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Functional Role of the PE Domain and Immunogenicity of the *Mycobacterium tuberculosis* Triacylglycerol Hydrolase LipY▽

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PE and PPE proteins appear to be important for virulence and immunopathogenicity in mycobacteria, yet the functions of the PE/PPE domains remain an enigma. To decipher the role of these domains, we have characterized the triacylglycerol (TAG) hydrolase LipY from *Mycobacterium tuberculosis*, which is the only known PE protein expressing an enzymatic activity. The overproduction of LipY in mycobacteria resulted in a significant reduction in the pool of TAGs, consistent with the lipase activity of this enzyme. Unexpectedly, this reduction was more pronounced in mycobacteria overexpressing LipY lacking the PE domain [LipY(ΔPE)], suggesting that the PE domain participates in the modulation of LipY activity. Interestingly, *Mycobacterium marinum* contains a protein homologous to LipY, termed $LipY_{mar}$, in which the PE domain is substituted by a PPE domain. As for LipY, overexpression of $LipY_{mar}$ in *Mycobacterium smegmatis* significantly reduced the TAG pool, and this was further pronounced when the PPE domain of $LipY_{mar}$ was removed. Fractionation studies and Western blot analysis demonstrated that both LipY and LipY(ΔPE) were mainly present in the cell wall, indicating that the PE domain was not required for translocation to this site. Furthermore, electron microscopy immunolabeling of LipY(ΔPE) clearly showed a cell surface localization, thereby suggesting that the lipase may interact with the host immune system. Accordingly, a strong humoral response against LipY and LipY(ΔPE) was observed in tuberculosis patients. Together, our results suggest for the first time that both PE and PPE domains can share similar functional roles and that LipY represents a novel immunodominant antigen.

The determination of the complete genome sequence of *Mycobacterium tuberculosis* has provided crucial information with respect to the physiology of this bacterium and the pathogenesis of tuberculosis (TB) (16). One important outcome of the genome-sequencing project was the discovery of two new multigene families designated PE and PPE. About 10% of the *M. tuberculosis* coding capacity (167 genes) is devoted to the PE and PPE genes, named for the Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs near the N terminus of their gene products (16). In addition to these motifs, proteins of the PE family share N-terminal domains of approximately 100 amino acids, whereas the PPE proteins possess an N-terminal domain of about 180 amino acids. Many PE and PPE proteins are composed only of these homologous domains. However, other members possess an additional C-terminal segment of variable length, often composed of multiple copies of polymorphic GC-rich sequences (PGRS), which led to the hypothesis that these proteins have a structural role (9, 16). So far, no PE and PPE genes have been identified in nonmycobacterial species. Because the PE and PPE gene families encode numerous proteins in certain pathogenic mycobacterial species, including *M. tuberculosis* and *M. marinum*, it is tempting to assume that they fulfill important functions related to survival within different environmental niches.

Some of the PE proteins may play a role in immune evasion and antigenic variation (4, 9, 16, 20) or may be linked to virulence (15, 27, 34). PPE proteins are also known to induce a strong B-cell response in humans (13, 15, 44). Some of the members of the PE and PPE families have been found to associate with the cell wall (10, 21, 26, 30, 37). The PPE41-encoding gene, Rv2430c, together with the PE25-encoding gene, Rv2431c, have recently been found to be an operon (42). The structure of the heterodimeric complex formed by PPE41 and PE25 has been determined, and it was shown that the PE and PPE proteins mate along an extended apolar interface to form a four-α-helix bundle, where two of the α-helices are contributed by the PE protein and two by the PPE protein (41). These authors also proposed that some of the PE-PPE complexes may be involved in signal transduction either as mem-

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brane-associated or soluble proteins, although this remains to be clearly addressed. In general, although the PE and PPE families of mycobacterial proteins are the focus of intense research, no precise function has been unraveled for any member of these families. Interpretation of the importance of the PE and PPE domains is complicated by the paucity of potentially functional data on PE/PPE members.

However, one PE protein, termed PE30 (11) and encoded by Rv3097c, contains a carboxy-terminal polypeptide which expresses triacylglycerol (TAG) hydrolase activity (19). The C-terminal portion of PE30 (also designated LipY) is homologous to the hormone-sensitive lipase family characterized by the conserved GDSAG active-site motif (19). Interestingly, among the 24 *M. tuberculosis* putative lipases/esterases, LipY induced the highest breakdown of stored TAGs under conditions of starvation. In addition, TAG utilization was drastically decreased under nutrient-deprived conditions in a *lipY*-deficient mutant of *M. tuberculosis*. It was therefore proposed that LipY may be responsible for the utilization of stored TAG during dormancy and reactivation of the pathogen (19). Therefore, LipY is interesting not only because it is the only PE protein with a characterized enzymatic activity but also because it appears to be a key enzyme participating in mycobacterial dormancy.

We reasoned that because TAG hydrolysis can be easily monitored in vivo, *lipY* could be used as a "reporter" gene and would represent a powerful tool to scrutinize the contribution of the PE domain to the lipase activity. Herein, we investigated the role and effect of the PE domain on LipY activity in vivo and analyzed the subcellular localization of the protein in *Mycobacterium bovis* BCG. Finally, we analyzed the humoral response to LipY in *M. tuberculosis*-infected patients.

MATERIALS AND METHODS

Strains and media. *M. smegmatis* mc^{2}155 and *M. bovis* BCG 1173P2 cultures were usually grown at 37°C under constant shaking in Sauton's medium, in the presence of either 50 μg/ml hygromycin or 25 μg/ml kanamycin when required. Transformants were selected on Middlebrook 7H11 solid media supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco) and containing either 50 μg/ml hygromycin or 25 μg/ml kanamycin when necessary. Plates were incubated at 37°C for 3 to 4 days for *M. smegmatis* and for about 15 days for *M. bovis* BCG.

Plasmids and DNA manipulation. The *Escherichia coli* mycobacterial shuttle vector pMV261 containing the *hsp60* promoter was used as described previously (40). The acetamide-inducible vector pSD26 (18) was used as reported earlier (6). Restriction enzymes, T4 DNA ligase, and Vent DNA polymerase were purchased from New England Biolabs. All DNA manipulations were performed using standard protocols. For cloning of *lipY* and *lipY*(Δ*PE*) in pMV261, the *lipY* and *lipY*(Δ*PE*) genes were amplified by PCR using *M. tuberculosis* H37Rv genomic DNA and the following primers: upstream primers *lipY*-up 5′-TGT CTT ATG TTG TTG CG-3′ and *lipY*(Δ*PE*)-up 5′-TCG CCA GCG GTA TCG GGA ACG G-3′ and the downstream primer *lipY-lo* 5′-AAG GAT CCT CAG GCG GCG ATA CCG AGT TGC TG-3′ (contains a BamHI site [underlined]). Both PCR products were cut with BamHI and directly ligated into pMV261 restricted with MscI/BamHI. Cloning of *lipY* and *lipY*(Δ*PE*) into pSD26 was performed by amplifying the genes using the following primers: upstream primers *pSDlipY*-up 5′-AAA GGA TCC GTG TCT TAT GTT GTT GCG TTG-3′ and *pSDlipY*(Δ*PE*)-up 5′-AAA GGA TCC TTC GCC AGC GGT ATC GGG AAC GG-3′, respectively, and the downstream primer *pSDlipY*-lo 5′-AAG GAT CCG GCG GCG ATA CCG AGT TGC TG-3′ (all three primers contain BamHI sites [underlined]). Both PCR products were cut with BamHI and directly ligated in frame into pSD26 restricted with BamHI. A similar approach was used to clone *M. marinum lipY* (*lipY*$_{mar}$) and *lipY*(Δ*PPE*)$_{mar}$ in pSD26 by using *M. marinum* M strain genomic DNA and the following primers: upstream primers *pSDmarlipY*-up 5′-AAG GAT CCA GTT TCT GGA TGT GGC CGC CC-3′ and *pSDmarlipY*(Δ*PPE*)-up 5′-AAG GAT CCT TCG CCC GAG CCC TGG ACA ACG GTC-3′, respectively, and the downstream primer *pSDmarlipY*-lo 5′-AAG GAT CCG GCC GCG ATG CCG AGT TCC TGG TTG ATC-3′.

Site-directed mutagenesis of the S309 residue. A pMV261::*lipY*(Δ*PE*) derivative containing the S309A point mutation within the *lipY*(Δ*PE*) gene was generated by site-directed mutagenesis. pMV261::*lipY*(Δ*PE*) was used as a template for the QuikChange II site-directed mutagenesis kit (Stratagene) with the following primers: S309A-up 5′-GCG TGG TCG GGG AC**G** CCG CGG GCG GCA ACC-3′ and S309A-lo 5′-GGT TGC CGC CCG CGG **C**GT CCC CGA CCA CGC-3′ (the nucleotide changes that allow specific amino acid replacement are indicated in bold). Mutant clones were verified by automated DNA sequencing.

Subcellular fractionation of mycobacteria. Cells were disrupted using a French pressure cell and subcellular fractionation was performed essentially as described previously (31). Briefly, cell lysates were centrifuged twice at 1,000 × *g* to remove unbroken cells and insoluble material. Lysate supernatants were then centrifuged at 20,000 × *g* at 4°C to separate the soluble cytoplasmic fraction from the cell wall. The cell wall fraction was washed three times with phosphate-buffered saline (PBS) in order to remove cytoplasmic contaminants. Both fractions were resuspended in identical volumes of buffer.

Gel overlay assay. Tributyrin agar plates were obtained by dissolving tributyrin agar (Fluka) in distilled water at a final concentration of 20 g/liter. After sterilizing at 121°C for 15 min, 10 g/liter of tributyrin (Sigma) was added to the hot agar and the medium was sonicated in a water bath sonicator for a few seconds and stirred with a magnetic stirrer to obtain a homogenous emulsion before being poured into plates. Recombinant transformants expressing lipase activity were identified by their ability to form clear zones in an otherwise turbid and yellowish medium. Lipase activity was also directly determined on crude mycobacterial lysates and/or cell wall preparations. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. After electrophoresis, the gels were washed twice for 15 min in 25% isopropanol and then twice for 5 min in 20 mM Tris-HCl, pH 8.0. The gels were then laid on top of a tributyrin agar plate and incubated overnight at room temperature (RT). A protein with lipolytic activity generates a clear halo on plates.

