Conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) Protein Domains Target LipY Lipases of Pathogenic Mycobacteria to the Cell Surface via the ESX-5 Pathway


published in
Journal of Biological Chemistry
2011

DOI (link to publisher)
10.1074/jbc.M110.204966

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:
vuresearchportal.ub@vu.nl

Download date: 10. May. 2022
Conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) Protein Domains Target LipY Lipases of Pathogenic Mycobacteria to the Cell Surface via the ESX-5 Pathway

Received for publication, November 23, 2010, and in revised form, April 5, 2011 Published, JBC Papers in Press, April 6, 2011, DOI 10.1074/jbc.M110.204966

María H. Daleke*, Alessandro Cascioferro†, Karin de Punder‡, Roy Ummels§, Abdallah M. Abdallah¶, Nicole van der Wel*, Peter J. Peters*, Joen Luijink§, Riccardo Manganelli§, and Wilbert Bitter*†‡

From the *Department of Medical Microbiology and Infection Control, VU University Medical Centre, 1081 BT Amsterdam, The Netherlands, the †Department of Molecular Microbiology, VU University, 1081 HV Amsterdam, The Netherlands, the ‡Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, 35121 Padua, Italy, and the §Department of Medical Microbiology and Infection Control, VU University Medical Centre, 1081 BT Amsterdam, The Netherlands

The type VII secretion system ESX-5 is a major pathway for export of PE and PPE proteins in pathogenic mycobacteria. These mycobacteria-specific protein families are characterized by conserved N-terminal domains of 100 and 180 amino acids, which contain the proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs after which they are named. Here we investigated secretion of the triacylglycerol lipase LipY, which in fast-growing mycobacteria contains a signal sequence, but in slow-growing species appears to have replaced the signal peptide with a PE or PPE domain. Selected LipY homologues were expressed in wild-type peptide with a PE or PPE domain. Selected LipY homologues were expressed in wild-type Mycobacterium marinum and its corresponding ESX-5 mutant, and localization of the proteins was investigated by immunoblotting and electron microscopy. Our study shows that Mycobacterium tuberculosis PE-LipY (LipYsub) and M. marinum PPE-LipY (LipYmar) are both secreted to the bacterial surface in an ESX-5-dependent fashion. After transport, the PE/PPE domains are removed by proteolytic cleavage. In contrast, Mycobacterium gilvum LipY, which has a signal sequence, is not transported to the cell surface. Furthermore, we show that LipYsub and LipYmar require their respective PE and PPE domains for ESX-5-dependent secretion. The role of the PE domain in ESX-5 secretion was confirmed in a whole cell lipase assay, in which wild-type bacteria expressing full-length LipYsub but not LipYsub lacking its PE domain, were shown to hydrolyze extracellular lipids. In conclusion, both PE and PPE domains contain a signal required for secretion of LipY by the ESX-5 system, and these domains are proteolytically removed upon translocation.

Mycobacteria such as Mycobacterium tuberculosis, the etiological agent of tuberculosis, have a highly unusual and complex cell envelope (1). To secrete virulence factors across the cell envelope, these bacteria use specialized protein secretion systems, known as ESX or type VII secretion systems (2–8). Mycobacterial genomes contain up to five genetic loci coding for type VII secretion systems, named ESX-1 to ESX-5 (9, 10). The most well studied system, ESX-1, is responsible for the secretion of 10 substrates, including the important T-cell antigens ESAT-6 and CFP-10, and is required for full virulence of M. tuberculosis (7, 11–17). Phylogenetic analyses and comparative genomics suggest that the five ESX clusters have evolved by gene duplication and that ESX-5 is the result of the most recent duplication event (9). Interestingly, ESX-5 is restricted to a group of mycobacterial species known as the slow-growing mycobacteria, which include all major pathogens, such as M. tuberculosis, Mycobacterium leprae, and Mycobacterium ulcerans, and the fish pathogen Mycobacterium marinum (9). Four of the ESX loci contain both PE and PPE genes (named after the conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) motifs near the N termini of their respective gene products) (9, 10), and the appearance of ESX-5 predates the huge expansion of these gene families in slow-growing mycobacteria (18). Intriguingly, although fast-growing, non-pathogenic mycobacteria encode only a small number of PE and PPE proteins, pathogens such as M. marinum and M. tuberculosis dedicate nearly 10% of the coding potential of their genomes to members of the PE and PPE gene families (19). Although the precise function of these proteins is largely unknown, members of both families are important for mycobacterial virulence (20–24). Moreover, various PE and PPE proteins are located on the cell surface, where they can interact with the host during infection (21, 25–28). However, because the PE and PPE proteins lack detectable secretion signals, the route of translocation across the cell envelope remained unclear until members of the two protein families were recently shown to be secreted by the ESX-5 system in M. marinum (3, 29).

PE and PPE proteins are characterized by unrelated, conserved N-terminal domains2 of ~100 (PE) and ~180 (PPE)

2 The abbreviations used are: PE/PPE domains, conserved 100 aa (PE) and 180 aa (PPE) N-terminal domains that contain the proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs after which the mycobacterial protein families are named; aa, amino acids; TAG, triacylglycerol; PE, PGRS, polymorphic GC-rich repetitive sequence subfamily of PE proteins; BCG, bacille Calmette-Guérin; Tn, transposon; OD, optical density; PPE_MPTR, major polymorphic tandem repeat subfamily of PPE proteins.

