDTSVDA}^d \\
\text{LipC34} & \text{GRSDCVPEVQARFVERL}^e \\
\text{LipC39} & \text{AHAIALFLNQVHRSAQFAK}^f \\
\hline
\end{array}
\]

\(^a\) Shown in red in Fig. 1.
\(^b\) Shown in orange in Fig. 1.
\(^c\) Shown in blue in Fig. 1.
\(^d\) Shown in green in Fig. 1.
\(^e\) Shown in yellow in Fig. 1.
\(^f\) Shown in magenta in Fig. 1.
Cytokine production by THP1 and A549 cells in response to rLipC. (A) Viability of A549 and THP-1 cells at 24 h with medium alone, medium containing 1 μg/ml rLipC, and medium containing 0.00025% SDS. The percent viability calculated by considering cells exposed to medium alone as 100% is plotted. (B) Production of IL-12 and TNF-α by THP-1 cells exposed to LPS alone (1 ng/ml), LPS (1 ng/ml) preincubated with polymyxin B (10 ng/ml), rLipC (1 μg/ml), and rLipC (1 μg/ml) preincubated with polymyxin B (10 ng/ml). (C) Induction of IL-12, TNF-α, IL-8, and MCP-1 in THP-1 cells after incubation with various concentrations of rLipC and 1 μg/ml LPS. (D) Production of IL-8 and MCP-1 by A549 cells exposed to different concentrations of rLipC and 1 μg/ml LPS.
The enzymatic activity of rLipC was measured by using p-nitrophenyl esters with carbon chain lengths ranging from C$_2$ to C$_{16}$ as substrates using various concentrations of NaTDC. The means ± SD of specific activities (mU/mg) obtained with 3 independent replicates are plotted.

**FIG 7** Enzymatic activity of rLipC. The enzymatic activity of rLipC was measured by using p-nitrophenyl esters with carbon chain lengths ranging from C$_2$ to C$_{16}$ as substrates using various concentrations of NaTDC. The means ± SD of specific activities (mU/mg) obtained with 3 independent replicates are plotted.

LipC is an Immunogenic Cell Surface Esterase

M. tuberculosis antigens have been delineated (11, 34, 36, 39, 43, 44). Since combinations of multiple antigens provide an increased sensitivity of antibody detection (11, 15, 17, 42, 53), it is likely that combinations of immunodominant epitopes from multiple highly immunogenic proteins of *M. tuberculosis* will enable the development of a peptide-based rapid test for TB. One or more of the six epitopes of LipC defined in these studies could make significant contributions to such a diagnostic test.

The use of peptides is economically advantageous over recombinant purified proteins, which are expensive to produce and difficult to purify and pose considerable challenges for maintaining batch-to-batch consistency and long-term stability. Moreover, the use of peptides eliminates the nonimmunogenic and sometimes cross-reactive portions of the antigens. The use of biotin-conjugated peptides enables the equal binding of individual peptides to streptavidin-coated plates, thus eliminating differences between their binding capacities when being tested by ELISA. Despite equal binding, some peptides that are recognized by antibodies in a high proportion of TB patients during the first screen performed poorly when retested. This was also observed when epitope mapping of other immunogenic proteins was performed (39, 43). In some cases, solubilized peptides were seen to gradually aggregate and precipitate during storage, suggesting that they undergo conformational changes in solution; for other peptides, it was unclear why the reactivity decreased during retesting. It is for this reason that the reactivity of each peptide needs to be evaluated multiple times so that only stable and highly reactive peptides are finally selected for test development.

Antibodies in sera from a few (<10%) of the HIV$^+$ TB$^-$ subjects showed reactivity with the 6 LipC peptides. Since these HIV$^+$ TB$^-$ patients were from the United States and were asymptomatic and on antiretroviral therapy (ART), it is unlikely that they were infected with any nontuberculous mycobacteria (NTM). The cross-reactivity observed with some of these sera is likely due to the hypergammaglobulinemia caused by HIV infection and may be eliminated during the optimization of conditions during the development of the rapid test.

As expected from B cell epitopes, 5 of the 6 immunodominant peptides mapped to the exposed surface of the modeled protein (Fig. 1). The presence of antibodies to LipC24, which is not surface exposed, indicates that in vivo, the shedding of the bacterial capsule may result in the release of LipC in a somewhat unfolded conformation, providing the B cells with access to this region.