In vitro lipase assay. Lipolytic activity was assayed using cell wall preparations from *M. smegmatis* strains carrying the various lipase-expressing derivatives following induction with acetamide. Cell wall fractions from *M. smegmatis* harboring empty pSD26 was used as an internal control, so that the activity observed can be attributed only to the overexpressed lipases. *para*-Nitrophenyl stearate (Sigma) was used as the substrate, as it is specifically hydrolyzed by lipases and not by carboxyl esterases, which can hydrolyze substrates with short-chain acyl groups only (45). Assays were performed in 96-well plates (in a 100-μl reaction mixture) containing increasing cell wall concentrations and a final concentration of 0.5 mM *p*-nitrophenylstearate (by diluting a 20 mM stock solution in acetonitrile with 100 mM Tris-HCl, pH 8.0). The mixture was incubated at 35°C, and the release of *p*-nitrophenol was measured spectrophotometrically at 405 nm after 40 min of reaction. The lipase activity was expressed as the difference between absorbance at 40 min and that at 0 min. Reactions were done in triplicate.

Protein purification. Recombinant LipY and LipY(ΔPE) were extracted from cell wall preparations of acetamide-induced *M. smegmatis* cultures harboring either pSD26::*lipY* or pSD26::*lipY*(Δ*PE*). The proteins were purified using Ni-nitrilotriacetic beads under denaturing conditions by solubilizing the cell wall preparations in 8 M urea. Elution fractions were then further subjected to preparative SDS-PAGE, and the bands corresponding to LipY or LipY(ΔPE) were recovered from the gel by electroelution. Recombinant His-tagged Rv1818c protein was expressed in *E. coli* harboring pET15b-Rv1818c (20), kindly provided by M. J. Brennan, and purified by nickel chromatography as reported previously (20). The protein concentration was determined using the bicinchoninic acid protein assay reagent kit (Pierce).

Preparation of anti-LipY(ΔPE) antiserum. Purified recombinant LipY(ΔPE) produced in *M. smegmatis* was used to prepare anti-LipY(ΔPE) immune sera. Rats were injected four times with 25 μg of antigen mixed with an equal volume of adjuvant (monophosphoryl lipid A and trehalose dicorynomycolate; Sigma) at days 1, 15, 30, and 60. Sera were collected at various time points after the last injection, and the specificity of the antibodies was tested by Western blotting.

Electrophoresis and immunoblotting. Mycobacterial cultures (10 ml) were harvested, and the cells were resuspended in 0.8 ml of PBS and disrupted using a bead beater (MM301; Retsch). Protein concentrations in crude cell lysates were determined using the bicinchoninic acid protein assay reagent kit (Pierce). Equal amounts of protein (10 or 20 μg) were then separated by 12% SDS-PAGE and transferred onto a Protran nitrocellulose transfer membrane (Schleicher &

Schuell). The membranes were then saturated with 1% bovine serum albumin (BSA) in PBS, 0.1% Tween 20 and probed by incubation overnight with either rat antiserum raised against LipY(ΔPE) at a 1/2,500 dilution or rabbit anti-OmpATb antiserum raised against the *M. tuberculosis* protein (38) at a 1/25,000 dilution. After extensive washing, the membranes were then incubated with anti-rat or anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase, respectively (dilution, 1/7,000; Promega). Monoclonal antibodies against *M. tuberculosis* KatG (IgG1; clone IT-57) were also used at a 1/1,000 dilution and served as a marker for the detection of cytoplasmic contamination in the cell wall fraction (35).

Labeling experiments and analysis of apolar lipids. Mycobacterial cultures were grown in Sauton's medium and labeled with 1 μCi/ml [1, 2-^{14}C]acetate (50 to 62 mCi/mmol; Amersham) for 4 h (*M. smegmatis*) or 24 h (*M. bovis* BCG). ^{14}C-labeled apolar lipids were then extracted by adding 2 ml of CH_3OH:0.3% NaCl (100:10) and 2 ml petroleum ether to the cell pellet. After centrifugation, the upper petroleum ether layer was removed, and 2 ml of petroleum ether was added. The combined petroleum ether extracts were then evaporated under nitrogen to yield apolar lipids that were resuspended in CH_2Cl_2 prior to autoradiography–thin-layer chromatography (TLC) analysis. Total apolar lipids were analyzed by two-dimensional (2D) TLC using silica gel plates (5735 silica gel $60F_{254}$; Merck, Darmstadt, Germany) as reported earlier (5) and as follows: first dimension, petroleum ether-ethyl acetate (98:2; ×3); second dimension, petroleum ether-acetone (98:2). To visualize free fatty acids, plates were resolved using chloroform-methanol (96:4) in the first dimension and toluene-acetone (80:20) in the second dimension. TLC plates were exposed overnight to Kodak X-Omat film.

Electron microscopy (EM) immunolabeling of LipY(ΔPE). *M. bovis* BCG strains were grown at 37°C without any shaking. The immunolocalization of LipY was performed on thin sections of cryosubstituted bacteria. In this case, bacteria were fixed for 1 h at RT with a mixture of 2.5% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate (Sorensen) buffer, rapidly washed with buffer, and postfixed at 4°C with 1% paraformaldehyde in the same buffer. After two washes with 50 mM NH_4Cl-containing buffer and three washes with NH_4Cl-free Sorensen buffer, bacteria were concentrated in 2% agarose in the same buffer. Samples were then incubated in successive baths of 10%, 20%, and 30% sucrose for 1 h each at 4°C and then overnight at 4°C in 2.3 M sucrose. Samples were then frozen in ethane by the rapid plunging method and processed for cryosubstitution and embedding in Lowicryl HM20 as described previously (29). All immunolabeling steps were carried out at RT. Thin sections of even thickness (70 nm) were collected on nickel grids and sequentially incubated on drops of (i) PBS containing 5% BSA and 0.1% Tween 20 for 15 min, (ii) rat anti-LipY(ΔPE) antibody for 2 h, (iii) rabbit anti-rat IgG (Dako) for 1 h, and (iv) protein A coupled to gold particles of 5 nm in diameter (PAO) (University of Utrecht, Utrecht, The Netherlands) for 30 min. Antibodies and PAO were diluted in PBS containing 5% BSA and 0.1% Tween 20. In between the labelings and after exposure to PAO, bacterial sections were washed with PBS containing 0.5% BSA and 0.1% Tween 20. Sections were then washed with PBS only followed by distilled water. Sections were then stained with uranyl acetate and lead citrate prior to observation under the EM. The number of gold particles within the cytoplasm or associated with the bacterial surface was determined for 50 (five groups of 10) different longitudinal bacterial profiles observed at random. Results are expressed as means ± standard errors of the means.

For a more precise surface localization of LipY, immunolabelings were performed on whole bacteria either deposited onto EM Formvar-coated nickel grids or kept in suspension. In the latter case, bacteria were either unfixed or fixed prior to immunolabeling. The fixative consisted of 5% paraformaldehyde, EM grade (Electron Microscopy Science), and 0.25% glutaraldehyde, EM grade (Sigma), prepared in 0.1 M Na-cacodylate buffer, pH 7.2, containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. One volume of this fixative was added to an equal volume of the bacterial suspension, and bacteria were fixed for 30 min at RT. All immunolabeling steps were carried out at RT as described above. In the case of bacteria on grids, the grids were sequentially deposited onto drops of antibodies or washing medium. Bacteria kept in suspension were spun down at 10,000 rpm for 5 min and resuspended in the successive antibodies, in PAO, or in washing medium. As a control, bacteria were exposed to (i) preimmune rat serum instead of anti-LipY(ΔPE), (ii) rabbit anti-rat IgG and PAO only, or (iii) PAO only. Antibodies and PAO were diluted in PBS containing 5% FBS. In between the labelings and after exposure to PAO, bacteria were washed with PBS containing 0.5% FBS and then with PBS only. Bacteria on grids were then rinsed with doubly distilled water, dried for 2 h at RT, and observed immediately under the EM. Bacteria labeled in suspension were processed for conventional EM as described below.

Processing for conventional EM. Bacteria were fixed for 2 h at RT with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ and then washed twice with the same buffer and postfixed overnight at RT with 1% osmium tetroxide in the same buffer. Following concentration in 1% agarose in the same buffer and treatment for 1 h at RT with 1% uranyl acetate in Veronal buffer, bacteria were dehydrated in a graded series of ethanol and embedded in Spurr resin. Thin sections were stained with uranyl acetate and lead citrate. For quantifications, 200 to 600 different bacteria or bacterial profiles were examined to determine the percentage of positively labeled bacteria.

Patients and control subjects. The study population (n = 211) was comprised of TB-infected patients reporting to the National JALMA Institute of Leprosy and Other Mycobacterial Diseases in Agra, India. The patient population was categorized into different clinical groups as follows. (i) Group 1 (n = 94) consisted of patients diagnosed with pulmonary TB for the first time and no history of chemotherapeutic intervention. Group 1 had 69 adult and 25 child patients. (ii) Group 2 (n = 30) consisted of patients with relapsed infection, and all recruited patients were adults. After diagnosis, these patients had a full course of antitubercular chemotherapeutic treatment but had a recurrence of the infection and disease symptoms after the therapy. Pulmonary TB in groups 1 and 2 was confirmed by the presence of acid-fast bacilli in at least two initial sputum smear examinations and the growth of bacilli in BACTEC cultures. (iii) Group 3 (n = 31) consisted of 9 adult and 22 child patients with extrapulmonary TB infections. Members from group 3 were primarily abdominal TB and tubercular meningitis patients. The diagnosis of extrapulmonary TB was carried out by histological examination as well as with bacillus culture positivity in specimens obtained from extrapulmonary sites. The patients were categorized as per the guidelines of National TB Control Program, Central TB division, Government of India. The age ranges for adult and child patients were from 18 to 60 years and from 2 to 15 years, respectively. Sera were separated from blood and kept at −20°C until used. The samples were obtained from all donors upon entry into the study, and samples from human immunodeficiency virus-positive subjects were excluded from the study. The healthy donors (n = 56) included in the study were recruited after radiological and clinical examination to exclude individuals with active TB. Additionally, healthy donors included in the study were age and gender matched to the different clinical groups. The included subjects had given written consent, and the current study was carried out after approval from the institutional bioethics committee.