†‡ This work was supported by funding from an “Earth and Life Sciences (ALW) Open Program” grant from the Netherlands Organization for Scientific Research (NWO) (to M. H. D.) and from the European Community Seventh Framework Programme (FP7/2007–2013) under Grant Agreement 201762.

1 To whom correspondence should be addressed: van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. Fax: 31-20-4448318; E-mail: w.bitter@vumc.nl.
amino acids (aa) (19), and they can be divided into different subfamilies on the basis of their C-terminal domains (19, 30, 31). To date, little is known about the function of the various domains of the PE and PPE proteins. In fact, the M. tuberculosis PE protein LipY (hereafter referred to as LipY<sub>tub</sub>) is the only PE protein for which a function has been characterized (32). M. tuberculosis LipY<sub>tub</sub> is involved in the degradation of triacylglycerols (TAGs) and is the major active lipase under nutrient-deprived conditions. This led to the hypothesis that LipY<sub>tub</sub> plays a role in fatty acid metabolism during the dormancy and reactivation stages of the <i>M. tuberculosis</i> infection cycle (32). Although the lipolytic activity of LipY<sub>tub</sub> is expressed by the C-terminal part of the protein (32), the function of its N-terminal PE domain is less clear. It has been shown that the PE domain of LipY<sub>tub</sub> has an inhibiting effect on the lipase activity (33). Furthermore, cell wall and cell surface localization of LipY was shown to occur independently of the presence of the PE domain (33). Similar results were obtained for <i>M. marinum</i> LipY (hereafter referred to as LipY<sub>mar</sub>), which contains a PPE domain (33). Similar results were obtained for <i>M. marinum</i> protein for which a function has been characterized (32).

<i>LipY</i> (hereafter referred to as LipY<sub>mar</sub>), which contains a PPE domain (33). Similar results were obtained for was shown to occur independently of the presence of the PE domain (33). Similar results were obtained for for mycobacteria and 100 micrograms per ml. In this study, we show that, like many other PE and PPE proteins, the <i>M. tuberculosis</i> and <i>M. marinum</i> LipY homologues are secreted by ESX-5. Using a combination of molecular techniques and analysis of the lipolytic activity of LipY, we show that ESX-5-mediated secretion of the LipY homologues is dependent on the respective PE and PPE domains.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**<i>M. marinum</i> wild-type strains E11 (34) and M<sup>ac</sup> (35) and their respective ESX-5 mutants 7CI (29) and Mx2 (3) and <i>Mycobacterium gilvum</i> PYR-CGK (ATCC 700033) (36) were grown at 30 °C with shaking at 90 rpm, whereas <i>Mycobacterium bovis</i> bacille Calmette-Guérin (BCG) Copenhagen (37), <i>M. bovis</i> BCG Tice (38), and <i>M. tuberculosis</i> mc<sup>6020</sup> (39) were grown standing at 37 °C. <i>M. smegmatis</i> mc<sup>155</sup> (40) was grown at 37 °C with shaking at 90 rpm. All mycobacterial strains were grown in Middlebrook 7H9 broth (Difco-BD Biosciences), supplemented with Middlebrook ADC and 0.05% Tween 80 (Sigma-Aldrich). In secretion experiments, mycobacteria were grown to mid-logarithmic phase, at which they were washed to remove BSA (part of the ADC supplement) and subsequently grown in Middlebrook 7H9 supplemented with 0.2% dextrose and 0.05% Tween 80 for another 24 h. If cultures were grown for electroporation, glycine was added to a final concentration of 2.5 mg/ml during the early exponential phase to increase electroporation efficiency (3). Transformants were selected on Middlebrook 7H10 agar plates and supplemented with Middlebrook OADC and appropriate antibiotics. <i>Escherichia coli</i> DH5α was grown at 37 °C in LB medium (Difco-BD Biosciences) for cloning experiments. Hygromycin was used at a concentration of 50 μg/ml for mycobacteria and 100 μg/ml for <i>E. coli</i>, and kanamycin was used at a concentration of 25 μg/ml.

<i>Plasmid Construction—**All DNA manipulations were carried out according to standard protocols. Pfu DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from Fermentas. Chromosomal DNA from <i>M. marinum</i> E11 and <i>M. gilvum</i> PYR-CGK was used as template in PCR reactions. Plasmids and primers are described in supplemental Tables S1 and S2, respectively.

Cloning of <i>Rv3097c</i> (gene encoding LipY<sub>tub</sub>), modified to express a C-terminal HA tag, in the <i>E. coli</i>-mycobacterial shuttle vector pMV10–25 to generate pAL36 is described in Ref. 26. pMV10–25 contains a minimal pAL5000 origin of replication, which lacks <i>orfs</i> that is required for initiating stable replication (41). Therefore, a 1749-bp fragment containing the <i>his60</i> promoter sequence, the <i>Rv3097c</i> gene, and the in-frame sequence encoding an HA tag followed by a stop codon was isolated from pAL36 by digestion with HindIII and XbaI. The fragment was ligated into the pSMT3 vector (42), which contains a full pAL5000 origin of replication, digested with HindIII and XbaI, to generate pSMT3:<i>LipY<sub>tub</sub></i>. To construct pSMT3:<i>LipY<sub>tub</sub></i>-ΔPE, a gene fragment encoding LipY<sub>tub</sub> without the first 125 aa was amplified from pAL36, cloned in pMV10–25 to generate pAL45, and subsequently transferred to pSMT3 by digestion with HindIII and XbaI.