While macrophages are well known to be the host cells for *M. tuberculosis*, there is increasing evidence for important roles for alveolar epithelial cells in TB (2, 19, 20, 23, 52). We therefore evaluated the ability of the purified LipC protein to elicit cytokines from both THP-1 and A549 cells. While MCP-1 was the most highly induced cytokine, IL-8 was also expressed by the two cell types (Fig. 6C and D). Both MCP-1 and IL-8 are involved in the recruitment of macrophages, neutrophils, dendritic cells, and T cells and play important roles in the formation of granulomas (51). Importantly, IL-8 and MCP-1 were also demonstrated to be present in the bronchoalveolar lavage fluid of TB patients (24). LipC also induced the expression of TNF-$\alpha$ and IL-12 from THP-1 cells. TNF-$\alpha$ is known to play a key role in granuloma formation and induce macrophage activation and has immunoregulatory properties (51). IL-12 is known to play a crucial role in the induction of gamma interferon (IFN-$\gamma$), which activates macrophages to kill the bacteria (7). The *in vivo* expression of LipC in rabbits at 4 to 5 weeks postaerosol infection, when granuloma formation occurs, and the ability of LipC to stimulate proinflammatory cytokine and chemokine production suggest that the precise role of LipC in granuloma formation upon aerosol infection with *M. tuberculosis* warrants investigation in animal models. In this regard, previous studies showed that another carboxylesterase (Rv2224c), which was also anchored to the cell wall, modulates innate immune responses to *M. tuberculosis* in mice (25).

Previous studies have shown that *M. tuberculosis* stores energy in the form of triacylglycerol (TAG) as it goes into the latent state, which is utilized for survival during dormancy and reactivation when bacterial replication is resumed (9). TAG is also the common form of stored energy in hibernating animals and oil seeds when the level of metabolic activity is low (30). TAG is hydrolyzed via the glyoxylate cycle, which has been shown to be used by *M. tuberculosis* during intracellular survival (29). Interestingly, in *M. tuberculosis*, malate synthase, which participates in the glyoxylate cycle, is present not only in the cytoplasm but also on the cell wall and in the capsule (19), and antibodies to malate synthase are strongly associated with clinical TB but not latent TB, suggesting...
enhanced expression when bacteria are replicating actively in vivo. Previous studies have also demonstrated that LipY hydrolyzes TAG (10), and LipF hydrolyzes both short-chain esters and phosphatidycholine, which is the major constituent of biological membranes (45, 60). Both LipY and LipF also localize to the bacterial cell wall and elicit antibodies in patients with active TB (8, 10, 28, 45, 60). Together, these data suggest that LipY, LipF, and LipC may be used in tandem by Mycobacterium tuberculosis for the utilization of host lipid substrates during active replication and growth in vivo.

There is increasing evidence that bacterial cell wall/surface-localized enzymes also multitask as antigenic proteins that play roles in the modulation of immune responses and/or in the invasion of host cells (4, 19, 32, 38, 49). For example, besides LipC, LipY, LipF, malate synthase, MPT51, Ag85B, Ag85C, and Rv2224c are all cell wall/cell surface-localized enzymes of Mycobacterium tuberculosis that elicit humoral, cellular, and/or inflammatory immune responses (10, 19, 21, 22, 25, 53, 57). Ag85B, malate synthase, and MPT51 were also reported previously to be adhesins of Mycobacterium tuberculosis that bind to extracellular matrix proteins like fibronectin and laminin (14, 19, 21, 57). Interestingly, the GehD lipase of Staphylococcus epidermidis is a collagen-binding protein (4); the GbpD protein of Streptococcus mutans has both lipase and glucan-binding activities (38), and an extracellular lipase of Pseudomonas aeruginosa participates in the release of inflammatory mediators from granulocytes and monocytes (32). The current studies demonstrate that LipC is a cell surface-associated esterase of Mycobacterium tuberculosis that is highly immunogenic and elicits both antibodies and cytokines/chemokines. We have identified immunodominant regions of LipC, which may be important constituents of a peptide-based serodiagnostic test for TB. The role of LipC in infection and disease progression in TB merits further investigation.

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