Serological characterization of LipY, LipY(ΔPE), and Rv1818c. Enzyme-linked immunosorbent assays (ELISAs) were carried out with 96-well microtiter plates (Nunc) by use of LipY and LipY(ΔPE) proteins purified from recombinant *M. smegmatis* strains and Rv1818c purified from recombinant *E. coli*. The plates were incubated with proteins (1.25 μg/ml) overnight at 4°C, followed by three washes with PBS-Tween 20 (0.05%) buffer. After blocking with 3% BSA in PBS, wells were incubated with human sera (1:400 dilution in blocking buffer) for 1 h at 37°C prior to washing with PBS-Tween 20. The serum dilutions were selected following preliminary titration assays. Plates were then incubated with anti-human antibodies conjugated to horseradish peroxidase (HRP) and developed with *O*-phenylenediamine tetrahydrochloride. The absorbance values were measured at 492 nm in an ELISA reader.

Statistical analysis. Student's t test was used for the analysis of statistical significance (P values). The data from serological reactivities of different categories of patients were compared with the data from healthy controls. GraphPad Quickcalcs (online t test calculator [http://www.graphpad.com/quickcalcs/ttest1.cfm]) was used for this purpose. Only P values of <0.05 were considered significant.

RESULTS

Overexpression of LipY or LipY(ΔPE) in *M. smegmatis* decreases the TAG pool. TAGs are major lipid storage molecules. TAG accumulation is uncommon in prokaryotes but has been reported for different bacteria belonging to the actinomycetes genera (3), including *Mycobacterium* (22). We have recently shown that TAGs increased significantly during growth, especially during the late log phase of growth of *M. kansasii* (25). TAGs are also known to accumulate in *M. tuberculosis* cells entering the nonreplicative state (17). These results suggest that the production of TAGs is growth state dependent and that the accumulation of these lipids in intracellular lipid inclusions (22) or within the cell wall (32) may be advantageous

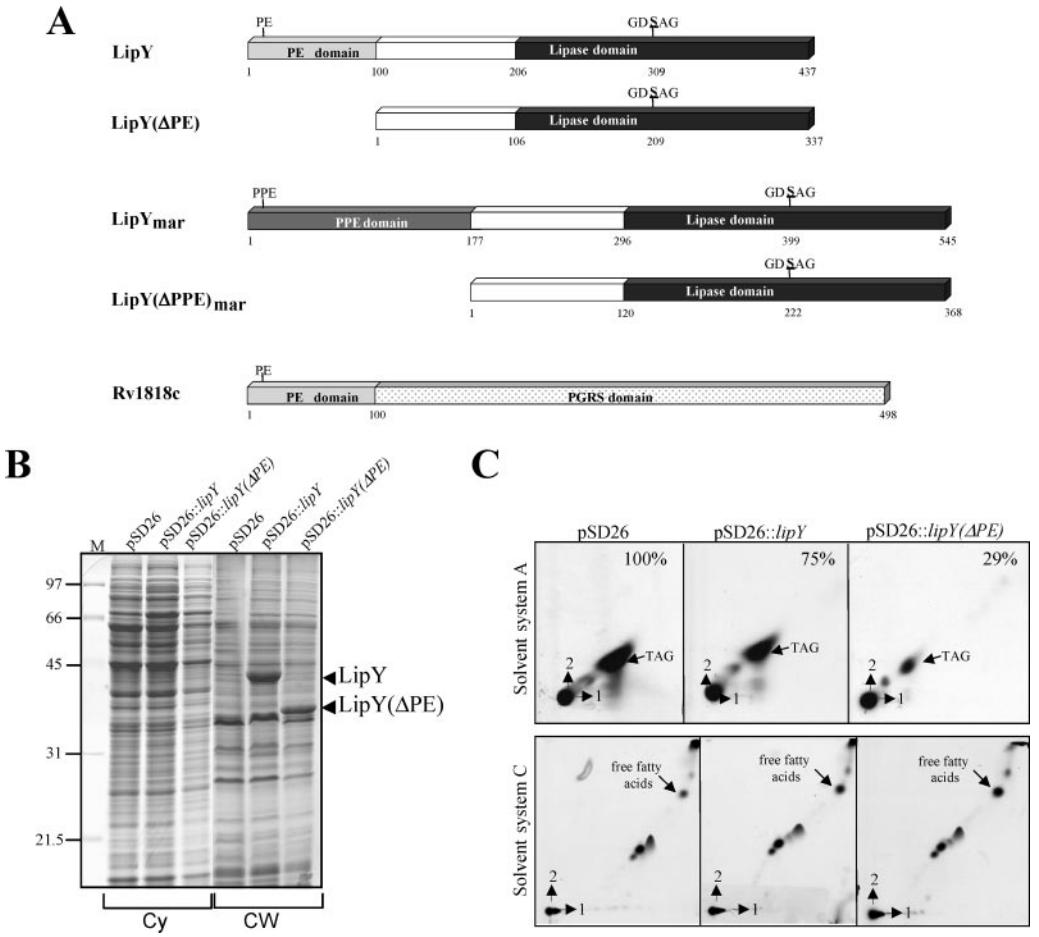

FIG. 1. Expression of *lipY* in *M. smegmatis* and in vivo effect on TAG hydrolysis. (A) Schematic representation of LipY and LipY(ΔPE) from *M. tuberculosis*, $LipY_{mar}$ and $LipY(\Delta PPE)_{mar}$ from *M. marinum*, and the PE-PGRS protein Rv1818c. The conserved PE and PPE domains are shown in gray, along with the Pro-Glu (PE) and Pro-Glu-Glu (PPE) signatures. The Gly-Ala-rich PGRS domain of Rv1818c is shown as a dot-filled rectangle. The lipase domains of LipY and $LipY_{mar}$ are represented in black, whereas the adjacent domains (100 to 206 in LipY and 177 to 296 in $LipY_{mar}$) with no homology to known sequences are shown in white. The catalytic lipolytic serine is represented in bold. (B) Mid-log-phase cultures of *M. smegmatis* carrying either pSD26, pSD26::*lipY*, or pSD26::*lipY*(Δ*PE*) were induced with 0.2% acetamide for 4 h. Cells were harvested and fractionated to separate the cytosol (Cy) from the cell wall (CW). Equal protein amounts (50 μg) were separated by 10% SDS-PAGE. Positions of LipY and LipY(ΔPE) are indicated by arrowheads. M, molecular mass marker. (C) *M. smegmatis* cultures were grown to mid-log phase and induced with 0.2% acetamide for 4 h prior to labeling with 1 μCi/ml [1,2-^{14}C]acetate for an additional 4 h. Apolar lipids were extracted as described in Materials and Methods. Equal counts (250,000) were applied onto silica gel plates and separated using two different solvent systems: solvent system A, which allows visualization of the TAGs (petroleum ether-ethyl acetate [98:2 {vol/vol}] in the first dimension [×3] and petroleum ether-acetone [98:2 {vol/vol}] in the second dimension), and solvent system C, which allows visualization of free fatty acids (chloroform-methanol [96:4 {vol/vol}] in the first dimension and toluene-acetone [80:20 {vol/vol}] in the second dimension). TLC plates were exposed overnight to film. The percentage of TAGs present in each strain (with regard to the reference strain presence, which was arbitrarily set to 100%) was determined by densitometry and is indicated in the upper right corner of each TLC plate.

for mycobacteria in vivo and be relevant to survival during dormancy.

The LipY protein, encoded by Rv3097c, can be dissected into three domains; the N-terminal PE domain and the C-terminal catalytic domain are interspersed by another domain which has no similarity with domains in any other known proteins (Fig. 1A). To date, LipY appears to be the only PE protein with a predicted function. We took advantage of this feature to analyze the effect of the PE domain on enzyme activity. We first analyzed the lipolytic activity of LipY in *M. smegmatis* overexpressing the *lipY* gene, which was placed under the control of a strong acetamide-dependent inducible promoter (18). A major band corresponding to LipY, which was absent in the control strain (the *lipY* gene being absent from *M. smegmatis*), was observed in crude lysates of *M. smegmatis* carrying pSD26::*lipY* following induction with acetamide (data not shown). Cells were then fractionated to separate the cytoplasmic compartment from the cell wall. Proteins from each compartment were separated by SDS-PAGE and stained with Coomassie blue. Figure 1B clearly shows that the majority of LipY was present in the cell wall fraction. To analyze the contribution of the N-terminal PE domain in the activity of LipY, the *lipY* gene devoid of its PE-encoding segment was cloned into the pSD26 to generate pSD26::*lipY*(Δ*PE*). The truncated LipY protein [LipY(ΔPE)] was also readily detected in the cell wall fraction (Fig. 1B).