For cloning of sequences encoding LipY<sub>mar</sub> and LipY<sub>gilv</sub>, or truncated forms of LipY<sub>tub</sub> and Lip<sub>mar</sub>, forward primers were designed to contain an NheI site immediately upstream of the start codon, and reverse primers contained an HA tag encoding sequence in-frame with the coding sequence, a stop codon, and a BamHI or BglII site. PCR fragments were inserted into pSMT3:<i>LipY<sub>tub</sub></i>-ΔPE digested with NheI and BamHI, thereby replacing the <i>Rv3097c</i> gene and HA-encoding sequence.

Plasmids for expression of fusions of LipY<sub>tub</sub> and LipY<sub>gilv</sub> were generated as follows. To replace the PE domain of LipY<sub>tub</sub> with the signal peptide of LipY<sub>gilv</sub>, a 145-bp fragment encoding the signal sequence was amplified from <i>M. gilvum</i> chromosomal DNA and digested with NheI and EcoRI. Then, a 1045-bp fragment coding for the linker and lipase domains of LipY<sub>tub</sub> and the HA tag was isolated from pSMT3:<i>LipY<sub>tub</sub></i>-ΔPE, digested with EcoRI and XbaI. The two fragments were ligated into pSMT3, digested with NheI and XbaI, to generate pSMT3:<i>LipY<sub>tub</sub></i>-ΔPE-Lip<sub>gilv</sub>. For the construct in which the signal peptide of LipY<sub>gilv</sub> was replaced by the PE domain of LipY<sub>tub</sub>, a 309-bp fragment encoding the LipY<sub>tub</sub> PE domain was isolated from pSMT3:<i>LipY<sub>tub</sub></i>-ΔPE-Lip<sub>gilv</sub> by digestion with NheI and EcoRI. In addition, a 1390-bp fragment encoding the linker and lipase domains of LipY<sub>gilv</sub>, or truncated forms of LipY<sub>tub</sub> and LipY<sub>mar</sub>, forward primers were designed to contain an NheI site immediately upstream of the start codon, and reverse primers contained an HA tag encoding sequence in-frame with the coding sequence, a stop codon, and a BamHI or BglII site. PCR fragments were inserted into pSMT3:<i>LipY<sub>tub</sub></i>-ΔPE-Lip<sub>gilv</sub> digested with NheI and BamHI, thereby replacing the <i>Rv3097c</i> gene and HA-encoding sequence.
hsp60 promoter in the pBH10 vector (3, 43). A 1355-bp fragment containing Rv3097c, the in-frame HA sequence, and the stop codon was cut out from pSMT3::LipYtub, with BspE1 and BamHI, and ligated into pBH10 digested with the same enzyme. Constructs were verified by sequencing.

Site-directed Mutagenesis of the LipYtub Cleavage Site—Derivatives of pSMT3::LipYtub containing point mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Because pSMT3::LipYtub was too large for the Pfu Turbo DNA polymerase to amplify, the smaller pAL36 was used as a template. The 1749-bp HindIII-XbaI fragments containing the hsp60 promoter and the mutated gene were transferred into pSMT3 as described above. The mutations were verified by sequencing.

SDS-PAGE and Immunoblotting—Mycobacterial cells grown to mid-logarithmic phase were separated from culture supernatants by centrifugation. The cells were washed with PBS and thereafter disrupted by sonication. Surface-exposed proteins were isolated by incubating intact cells with 0.5% Genapol X-080 (w/v; Sigma-Aldrich) for 30 min at room temperature with head-over-head rotation, prior to sonication (26). Secreted proteins were precipitated from culture supernatants with 10% TCA (w/v; Sigma-Aldrich). Protein samples were boiled, separated by SDS-PAGE, and thereafter transferred to nitrocellulose membranes (Amersham Biosciences) by Western blotting. The membranes were incubated with mouse monoclonal antibodies directed against the influenza hemagglutinin epitope (HA.11; Covance), GroEL2 (CS44; John Belisle, National Institutes of Health, Bethesda, MD, Contract AI-75320) or ESAT-6 (Hyb 76-8; Statens Serum Institut, Copenhagen, Denmark) or with rabbit polyclonal serum recognizing PE41 (3). Secondary horseradish peroxidase conjugated goat anti-mouse IgGs (A106PS, American Qualex) or goat anti-rabbit IgGs (11-1302, Rockland) were detected with 4-chloronaphthol/3,3-diaminobenzidine staining or ECL (Pierce).

N-terminal Sequencing—To identify the LipYtub processing site, we determined the N-terminal amino acid sequence of the cleaved form of the protein. Surface-exposed proteins were extracted with Genapol as described above and separated by SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore) and stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad) in 1% acetic acid, 40% methanol. After destaining with 50% methanol, the band corresponding to processed LipYtub was cut out from the membrane, and the sequence of the first seven amino acids was determined by Edman degradation (Eurosequence, Groningen, The Netherlands).

Immunogold Electron Microscopy (EM)—Fixation of bacterial cells and immunogold labeling of whole cells were done as described in Ref. 17. The HA.11 mouse monoclonal (Covance) was used to visualize the HA-tagged LipY homologues.