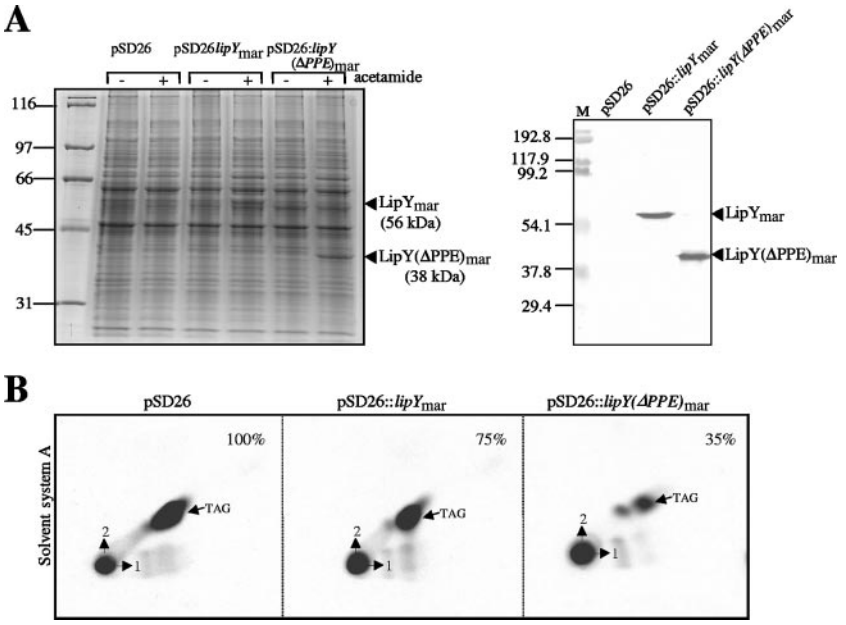

FIG. 2. Lipolytic activity of $LipY_{mar}$ expressed in *M. smegmatis*. (A) Mid-log-phase cultures of *M. smegmatis* carrying either pSD26, pSD26::*lipY*$_{mar}$, or pSD26::*lipY*(Δ*PE*)$_{mar}$ were induced with 0.2% acetamide for 4 h, centrifuged, resuspended in PBS, and lysed. Equal amounts of total proteins (20 μg) were separated by 10% SDS-PAGE. The gel was stained with Coomassie blue to reveal the presence of $LipY_{mar}$ or $LipY(\Delta PPE)_{mar}$. A comparison of the expression levels of $LipY_{mar}$ and $LipY(\Delta PPE)_{mar}$ was done following Western blot analysis. Proteins were transferred to a nitrocellulose membrane, probed with rat anti-LipY(ΔPE) antibodies, and then incubated with anti-rat antibodies conjugated to alkaline phosphatase. (B) Cultures of *M. smegmatis* carrying either pSD26, pSD26::*lipY*$_{mar}$, or pSD26::*lipY*(Δ*PE*)$_{mar}$ were grown to mid-log phase and induced with 0.2% acetamide for 4 h prior to labeling with 1 μCi/ml [1,2-^{14}C]acetate for an additional 4 h. Apolar lipids were extracted and equal counts were applied onto TLC plates. Separation was done using solvent system A and plates were exposed overnight to a film. The percentage of TAGs present in each strain (with regard to the reference strain presence, which was arbitrarily set to 100%) was determined by densitometry and is indicated in the upper right corner of each TLC plate.

To investigate whether the overexpression of LipY or LipY(ΔPE) influences TAG metabolism in *M. smegmatis*, strains harboring either the empty plasmid, pSD26::*lipY*, or pSD26::*lipY*(Δ*PE*) were treated with acetamide for 4 h and then labeled for an additional 4 h with 1 μCi/ml [1,2-^{14}C]acetate. Apolar lipids were then extracted, separated using different solvent systems, and analyzed by 2D TLC-autoradiography (5, 25). Figure 1C, top, reflects TAG production in the three mycobacterial strains. The overexpression of LipY was accompanied by a significant reduction of the TAG pool (about a 25% decrease with respect to the control strain), indicating that LipY hydrolyzes TAGs in vivo. This reaction was accompanied by a concomitant increase of the free fatty acid ratio; free fatty acids are released by the hydrolyzed TAGs (Fig. 1C, bottom). Unexpectedly, we found that the overexpression of LipY(ΔPE) dramatically reduced the TAG pool in the pSD26::*lipY*(Δ*PE*) strain (70% reduction with respect to the control strain). Consistently, the release of free fatty acids was also found to be more pronounced in this latter strain.

Since LipY and LipY(ΔPE) were expressed to comparable amounts in the two recombinant strains (Fig. 1B), the enhanced lipolytic activity of the LipY(ΔPE)-overexpressing strain cannot be attributed to an increased LipY(ΔPE) expression level. Together, these results suggest that, in vivo, LipY(ΔPE) hydrolyzes TAGs more efficiently than LipY.

Removal of the PPE domain of LipY from *M. marinum* affects the enzyme activity in vivo. Analysis of the mycobacterial genome databases allowed the identification of an orthologue gene of *lipY* in *Mycobacterium marinum*. Surprisingly, a BLAST search revealed that although the catalytic domain is well conserved between *M. marinum* and *M. tuberculosis*, the N-terminal domain of the *M. marinum* protein (named $LipY_{mar}$) showed no homologies with the PE domain of LipY but instead showed strong homology with the PPE domain of different proteins. PPE genes are widely present in pathogenic mycobacteria, such as *M. marinum* and *M. avium* (1, 27). As for the function of PE proteins, that of PPE proteins remains an enigma, although recent studies have suggested that they are involved in virulence (27). Figure 1A represents the different domains present in $LipY_{mar}$. Because it contains a PPE domain of about 180 amino acids, the *M. marinum* protein is longer than the *M. tuberculosis* protein. We reasoned that since in one pathogenic strain the lipolytic activity is fused to a PE domain and in another pathogenic strain the same activity is fused to a PPE domain, this would give us a unique opportunity to investigate/compare the roles of the PE and PPE domains with respect to enzymatic activity. Therefore, the *lipY*$_{mar}$ gene and the *lipY*(Δ*PPE*)$_{mar}$ gene were cloned into pSD26, and the resulting plasmids were introduced into *M. smegmatis*. Figure 2A shows that both $LipY_{mar}$ and $LipY(\Delta PPE)_{mar}$ are highly expressed at the expected sizes after the addition of acetamide to the culture medium. Western blot analysis using the rat anti-LipY(ΔPE) antiserum, which strongly cross-reacted with $LipY_{mar}$ and $LipY(\Delta PPE)_{mar}$, showed that both proteins were expressed at comparable levels (Fig. 2A), which allowed us to analyze the influence of the PPE domain on the

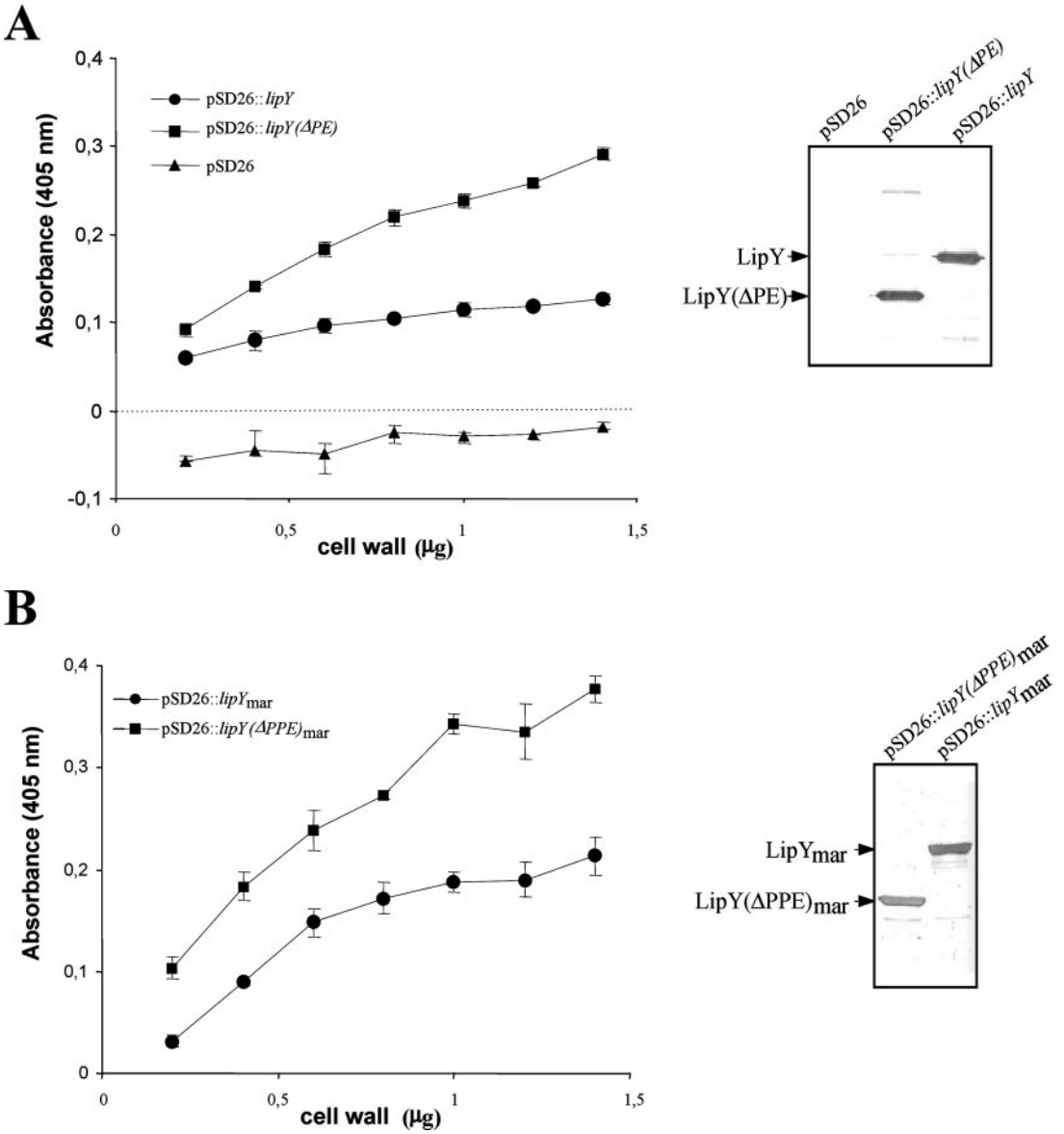

FIG. 3. In vitro lipase assay. An in vitro assay was developed using cell wall fractions from *M. smegmatis* strains overproducing the various lipases following induction with 0.2% acetamide. Cell wall preparations from *M. smegmatis* harboring empty pSD26 were used as an internal control, so the activity observed can be attributed only to the overexpressed lipases. Western blot analysis of the cell wall fractions using anti-LipY(ΔPE) antibodies confirmed the presence of equal amounts of the overexpressed lipases. Increasing concentrations of cell wall fractions (in micrograms) were incubated with *para*-nitrophenyl stearate at 35°C for 40 min. The release of *para*-nitrophenol was monitored spectrophotometrically at 405 nm. (A) Lipolytic activity of LipY versus LipY(ΔPE) and (B) lipolytic activity of $LipY_{mar}$ versus $LipY(\Delta PPE)_{mar}$. Each data point is the mean of triplicates, and error bars correspond to standard deviation (±). The data are representative of three experiments performed with independent cell wall preparations.

activity. The pool of TAG was significantly reduced in the strain overexpressing $LipY_{mar}$ (around 25% decrease) (Fig. 2B). More importantly, removal of the PPE domain drastically reduced the TAG pool in the strain harboring pSD26::*lipY* $(\Delta PPE)_{mar}$ (about 65% decrease). This indicates that $LipY_{mar}$ is an active enzyme involved in TAG hydrolysis and that its activity was strongly increased following the removal of the PPE domain, similar to what was seen relative to removal of the PE domain in LipY. Together, these results suggest that although they do not share sequence conservation, the PE and PPE domains share similar function(s).