Lipase Activity Assay—The lipolytic activity of surface-exposed LipYtub was analyzed with a modified method of the assay described in Ref. 33. Intact M. marinum cells grown in the dark (to prevent production of yellow pigment that might interfere with the absorbance measurements) were centrifuged, and an equivalent of 1 OD unit was resuspended in Tris-HCl buffer (100 mM, pH 8.0). p-Nitrophenyl stearate was added from a 20 mM stock solution prepared in isopropyl alcohol to reach a final concentration of 0.5 mM in 200 μl, and the mixture was vortexed and incubated for 15 min at 37 °C. Thereafter the bacteria were centrifuged, and the supernatant was transferred to a 96-well plate before measuring the production of p-nitrophenol with a spectrophotometer at 405 nm. Reactions were carried out in triplicate.

Bioinformatic Analyses—Nucleotide and protein sequences were retrieved from the MarinoList, TubercuList, and National Center for Biotechnology Information (NCBI) databases. Linear genomic comparisons were performed with the Artemis Comparison Tool software release 9 (44), with a cutoff value of 100 bp.

Cells Lines and Culture Conditions—For LipY macrophage infection experiments, the human acute pro-monocytic leukemia THP-1 cell line was used. Cells were cultured in RPMI 1640 with Glutamax-1 medium (Invitrogen) supplemented with 10% FCS, streptomycin, and penicillin and differentiated into macrophage-like cells in the presence of 10 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich).

LipY Processing by Mycobacteria in Macrophages—For infection, THP-1 cells were used between passage 13 and 20. Cells were seeded at 10 × 10^4 per flask in 75-cm² diameter flasks. Mononuclear cells (3–5 × 10^7) were infected with M. tuberculosis or M. bovis BCG (multiplicity of infection 10) and incubated for 3 h at 37 °C and 5% CO₂. The supernatant was removed, and the infected cells were washed three times with medium to remove extracellular bacteria. Subsequently, the cells were incubated in fresh medium with 10% FCS at 37 °C and 5% CO₂ for the indicated time periods. Enrichment of mycobacterial proteins was performed as follows. Briefly, infected THP-1 cells were collected and washed twice with PBS. Cellular membranes were solubilized by treatment with 1% Triton X-100 for 15 min at room temperature. Intact mycobacteria were sedimented at 25,000 × g for 30 min and subsequently disrupted by sonication. Proteins were separated by SDS-PAGE and visualized by immunoblotting using the HA.11 mouse monoclonal (Covance).

RESULTS

Homologues of LipY Have Evolved Different N-terminal Domains—M. tuberculosis LipY consists of three domains: a typical PE domain at the N terminus followed by a linker domain of unknown function and a C-terminal domain containing the triacylglycerol lipase motif (for a schematic view, see Fig. 1A). Nearly identical orthologues of LipYtub are found in various other species of the M. tuberculosis complex, such as M. bovis. However, with the exception of a pseudogene in M. leprae (32), only one orthologue has been reported in a mycobacterial species outside the M. tuberculosis complex (33). This gene is present in M. marinum and codes for a protein with a different domain organization. Remarkably, although the linker and lipase domains of LipYtub are 65% identical to LipYtub, the N-terminal PE domain is replaced by a PPE domain (Fig. 1A).

We performed a BLAST search to identify additional homologues of LipY in mycobacterial species. Two lipY copies were found in Mycobacterium kansasii. Interestingly, one of these
PE and PPE Domains Target LipY Homologues to ESX-5

M. marinum (Fig. 1B). Short stretches of non-homologous DNA immediately up- and downstream of the lipY genes indicate that some insertions and/or deletions have occurred in this region. When the BLAST analysis was extended to the fast-growing mycobacteria, two additional putative LipY proteins were identified in M. gilvum (hereafter referred to as LipY_gilv) and Mycobacterium vanbaalenii that shared 44 and 43% identity, respectively, with the C-terminal half of the linker domain and the lipase domain of LipY_{tub} (NCBI accession numbers YP_001135683 and YP_952650). Strikingly, the N termini of these sequences do not share homology with PE or PPE domains but instead appear to contain classical signal peptides (Fig. 1A), as indicated by SignalP analysis.

To gain insight in the evolutionary relationships between the LipY homologues, a phylogenetic tree rooted to the Nocardia farcinica lipase Nfa23150 (NCBI accession number YP_118526) was constructed based on the conserved part of the lipase domains, using the MEGA4 software (45) (not shown). The tree topology is similar to that of the mycobacterial species tree, suggesting that the original lipY gene was modified to contain PE/PPE domains. The alignment tree also suggests that PE-LipY evolved before PPE-LipY. This implies that the signal sequence was initially replaced by a PE domain. Subsequently, PE-LipY was (partially) duplicated and modified to contain an N-terminal PPE domain.

LipY_{tub} and LipY_{mar} Are Substrates of ESX-5 in M. marinum—Because several PE and PPE proteins have been shown to be secreted by the ESX-5 secretion system (3, 29), we hypothesized that the LipY homologues with PE/PPE domains are also ESX-5 substrates. To investigate this, the genes encoding LipY_{tub} and LipY_{mar} were each cloned under control of the hsp60 promoter and modified to express a C-terminal HA tag (Fig. 1A). The two constructs were subsequently introduced into wild-type M. marinum strain E11 and its ESX-5 mutant derivative 7C1, and the localization of the LipY-HA fusions was analyzed. Surface-exposed proteins were extracted by treating intact cells with the mild detergent Genapol X-080 (17, 26). For the wild-type strain, a minor fraction of both LipY_{tub} and LipY_{mar} was detected in the culture supernatant (Fig. 2, A and B, lane 4), whereas substantial amounts of both proteins were detected in the surface extracts (Fig. 2, A and B, lane 3). GroEL2 was absent from the surface extracts and culture supernatants, confirming the integrity of the cells. Furthermore, ESAT-6 was included in the analysis as a positive control for secretion, and this protein was normally secreted in all conditions. LipY_{tub} and LipY_{mar} were undetectable in the surface extracts and culture supernatants of the ESX-5 mutant (Fig. 2, A and B, lanes 7 and 8), which confirms our hypothesis that these two proteins are substrates of ESX-5. In addition, the LipY protein levels were markedly lower in the ESX-5 mutant as compared with in the wild-type lysates. Similar results have previously been obtained for the ESX-5 substrates PPE41 and PE_PGRS (3, 29) and indicate that when secretion is blocked, stability of the ESX-5 substrates is affected. The ESX-5-dependent secretion and surface localization of LipY_{tub} was confirmed in M. marinum strain MVU and its ESX-5 mutant Mx2 (not shown).

In the total cell lysate of the wild-type strain expressing LipY_{tub}, two bands are detected (Fig. 2A, lane 1). The upper 40-kDa

<table>
<thead>
<tr>
<th>LipY homologous</th>
<th>PE/LipYtub</th>
<th>PE/LipYmar</th>
<th>LipY_{tub}_APE</th>
<th>LipY_{mar}_APE</th>
<th>SP_{gilv}_LipYtub</th>
<th>PE_{tub}_LipY_{gilv}</th>
<th>PE/linke_LipY_{gilv}</th>
</tr>
</thead>
<tbody>
<tr>
<td>LipYtub</td>
<td>PE</td>
<td></td>
<td>link, lipase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LipYmar</td>
<td>PPE</td>
<td></td>
<td>link, lipase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LipY_{gilv}</td>
<td></td>
<td></td>
<td>link, lipase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LipY_{tub}</td>
<td></td>
<td></td>
<td>link, lipase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LipY_{mar}</td>
<td></td>
<td></td>
<td>link, lipase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LipY_{gilv}</td>
<td></td>
<td></td>
<td>link, lipase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 1. Mycobacterial LipY homologues have evolved different N-terminal domains. A, schematic representation of wild-type, truncated, and chimeric forms of M. tuberculosis LipY (LipY_{tub}), M. marinum LipY (LipY_{mar}), and M. gilvum LipY (LipY_{gilv}). The PE, PPE, and signal peptide (SP) domains are colored in shades of gray, the linker regions are colorless, and the lipase domains are black. The HA tag is depicted as a striped box. Domain boundaries are indicated for the wt proteins. B, comparisons of 30-kb regions surrounding the lipY genes, using the Artemis Comparison Tool, in the genomes of M. tuberculosis, M. marinum, and M. kansasii. Conserved regions are shown in black. The cut-off value for sequence homology was set to 100 bp.

has a PE domain (NCBI accession number ZP_04747303), whereas the other has a PPE domain (NCBI accession number ZP_04747310). The latter variant is probably not functional because its lipase domain is disrupted by a stop codon so that this PPE-LipY protein lacks the histidine residue of the Ser-Asp-His catalytic triad. The two M. kansasii lipY genes are located six genes apart in a region of conserved synteny with the sequences surrounding the lipY genes in M. tuberculosis and
band likely represents the full-length protein, which has a calculated molecular mass of 45.9 kDa, whereas the lower band at \(~28\) kDa probably represents an N-terminally processed or degraded form. Only the smaller form is detectable in the surface extract and in the culture supernatant (Fig. 2A, lanes 3 and 4), whereas full-length LipYtub remains in the

FIGURE 2. LipYtub and LipYmar but not LipYgilv are secreted by ESX-5. A–D, immunoblot analysis of fractions containing equivalent OD units of cell pellets (P -), cells treated with the mild detergent Genapol (S +), and 2-fold more OD units of culture supernatants (S -) from M. marinum wild-type E11 (wt) and ESX-5 mutant 7C1 (ESX-5::Tn) (A–C), or M. smegmatis strain mc^{+}155 (D), expressing LipYtub (A), LipYmar (B), and LipYgilv (C and D). LipY is detected with an antibody recognizing the HA epitope. GroEL2 is used as control for cell lysis, and ESAT-6 is used as control for protein secretion. Processed forms of LipYtub and LipYmar are marked with arrowheads. E–J, whole bacteria fixed and probed with HA antiserum followed by goat anti-mouse conjugate labeled with 10-nm gold particles, analyzed with EM to detect surface-exposed LipYtub (E and F), LipYmar (G and H), and LipYgilv (I and J) in M. marinum wt E11 (E, G, and I) and the ESX-5 mutant 7C1 (F, H, and J). The scale bar represents 100 nm.
Genapol-treated pellet and thus appears to be intracellular (Fig. 2A, lane 2). In contrast, LipYtub was detected as a single, 40-kDa band in the total cell lysate of the ESX-5 mutant, indicating that processing only occurs when LipYtub is secreted (Fig. 2A, lane 5). For LipYmar, three bands are detected, a minor band of 56 kDa probably representing full-length LipYmar and two processed or degraded forms at 39 and 36 kDa (Fig. 2B, lane 1). As for LipYtub, only the full-length band is detected in the ESX-5 mutant strain (Fig. 2B, lane 5). However, unlike for LipYtub, all three forms are represented in the surface extract of the wild-type strain (Fig. 2B, lane 3).