To confirm whether the removal of the PE/PPE domains influences the lipolytic activity of LipY or $LipY_{mar}$, we have developed a cell-free assay using cell wall fractions from *M. smegmatis* strains overproducing the lipases or their respective truncated enzymes. Increasing concentrations of cell wall fractions were incubated with *para*-nitrophenyl stearate, and the lipolytic activity measured spectrophotometrically at 405 nm. Cell wall fractions from *M. smegmatis* overexpressing LipY(ΔPE) were reproducibly found to be more active than those from *M. smegmatis* overexpressing LipY in hydrolyzing *para*-nitrophenyl stearate, whereas cell wall preparations from

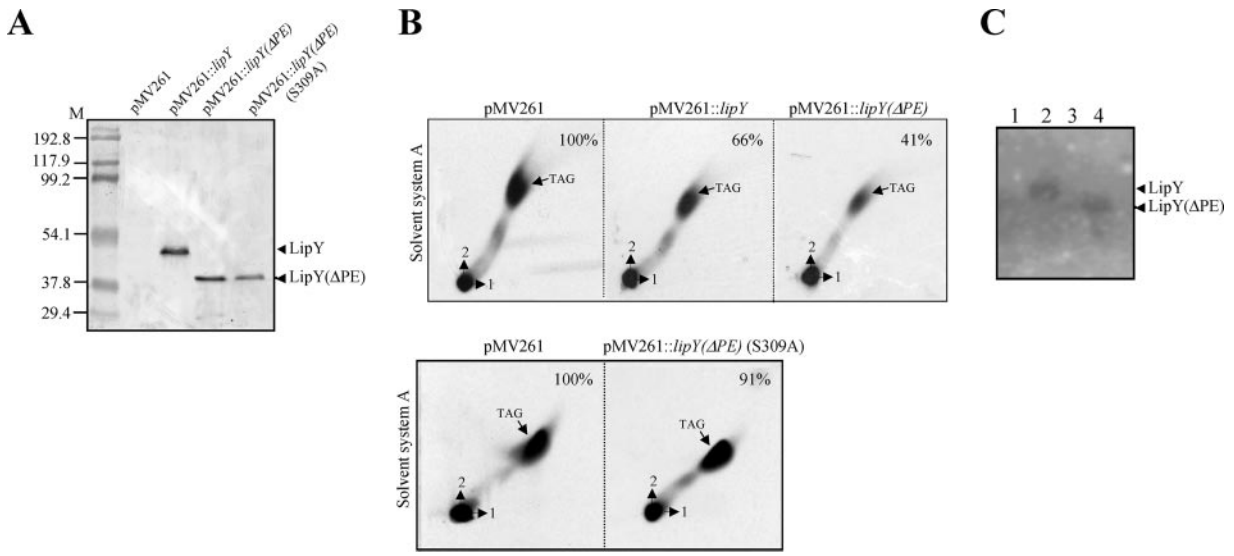

FIG. 4. Overexpression and lipolytic activity of LipY and LipY(ΔPE) in *M. bovis* BCG. (A) *lipY* and *lipY*(Δ*PE*), as well as a version of *lipY*(Δ*PE*) carrying the S309A mutation [encoding LipY(ΔPE)(S309A)], were cloned in pMV261, and the resulting plasmids were introduced into *M. bovis* BCG. The expression of the proteins in each lysate was assayed by Western blotting using rat anti-LipY(ΔPE) antibodies. Equal protein amounts (20 μg) were separated on a 10% SDS-PAGE gel. (B) Recombinant *M. bovis* BCG cultures were labeled with 1 μCi/ml [1,2-^{14}C]acetate for 24 h. Apolar lipids were extracted and equal counts were applied onto silica gel plates. Separation was done using solvent system A and plates were exposed overnight to film. The percentage of TAGs present in each strain (with regard to the reference strain presence, which was arbitrarily set to 100%) was determined by densitometry and is indicated in the upper right corner of each TLC plate. (C) Gel overlay assay. *M. bovis* BCG lysates were separated by SDS-PAGE. Following renaturation, the gel was laid on top of an agar plate containing tributyrin as a substrate. A clear halo forms when the overexpressed lipase is active. Lanes: 1, *M. bovis* BCG pMV261; 2, *M. bovis* BCG pMV261::*lipY*; 3, *M. bovis* BCG pMV261::*lipY*(Δ*PE*)(S309A); 4, *M. bovis* BCG pMV261::*lipY*(Δ*PE*).

M. smegmatis harboring the empty vector did not express any activity (Fig. 3A). Western blot analysis of the cell wall fractions with anti-LipY(ΔPE) antibodies confirmed the presence of equivalent amounts of the overexpressed lipases, thus ruling out the possibility that the differences in activity were due to different expression levels of LipY and LipY(ΔPE). Consistent with these results, cell wall fractions from *M. smegmatis* overexpressing LipY(ΔPPE)$_{mar}$ exhibited an enhanced lipolytic activity compared to those from *M. smegmatis* overexpressing LipY$_{mar}$ (Fig. 3B).

Together, these results support the hypothesis that the PE/PPE domains modulate the lipolytic activities of LipY and LipY$_{mar}$, respectively.

The lipase Ser309 residue is required for the reduction of the TAG pool in *M. bovis* BCG. The *lipY* gene appears to be restricted to members of the *M. tuberculosis* complex, whereas the corresponding orthologue gene is a pseudogene in *M. leprae* and is absent in *M. smegmatis*. We therefore chose to overexpress the gene in *M. bovis* BCG. Both *lipY* and *lipY*(Δ*PE*) were placed under the control of the constitutive *hsp60* promoter in the pMV261 vector (40). Western blot analysis of the crude lysates of the recombinant *M. bovis* BCG strains showed that comparable amounts of LipY and LipY(ΔPE) were expressed in strains carrying pMV261::*lipY* and pMV261::*lipY*(Δ*PE*), respectively (Fig. 4A). Interestingly, LipY could not be detected in protein extracts of the control strain, indicating that it is not expressed or is expressed only poorly during normal in vitro growth conditions.

Following labeling with acetate, apolar lipids were extracted and resolved by 2D TLC. Figure 4B shows a decreased intensity of the spot corresponding to TAGs in the strain overproducing LipY, which was further decreased in the LipY(ΔPE)-overexpressing strain (about 60% decrease with respect to the control strain). Since similar levels of LipY and LipY(ΔPE) are produced in these strains, the reduced pool of TAGs present in the LipY(ΔPE)-overexpressing strain cannot be attributed to an excess LipY(ΔPE) expression. Similar results were obtained when the *M. bovis* BCG strains were labeled with [^{14}C]stearate (data not shown).

LipY possesses the conserved active-site motif GDSAG, a characteristic motif of the hormone-sensitive lipase family that contains an active serine residue (19). In addition, LipY and LipY(ΔPE) contain a domain adjacent to the catalytic domain [residues 100 to 206 in LipY and 1 to 106 in LipY(ΔPE)] (Fig. 1A) to which no functions have been assigned. We therefore investigated whether the effect of TAG reduction could be attributed to the presence of this domain or whether it was directly linked to increased TAG hydrolase activity. To test this hypothesis, we mutated the active Ser309 residue of the GDSAG motif into an Ala residue. Autoradiography-TLC analysis showed only a minor decrease in TAG biosynthesis (about 9%) (Fig. 4B). The activity of the LipY(ΔPE)(S309A) mutant was directly examined using a gel overlay assay on tributyrin-containing plates (Fig. 4C). A clear halo could be seen on the top of the plates only with the *M. bovis* BCG strain overexpressing LipY or LipY(ΔPE), thereby indicating that both proteins were able to use tributyrin as a substrate. However, no halo formation was observed for the control strain or for the strain overexpressing the S309A mutant protein, thereby indicating that the Ser309 represents a crucial catalytic residue, as its

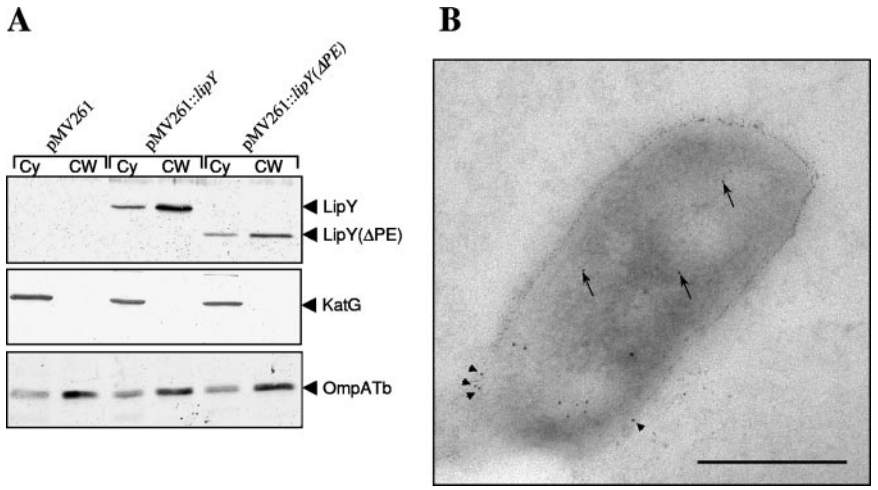

FIG. 5. Localization of LipY and LipY(ΔPE) in *M. bovis* BCG. (A) Subcellular localization of LipY and LipY(ΔPE) in *M. bovis* BCG strains. Cultures were lysed and fractionated to separate the cytoplasm (Cy) from the cell wall (CW). Equal amounts of proteins (10 μg) of each fraction were subjected to SDS-PAGE, electroblotted onto a nitrocellulose membrane, and probed with either rat anti-LipY(ΔPE) antiserum (top), monoclonal anti-KatG antibodies (middle), or rabbit anti-OmpATb antiserum (bottom). (B) EM immunolocalization of LipY. Thin sections of cryosubstituted *M. bovis* BCG LipY(ΔPE) were sequentially incubated on drops of (i) PBS containing 5% BSA and 0.1% Tween 20 for 15 min, (ii) rat anti-LipY(ΔPE) antibody for 2 h, (iii) rabbit anti-rat IgG for 1 h, and (iv) PAO for 30 min. Intracytoplasmic labeling is indicated by arrows and surface labeling by arrowheads. Bar = 0.5 μm.

replacement abolished the enzyme activity. This is consistent with the observation that the organophosphorous compound E-600, known to irreversibly inhibit serine esterases/lipases, completely blocked LipY activity at 0.5 μM (19). Overall, these results indicate that the reduction of the TAG pool is directly correlated with the catalytic activity of the lipase.