To confirm the surface exposure of LipYtub and LipYmar, the localization of the proteins was analyzed by immunogold EM on whole cells. Wild-type cells expressing LipYtub were efficiently labeled (Fig. 2E), whereas the ESX-5 mutant showed little or no labeling (Fig. 2F), consistent with the immunoblot results (Fig. 2A). Wild-type M. marinum expressing LipYmar also showed surface labeling (Fig. 2G), although to a lesser extent than LipYtub. Again, the ESX-5 mutant showed little or no labeling (Fig. 2H), confirming that LipYmar is also secreted by ESX-5. M. marinum harboring the empty plasmid showed no reaction with the HA antiserum in immunoblots or whole cell EM (not shown).

To investigate whether surface-exposed LipYtub is functional, bacterial cells were incubated with p-nitrophenyl stearate, after which the bacteria were removed by centrifugation. Lipolytic activity resulted in the release of yellow p-nitrophenol in the supernatants, which could be measured spectrophotometrically. The wild-type strain expressing LipYtub showed strongly increased activity as compared with the wild-type strain harboring the empty plasmid pSMT3, confirming that LipYtub is secreted to the cell surface where it is enzymatically active (Fig. 3). Interestingly, the empty wild-type strain also exhibited lipolytic activity, which was completely abolished in the ESX-5 mutant strain, with or without lipY-containing plasmids. This activity could be due to chromosomally encoded LipYmar.

LipYgilv is Not Secreted in M. marinum or M. smegmatis—The Sec-dependent secretion pathway in mycobacteria is, as in other bacteria, responsible for translocation of proteins with signal sequences across the inner membrane (4). Because LipYgilv is predicted by the SignalP program to have a signal peptide, we set out to determine its subcellular localization. To this end, we amplified and cloned the lipYgilv gene with a C-terminal HA tag under control of the hsp60 promoter (Fig. 1A). Upon introduction into M. marinum wild-type E11 and its ESX-5 mutant, no bands could be detected in the culture supernatant or Genapol extract fractions (Fig. 2C, lanes 3 and 4 and lanes 7 and 8). In the whole cell lysate, several bands were detected of which the largest is ~65 kDa (Fig. 2C, lane 1). Because LipYgilv has a theoretical molecular mass of 51 kDa, this could indicate that the protein is post-translationally modified. However, because a similar band was observed upon expression of lipYgilv in E. coli (results not shown), this hypothesis is not likely to be correct. In agreement with the results obtained by immunoblotting, no labeling was observed of whole cells in immunogold EM (Fig. 2, I and J). We attempted to repeat the experiments in M. gilvum to rule out that LipYgilv is secreted by its natural host, but despite trying a variety of experimental settings, we failed to transform M. gilvum. Therefore, secretion was analyzed in another fast-growing species, i.e. M. smegmatis. Similar to M. marinum, several bands were observed in the cell pellet (Fig. 2D, lane 1), but also, in this host, LipYgilv is neither secreted nor surface-exposed (Fig. 2D, lanes 3 and 4). In conclusion, LipYgilv is neither exposed on the bacterial surface nor secreted to the extracellular environment.

The PE and PPE Domains Are Required for Secretion of LipYtub and LipYmar via ESX-5—To investigate the role of the conserved PE and PPE domains in ESX-5 secretion, N-terminally truncated forms of LipYtub and LipYmar lacking the first 125 and 176 aa, respectively (Fig. 1A), were studied. Both mutant proteins were stably produced in the cell lysates of the wild-type and mutant strains (Fig. 4, A and B, lanes 1 and 5). However, the absence of bands in the Genapol extracts and culture supernatants (Fig. 4, A and B, lanes 3 and 4 and lanes 7 and 8) indicated that these truncated proteins were not secreted. Detection of ESAT-6 in culture supernatant fractions confirmed that the lack of secretion was specific for the LipY homologues. To exclude the possibility that LipYtubΔPE and LipYmarΔPE were still secreted but could not be recognized by the antiserum, we analyzed the lipolytic activity of M. marinum wild-type E11 and its ESX-5 mutant expressing LipYtubΔPE. As shown in Fig. 3, E11 expressing LipYtubΔPE exhibits the same lipase activity as the wild-type cells. The ESX-5 mutant expressing LipYtubΔPE did not show any activity. Because LipYtub and LipYmar lacking their N-terminal PE or PPE domains have been shown to have higher lipase activity than the corresponding full-length proteins (33), these results confirm that our N-terminally truncated LipY homologues are indeed not secreted.

To test whether the signal peptide of LipYgilv could replace the PE domain and facilitate secretion of LipYtub to the cell surface, we exchanged the PE domain for the PPE domain of LipYtub (Fig. 4, E and F). As shown in Fig. 3, E11 expressing LipYtub/H9004-PPE exhibited a similar band was observed upon expression of lipYgilv in E. coli (results not shown), this hypothesis is not likely to be correct. In agreement with the results obtained by immunoblotting, no labeling was observed of whole cells in immunogold EM (Fig. 2, I and J). We attempted to repeat the experiments in M. gilvum to rule out that LipYgilv is secreted by its natural host, but despite trying a variety of experimental settings, we failed to transform M. gilvum. Therefore, secretion was analyzed in another fast-growing species, i.e. M. smegmatis. Similar to M. marinum, several bands were observed in the cell pellet (Fig. 2D, lane 1), but also, in this host, LipYgilv is neither secreted nor surface-exposed (Fig. 2D, lanes 3 and 4). In conclusion, LipYgilv is neither exposed on the bacterial surface nor secreted to the extracellular environment.

The PE and PPE Domains Are Required for Secretion of LipYtub and LipYmar via ESX-5—To investigate the role of the conserved PE and PPE domains in ESX-5 secretion, N-terminally truncated forms of LipYtub and LipYmar lacking the first 125 and 176 aa, respectively (Fig. 1A), were studied. Both mutant proteins were stably produced in the cell lysates of the wild-type and mutant strains (Fig. 4, A and B, lanes 1 and 5). However, the absence of bands in the Genapol extracts and culture supernatants (Fig. 4, A and B, lanes 3 and 4 and lanes 7 and 8) indicated that these truncated proteins were not secreted. Detection of ESAT-6 in culture supernatant fractions confirmed that the lack of secretion was specific for the LipY homologues. To exclude the possibility that LipYtubΔPE and LipYmarΔPE were still secreted but could not be recognized by the antiserum, we analyzed the lipolytic activity of M. marinum wild-type E11 and its ESX-5 mutant expressing LipYtubΔPE. As shown in Fig. 3, E11 expressing LipYtubΔPE exhibits the same lipase activity as the wild-type cells. The ESX-5 mutant expressing LipYtubΔPE did not show any activity. Because LipYtub and LipYmar lacking their N-terminal PE or PPE domains have been shown to have higher lipase activity than the corresponding full-length proteins (33), these results confirm that our N-terminally truncated LipY homologues are indeed not secreted.

To test whether the signal peptide of LipYgilv could replace the PE domain and facilitate secretion of LipYtub to the cell surface, we exchanged the PE domain (1–101 aa) of LipYtub for the PE domain (1–101 aa) of LipYtub for
PE and PPE Domains Target LipY Homologues to ESX-5

The N-terminal PE and PPE domains are required for ESX-5 secretion of LipYtub and LipYmar. Immunoblot analysis of truncated and chimeric forms of the LipY homologues, expressed in M. marinum wt E11 and its ESX-5 mutant 7C1 (ESX-5::Tn) (A–C) or in E11 alone (D), is shown. A–D, LipYtub lacking its PE domain (LipYtub-ΔPE) (A), LipYmar lacking its PPE domain (LipYmar-ΔPPE) (B), and the chimeras in which the PE domain of LipYtub was replaced by the signal peptide from LipYgilv (SPgilv-LipYtub) (C) or in which the signal peptide of LipYgilv was replaced by the PE domain of LipYtub (PEtub-LipYgilv) (D) were digested with HA antiserum. Equivalent OD units of cell pellets ($P^-$), Genapol-treated cells ($P^+$), Genapol surface extracts ($S^-$), and 2-fold more culture supernatant ($S^+$) are shown. GroEL2 and ESAT-6 were detected as controls for cell lysis and secretion, respectively.

The signal peptide encoding sequence (1–45 aa) of LipYgilv (Fig. 1A). This chimera was, however, neither secreted nor surface-exposed upon expression in M. marinum (Fig. 4C, lanes 3 and 4 and lanes 7 and 8). Finally, we also tested whether the PE and PPE domains of LipYtub and LipYmar without the linker and lipase domains could be secreted. To this end, we cloned the PE (1–99 aa) and PPE (1–176 aa) domains of LipYtub and LipYmar and modified them to express C-terminal HA tags (Fig. 1A) under the control of the hsp60 promoter. However, upon introduction into M. marinum, neither PE_LipYtub nor PE_LipYmar could be detected in immunoblots (results not shown), suggesting that they were not stable. In conclusion, these results show that LipYtub and LipYmar require their respective N-terminal PE and PPE domains for translocation to the cell surface by the ESX-5 secretion machinery.

LipYgilv, Fused to a PE Domain Is Not Secreted—To test whether the PE domain can target unrelated proteins for ESX-5 secretion, we replaced the signal peptide of LipYgilv, (1–45 aa) with an in-frame N-terminal fusion of the PE domain of LipYtub (1–103 aa) to the linker and lipase domains of LipYgilv (46–494 aa) (Fig. 1A). However, upon introduction into M. marinum, this chimeric protein was neither surface-exposed nor secreted (Fig. 4D, lanes 3 and 4). This led us to believe that (part of) the linker domain following the PE domain could be critical. Therefore, a second fusion was constructed in which the signal peptide and the linker domain of LipYgilv (1–236 aa) were replaced by the PE domain and the linker domain of LipYtub (1–205 aa) (Fig. 1A). However, this chimera also failed to be secreted (Fig. 4D, lanes 7 and 8). The first chimeric protein showed the aberrant molecular weight features of the LipYgilv protein, whereas the second construct migrated at the expected molecular weight (Fig. 4D, lanes 1 and 5), indicating that the linker domain is responsible for the multiple bands observed with LipYgilv in SDS-PAGE, of which some are larger than expected.