The PE domain is not required to anchor LipY to the cell wall. It has been suggested that the PE domain anchors the protein to the cell wall, allowing the antigenically variable PGRS domain of PE-PGRS proteins to gain access to the extracellular compartment (4). The PE domain has been shown to be required/essential for the cell wall localization of another PE protein, Rv1818c (PE-PGRS33) (21). Our results obtained with recombinant *M. smegmatis* strain suggest that LipY is a cell wall-associated protein (Fig. 1). To confirm this hypothesis, subcellular fractions of *M. bovis* BCG overproducing either LipY or LipY(ΔPE) were probed with anti-LipY(ΔPE) antisera for the presence of these proteins. Consistent with the results obtained for *M. smegmatis*, both LipY and LipY(ΔPE) were found to be present in the cell wall fraction (Fig. 5A) and also in the cytosolic preparations. The possible contamination of the cell wall fraction by cytoplasmic components was ruled out by probing the fractions with monoclonal antibodies raised against the cytosolic catalase-peroxidase KatG of *M. tuberculosis* (35). KatG was detected only in the cytosolic fraction and not in the cell wall one, thereby validating the purity of the cell wall preparations (Fig. 5A, middle). Since OmpATb, the major porin from *M. tuberculosis* and *M. bovis* BCG, has been shown to be associated with the cell wall (2), it was used as a marker of the cell wall proteins. Membranes were therefore probed with anti-OmpATb antibodies. Figure 5A, bottom, clearly shows that the porin was mainly present in the cell wall, although it could be detected in the cytoplasmic fraction, as reported earlier (2). Altogether, these fractionation studies establish that LipY and LipY(ΔPE) are present both in the cell wall and to a lesser extent in the cytoplasm and that the PE domain is not required for cell wall anchoring.

LipY and LipY(ΔPE) are located in the outermost layer of the *M. bovis* BCG cell wall. To confirm the location of LipY(ΔPE), immunolabelings were performed at the EM level. Immunolabelings of thin sections of cryosubstituted bacteria with rat antibodies raised against LipY(ΔPE) showed that LipY was located both in the cytoplasm and at the bacterial surface in *M. bovis* BCG overexpressing LipY (not shown) or LipY(ΔPE) (Fig. 5B). Thin sections of control *M. bovis* BCG displayed lower amounts of intracytoplasmic gold particles (5.9 ± 1.3 versus 9.5 ± 1.9) and, especially, three times fewer gold particles on the bacterial surface (1.1 ± 0.4 versus 3.4 ± 1.0) than seen for the LipY(ΔPE) strain. More precisely, thin sections of control bacteria displayed between zero and three gold particles at their surface, with 38.4% of the sections observed displaying no gold particles. In contrast, LipY(ΔPE)-overexpressing bacteria displayed between 0 and 12 gold particles at their surface, with only 11.5% of the sections displaying no gold particles and 34.6% of them displaying 5 particles or more. Although we cannot completely exclude that some of the gold particles might correspond to unspecific labeling, it is clear that the strain overexpressing LipY(ΔPE) is more strongly labeled, especially at the bacterial surface.

These results are in agreement with those of the fractionation studies which showed the presence of LipY or LipY(ΔPE) in both the cytoplasm and the cell wall. To confirm the presence of LipY(ΔPE) in the bacterial wall and gain more-precise information on its location, additional experiments were designed in such a way as to label only surface-exposed LipY(ΔPE). For such studies, it was therefore important to grow bacteria in conditions where shedding of the outer wall layer was kept minimal, i.e., without shaking of cultures and also without bead shearing methods used to separate

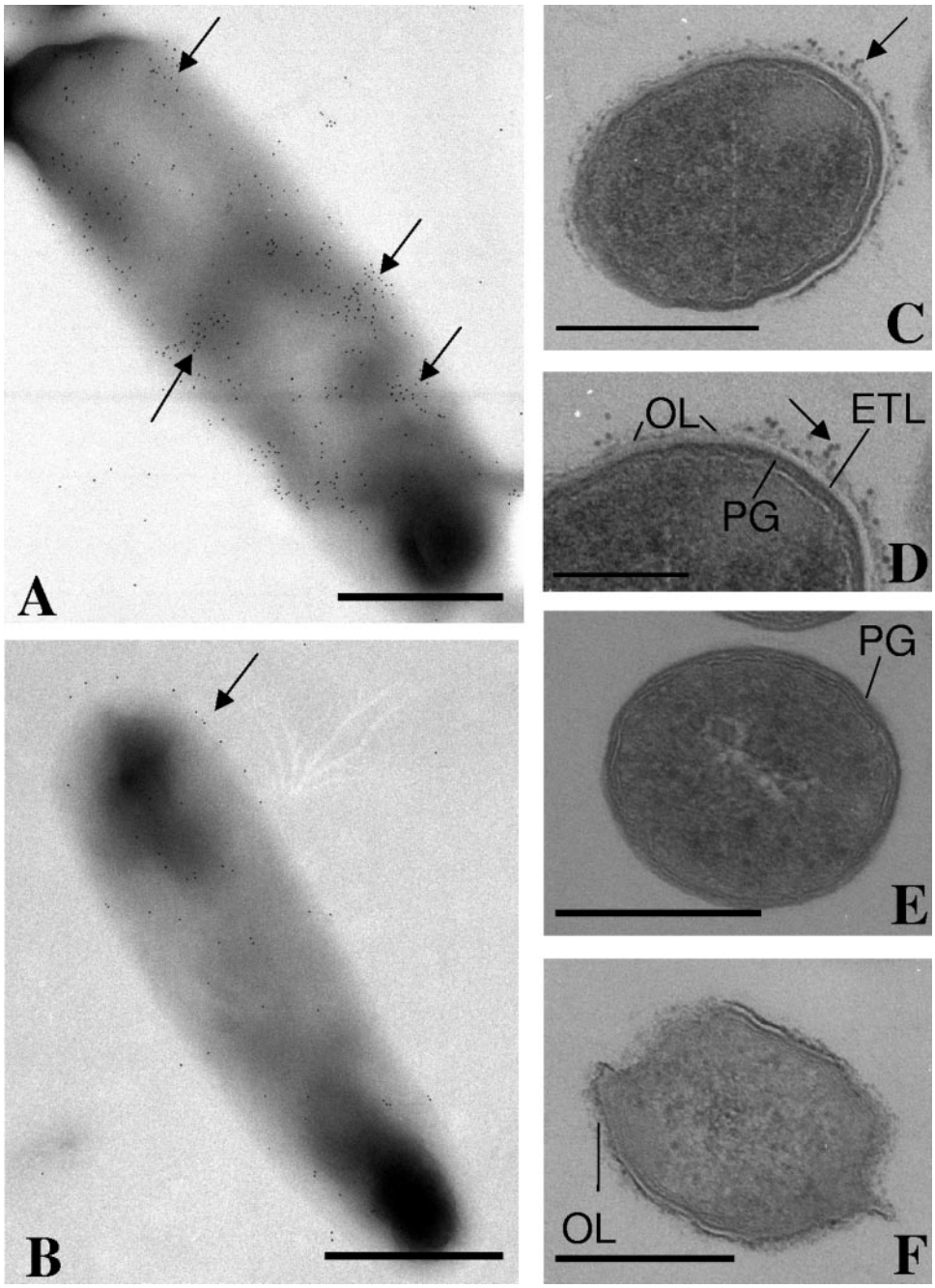

FIG. 6. Immunolocalization of LipY(ΔPE) at the surface of *M. bovis* BCG. Immunolabelings were performed on whole *M. bovis* BCG carrying pMV261::*lipY*(Δ*PE*), deposited on EM Formvar-coated nickel grids prior to labeling (A and B) or onto prefixed bacteria labeled in suspension prior to processing for conventional EM (C to F). In both cases, bacteria were sequentially exposed to rat anti-LipY(ΔPE) antibodies, rabbit anti-rat IgG, and PAO. (A) Whole bacteria on grids exposed to specific antibody, followed by rabbit anti-rat IgG and PAO: the gold particles (300 to 400 per bacterium) are distributed throughout the bacterial surface (arrows). (B) Whole bacteria on grids exposed to rabbit anti-rat IgG and PAO only as a control: bacteria display at most 40 gold particles on their surface (arrow). (C to E) Thin sections of bacteria exposed to specific antibody, followed by rabbit anti-rat IgG and PAO. (C) The outermost layer of the cell wall is labeled (arrow). This is particularly obvious in the enlarged view (D), where the peptidoglycan layer (PG), the thin electron-translucent layer (ETL), and the outermost fibrillar layer (OL) are clearly visible. (E) When the OL has been shed, bacteria are not labeled. (F) Thin sections of bacteria exposed to preimmune rat serum, followed by rabbit anti-rat IgG and PAO: bacteria are not labeled even when the OL is present. Bars in panels A and B = 0.5 μm; Bars in panels C, E, and F = 0.25 μm; Bar in panel D = 0.1 μm.

clumps of bacteria. Two approaches were used, namely, immunolabeling (i) of whole bacteria deposited onto EM grids for direct observation under the EM and (ii) of whole bacteria in suspension prior to conventional processing for EM. With the first method, the shedding of the outer layer of the wall is minimal, as bacteria are never centrifuged, but nonspecific binding of antibodies are more difficult to avoid, whereas when the second approach is used, bacteria are submitted to several

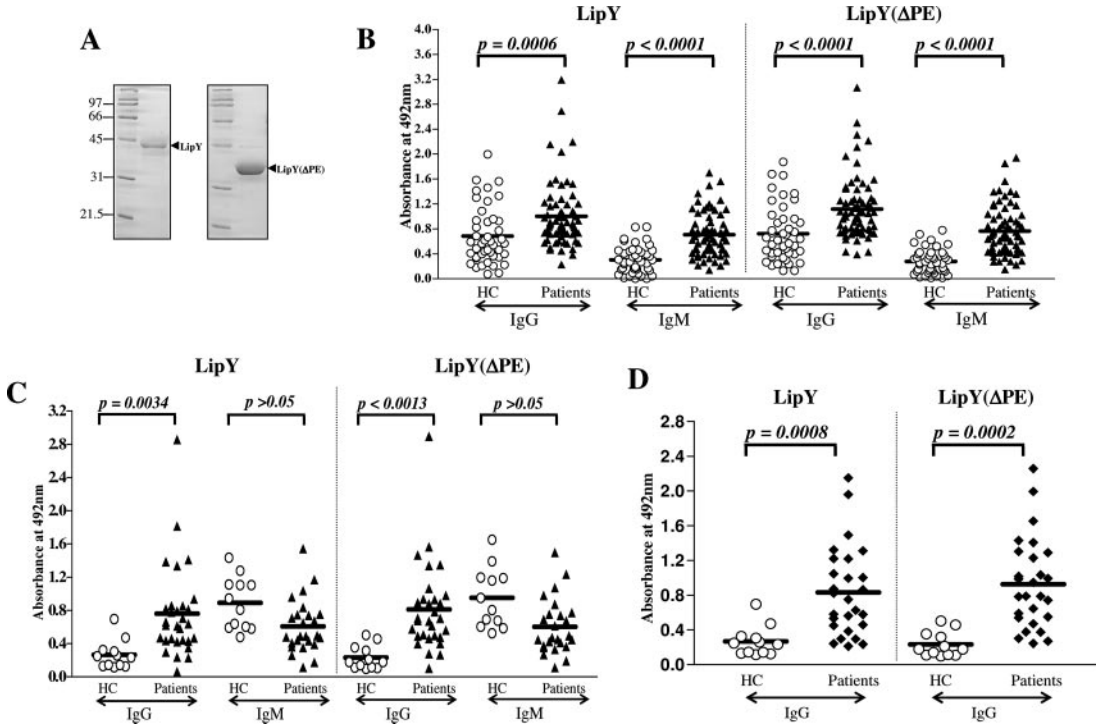

FIG. 7. Specific anti-LipY humoral responses in *M. tuberculosis*-infected patients as opposed to healthy controls. (A) Purification of the recombinant LipY and LipY(ΔPE) proteins expressed in *M. smegmatis* carrying either pSD26::*lipY* or pSD26::*lipY*(Δ*PE*). Proteins were extracted from *M. smegmatis* cell wall preparations, excised from preparative polyacrylamide gels, and then electroeluted to obtain pure proteins. (B) ELISA reactivities of IgG and IgM anti-LipY and anti-LipY(ΔPE) antibodies were assayed in sera of either *M. tuberculosis*-infected group 1 adult patients or healthy controls (HC) ($n = 44$ for healthy controls; $n = 69$ for patients). (C and D) ELISA reactivities of anti-LipY and anti-LipY(ΔPE) antibodies in two different categories of infected children. (C) IgG and IgM reactivities of sera from recently *M. tuberculosis*-infected children and from healthy controls ($n = 12$ for healthy controls; $n = 30$ for patients). (D) IgG reactivities for patients with extrapulmonary TB ($n = 12$ for healthy controls; $n = 27$ for patients).

centrifugations, which can cause shedding of the outer layer. It is noteworthy that the first method allows the labeling of a given molecule on the entire bacterial surface, whereas the second method shows labeling on a very small fraction of the bacterial surface. The location of the molecule under study is, however, more precise on thin sections. When the immunolabelings were performed on EM grids, over 95% of the bacteria were strongly labeled, with each bacterium displaying between 300 and 400 gold particles on its surface (Fig. 6A). If the specific antibody was omitted, i.e., incubation with rabbit ant-rat antibody followed by PAO only, bacteria displayed between 10 to 40 gold particles, indicating a low (less than 10%) background labeling due to unspecific binding of the secondary antibody (Fig. 6B). Bacteria incubated with PAO only (not shown) were completely negative. Labeling for LipY(ΔPE) on whole bacteria deposited onto grids clearly showed that the lipase was surface exposed. When bacteria were immunolabeled in suspension with antibodies raised against LipY(ΔPE), prior to fixation and processing for conventional EM, the cell wall was labeled (Fig. 6C). On thin sections, the gold particles were clearly restricted to the outermost fibrillar layer of the wall, as shown in the enlarged view (Fig. 6D). Although bacteria had been slightly fixed prior to immunolabeling, shedding of the outer layer proved to be unavoidable for about 80% of the bacterial population. The surface of these bacteria displayed no gold particles (Fig. 6E). As a control, bacteria were incubated with rat preimmune serum instead of rat antibodies raised against LipY(ΔPE). In this case, bacteria exhibiting an outer layer were unlabeled (Fig. 6F). Together, these results indicate that LipY, even when depleted for its PE domain, is located in the outermost layer of the wall. Interestingly, bacteria were stained only when the outermost layer was present, thereby indicating that more-interior layers of the wall do not seem to contain the lipase.

Recombinant LipY and LipY(ΔPE) display strong B-cell responses during TB infection. Our results indicate that LipY is strongly associated with the mycobacterial cell wall and more precisely with the outermost layer. Thus, we hypothesized that the protein may be accessible to the immune system of the infected host. We therefore evaluated the role of LipY as an antigen in a clinical setting. Experiments were designed to evaluate the potential immune response of TB patients to LipY and LipY(ΔPE) that were expressed and purified from recombinant *M. smegmatis* strains (Fig. 7A). Proteins were used to screen the TB patient sera by ELISA, using anti-human IgG-HRP and anti-human IgM-HRP as conjugates. The humoral immune responses directed against LipY and LipY(ΔPE) by patients with TB and by healthy controls were compared. As shown in Fig. 7B, sera from group 1 adult patients diagnosed with pulmonary TB mounted IgG and IgM antibody responses against LipY significantly higher than those from healthy controls. In addition, when assayed against puri-

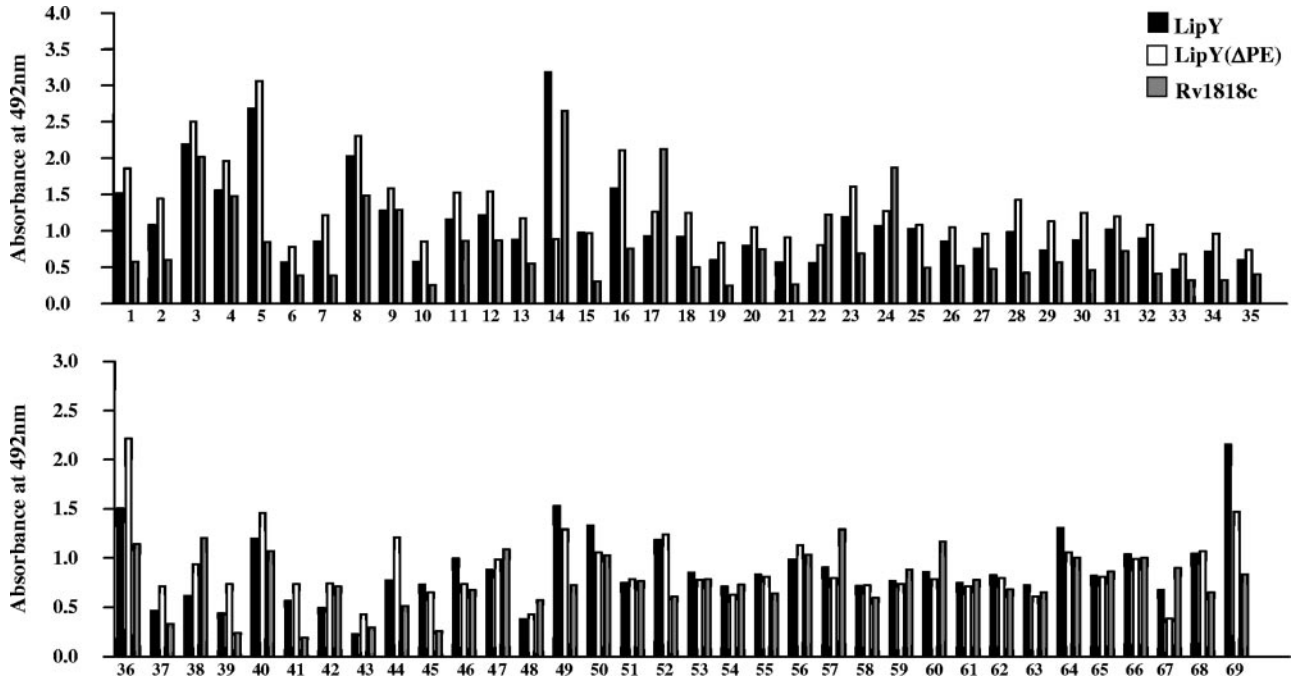

FIG. 8. Patient-by-patient analysis against LipY, LipY(ΔPE), and Rv1818c. Sera from 69 *M. tuberculosis*-infected group 1 adult patients (numbers below bars) were assayed individually by ELISA against LipY, LipY(ΔPE), and Rv1818c.

fied LipY(ΔPE), these sera displayed IgG and IgM antibody responses comparable to those obtained against LipY (Fig. 7B). This suggests that the humoral response elicited in pulmonary TB adult patients is directly against the C-terminal part of LipY (containing the catalytic domain) and not against the N-terminal PE domain of the protein. We next assayed sera from group 1 child patients against both LipY and LipY(ΔPE). The ELISA results show a strong IgG response against both proteins that was significantly higher than that seen for healthy controls (Fig. 7C). However, in contrast to what was seen for adults, there were no significant differences in the IgM responses in group 1 child patients (Fig. 7C). Furthermore, both LipY and LipY(ΔPE) exhibited significant IgG but not IgM (data not shown) immunoreactivity with sera from group 3 child patients with extrapulmonary TB infections (Fig. 7D). Since the *M. tuberculosis* genome encodes at least 24 putative esterases/lipases, one can hypothesize that the anti-LipY-specific B-cell response elicited in TB patients may also result from antibodies directed against other esterases/lipases that are expressed during infection. However, this appears unlikely, since LipY displays only 9 to 21% global amino acid identity with the other *M. tuberculosis* lipases/esterases (19). Taken together, these results indicate that LipY induces strong B-cell responses in infected patients, pointing to the immunodominant nature of the protein.

We next compared the serological sensitivities of LipY, LipY(ΔPE), and the PE-PGRS Rv1818c protein (Fig. 1A). This last protein was chosen because it represents the most widely studied PE-PGRS protein to date. Like LipY, Rv1818c has been shown to be associated with the mycobacterial cell surface (10, 21), and the immune response to this protein has been reported earlier (20). The reactivities of 69 sera from TB patients were assayed individually against LipY, LipY(ΔPE), and Rv1818c (Fig. 8). In most cases, the reactivities to LipY and LipY(ΔPE) were comparable, except for sera from patients 14 and 69, which presented much higher responses against LipY than against LipY(ΔPE). Conversely, sera from patients 16, 36, and 44 exhibited higher titers against LipY(ΔPE) than against LipY. In general, poor ELISA reactivity was observed against Rv1818c compared to LipY or LipY(ΔPE), with a few notable exceptions (sera 17, 24, 57, and 60).

DISCUSSION

There is an increasing body of evidence suggesting that fatty acids represent a source of energy for pathogenic mycobacteria during dormancy (17, 28, 36). Recently, it has been proposed that *M. tuberculosis* stores fatty acids in the form of TAGs as it enters in the nonreplicating persistence stage, similarly to hibernating animals and microbial spores which use TAGs for the long-term storage of energy (17). Utilization of the energy stored in TAGs requires their hydrolysis and the release of free fatty acids, which are subsequently catabolized by β-oxidation. In this study, we report that the overexpression of LipY in both the saprophytic *M. smegmatis* and the slow-growing *M. bovis* BCG species is accompanied by a significant decrease in the TAG pool, which was almost completely restored when the S309A mutation was introduced. This indicates that the decrease of TAGs was correlated with the expression of the TAG hydrolase activity of LipY. Deb et al. (19) have reported that among all the probable lipases present in *M. tuberculosis*, LipY showed the highest capacity to hydrolyze long-chain TAGs such as triolein. Our results indicate that LipY can hydrolyze

TAGs bearing a large panel of fatty acid chain lengths (ranging from C_4 to C_{18}). Indeed, analysis of the fatty acid profile from total TAGs purified from *M. bovis* BCG cultures harboring either pMV261, pMV261::*lipY*, or pMV261::*lipY*(Δ*PE*) did not reveal major changes in terms of chain length specificity, although the overall TAG content was dramatically reduced in the LipY(ΔPE)-overexpressing strain (data not shown). The gel overlay assay also clearly demonstrated that LipY and LipY(ΔPE) can utilize tributyrin as a substrate. This suggests that, in vivo, LipY has no selectivity for long fatty acyl chains, since it also hydrolyzes TAGs bearing short-chain fatty acids.

The roles of the PE and PPE domains from the respective *M. tuberculosis* and *M. marinum* LipY proteins with respect to enzymatic activity were rather unexpected. The overexpression of LipY and $LipY_{mar}$ lacking their respective PE and PPE domains was accompanied by a substantial decrease of TAGs in recombinant *M. smegmatis* strains. The role of the PE/PPE domains in influencing the activity of the lipases was also confirmed in in vitro assays using LipY and $LipY_{mar}$ lipases lacking their respective PE and PPE domains. This is the first study suggesting that PE and PPE domains can share similar functions. One hypothesis is that the lack of the PE domain could modify the network of interactions between LipY and other PE/PPE-containing partners (41, 42) which could directly take part in the regulation of LipY activity. Interactions of LipY with other PE/PPE partners could, for example, dampen its activity. Such a hypothesis is consistent with the fact that, in *M. marinum*, the PE domain has been replaced by a PPE domain. However, more studies are needed to strengthen this hypothesis.

An increasing body of evidence suggests that certain PE/PE-PGRS proteins are found at the cell surfaces of mycobacteria (9). Antisera raised against DNA vaccines expressing PE-PGRS proteins recognize surface antigens on mycobacteria (4, 10). In addition, fluorescence microscopy of *M. smegmatis* expressing the PE-PGRS Rv1818c gene fused to enhanced green fluorescent protein suggests that this protein is located on the bacterial surface and that the PE domain is required for its cell wall localization (21). According to previously published work, the PE domain anchors the protein to the cell wall, allowing the antigenically variable PGRS domain of PE-PGRS proteins to gain access to the extracellular compartment (4). An important message arising from our studies is that the PE/PPE domains may not always be required for cell wall anchoring/localization of the protein. It was recently shown that PPE41 is a secreted protein in *M. marinum* and that secretion is dependent on an ESAT-6-like-secretion system called ESX-5 (1). Apart from ESX-4, all ESX regions have one or more PPE genes (23), and it is possible that more PPEs are secreted via the ESX-5 machinery. It is therefore possible that $LipY_{mar}$ is translocated to the *M. marinum* cell wall through the ESX-5 machinery, a hypothesis that is further supported by the fact that both $LipY_{mar}$ and the ESX-5 secretion system are specific for pathogenic mycobacteria.

Our results are in agreement with a previous report showing that LipY is the enzyme with the highest potential for hydrolyzing TAGs stored inside *M. tuberculosis* (19). Cell wall fractionation studies and immunogold EM (Fig. 5 and 6) clearly demonstrate that LipY is associated with the cell wall and surface exposed, although the enzyme could be detected in the cytoplasmic fraction of *M. bovis* BCG. The location of LipY is therefore easy to reconcile with its activity, which consists of hydrolyzing TAGs considered to be located within intracellular lipid inclusions (22) and the mycobacterial cell wall (32) as well. It is noteworthy that *M. tuberculosis* grown in vivo shows a preference for fatty acids as the energy source, whereas it prefers to utilize carbohydrates when growing in vitro (7). In addition, macrophages infected with *M. bovis* BCG show a marked reduction in the total amount of TAGs (24). Due to its cell surface exposure and its accessibility to the host immune system, LipY is a potential candidate for hydrolyzing host macrophage TAGs, provided that this lipase manages to gain access to the host's lipids. This hypothesis is strengthened by microarray analysis of the response of *M. tuberculosis* PE genes to environmental changes which demonstrated that *lipY* was induced at least twofold inside gamma interferon-activated macrophages (43). Whether *M. tuberculosis* LipY is responsible for breakdown of host lipids during intracellular growth remains, however, unknown at present.

Probing LipY expression by Western blotting of crude lysates of *M. bovis* BCG grown in liquid culture did not reveal the presence of the protein, thereby suggesting that it is not expressed under these conditions or in amounts that are undetectable by this method. This is strengthened by a recent transcriptome analysis indicating that the *lipY* gene in *M. bovis* BCG (Mb3124c) is expressed at low levels (12). Similarly, the lipase could not be detected in crude lysates of *M. tuberculosis* H37Rv (data not shown). However, the induction of an important specific B-cell response against LipY requires that the antigen be expressed and properly presented to the immune system. This suggests that *lipY* is a regulated gene, the expression of which is upregulated during the course of *M. tuberculosis* infection. This hypothesis is strongly supported by a recent study aimed at identifying *M. tuberculosis* genes that are specifically upregulated in infected macrophages but not in in vitro growth conditions (39). Among these genes, *lipY* was found to be the most upregulated gene, and its expression was maximal after 24 h of infection. This is particularly interesting since studies with other pathogens have shown that genes which are upregulated during infection are often important for pathogenicity (14). Nevertheless, whether LipY expression may be associated with disease manifestation and progression remains to be addressed.

The lack of sufficient immune responses by TB patients against several serodiagnostic *M. tuberculosis* antigens has been emphasized. In many cases of patients with pulmonary TB, the immune response to *M. tuberculosis* antigens is not sufficiently primed to induce a potent humoral response. The surface-exposed localization of an antigen is particularly suited for the induction of a strong specific immune response. Here we show for the first time that a mycobacterial lipase induces a potent B-cell response in different clinical categories of TB patients with pulmonary or extrapulmonary TB infections. In addition, our results indicate that the immunogenic part of LipY is not provided by the PE domain, since both LipY and LipY(ΔPE) elicited comparable IgG and IgM responses. This result is consistent with earlier reports suggesting that the humoral response to PE-PGRS antigens is directed against the PGRS domain and not the PE domain. Studies with mice indicated that following immunization with PE-PGRS DNA vaccine an-

tibodies are produced only against the PGRS domain (20). Overall, LipY appears to be a B-cell target antigen with apparent diagnostic potential. To the best of our knowledge, humoral responses elicited by bacterial lipases are rather unusual, although some recent reports have suggested that lipases may be immunogenic proteins in *Staphylococcus* infections (8, 33). Therefore, LipY may provide an attractive candidate for future vaccine development in the form of recombinant *M. bovis* BCG expressing it alone or along with other immunodominant antigens. In addition, because it is also upregulated and presumably important for pathogenicity/dormancy, it may provide an attractive target for future drug development.

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