These results indicate that heterologous proteins cannot easily be secreted via the ESX-5 pathway. Failure to secrete even a closely related fusion sequence (44% identity on amino acid level) suggests that structure or folding kinetics of substrates may be of importance for ESX-5-dependent translocation.

Processing of Secreted LipYtub—As discussed above, secreted and surface-exposed LipYtub is detected in a processed form (Fig. 2A). This could mean either that LipYtub is processed during transport or that secreted LipYtub is sensitive to extracellular proteases. To gain more insight into the mechanism of processing, cell lysates of M. marinum wild-type strain E11 and its ESX-5 mutant 7C1 overexpressing LipYtub were incubated for 1, 2, 4, and 24 h at 30 °C. No additional processing or degradation of LipYtub was observed in either strain (Fig. 5A). These results suggest that LipY is not a protease-sensitive protein, which indicates that processing of LipYtub is a specific maturation event.

Next, processed LipYtub, was isolated from the Genapol supernatant (Fig. 5B), and the N-terminal sequence was determined by Edman degradation. The results showed that LipYtub is cleaved between Gly-149 and Ala-150 (Fig. 5C). The cleavage position indicates that the PE domain and part of the linker domain are removed, whereas the intact lipase domain is retained. The processed protein has a predicted mass of 30.3...
kDa, which correlates well with the observed protein band with an apparent molecular mass of 28 kDa.

To investigate the nature of the recognition sequence of the enzyme responsible for processing of LipYtub, we introduced point mutations in residues surrounding the cleavage site (Fig. 5C). Most strikingly, substitution of the alanine at position 150 for an aspartic acid residue resulted in a complete disappearance of processed LipYtub, whereas those that had no effect are marked with triangles. D, The S148D, GI49D, and A150D point mutations resulted in (partial) production of a surface-exposed slightly larger processed form of LipYtub, marked with an asterisk, when expressed in M. marinum strain M434. Equal OD units of cell pellets (P−), Genapol-treated cell pellet (P+), and Genapol surface extract (S−), and 2-fold more culture supernatant (S+) are shown.

Taken together, these results suggest that the entire PE domain and the first 50 amino acids of the linker domain of LipYtub are removed in a maturation event linked to secretion. Processing depends on the SGA motif encoded by residues 148–150, but if those residues are mutated, cleavage occurs at another adjacent site, and secretion and lipase activity are unaffected.

Expression and Processing of LipYtub in M. tuberculosis and M. bovis BCG—PE and PPE proteins have previously been detected on the surface of M. bovis BCG and M. tuberculosis (21, 25). To investigate whether LipYtub is also surface-exposed in members of the M. tuberculosis complex, we expressed HA-tagged LipYtub in M. bovis BCG Copenhagen, M. bovis BCG Tice, and the M. tuberculosis double auxotroph mutant mc26020. Although we observed secretion of endogenous PPE41, a protein that has previously been shown to depend on ESX-5 (3), LipYtub was not detected in the Genapol-extracted fraction or the culture supernatant (S−) of a culture of M. tuberculosis mc26020 harboring the pBH10::LipYtub plasmid. B, Immunoblot showing cell lysates of M. tuberculosis (M. tub) mc26020 carrying the empty pBH10 plasmid (lane 1) or pBH10::LipYtub (lane 2) isolated from THP-1 macrophages at day 2 after infection, and as a control for bacterial lysis in A and as a loading control in B, and the chromosomally encoded ESX-5 substrate PPE41 was detected as a secretion control in A.

FIGURE 5. The N terminus is proteolytically removed from secreted LipYtub. A and D, processing of wt and mutated forms of LipYtub was analyzed by immunoblotting using HA antiserum. GroEL2 was detected as a loading control in A and as a control for bacterial lysis in D. A, no additional processing or degradation was observed when cell lysates of M. marinum wt E11 or its ESX-5 mutant 7C1 (ESX-5::Tn) expressing LipYtub were incubated for 1, 2, 4, or 24 h after sonication. B, Coomassie Brilliant Blue-stained SDS-PAGE gel with Genapol surface extracts of wt M. marinum E11 or E11 expressing LipYtub. Proteins were transferred to a PVDF membrane, and the band representing processed LipYtub (marked with an arrow in the gel) was analyzed by Edman degradation. C, LipYtub is processed in the linker domain between residues Gly-149 and Ala-150. Residues altered by site-directed mutagenesis that affected processing are marked with asterisks, whereas those that had no effect are marked with triangles. D, The S148D, GI49D, and A150D point mutations resulted in (partial) production of a surface-exposed slightly larger processed form of LipYtub, marked with an asterisk, when expressed in M. marinum strain M434. Equal OD units of cell pellets (P−), Genapol-treated cell pellet (P+), and Genapol surface extract (S−), and 2-fold more culture supernatant (S+) are shown.

FIGURE 6. LipYtub processing in M. tuberculosis is triggered during infection. A, Immunoblot analysis of equal OD units of cell pellet (P−), Genapol-treated cell pellet (P+), Genapol surface extract (S+) fractions, and 2-fold more culture supernatant (S−) of a culture of M. tuberculosis mc26020 harboring the pBH10::LipYtub plasmid. B, Immunoblot showing cell lysates of M. tuberculosis (M. tub) mc26020 carrying the empty pBH10 plasmid (lane 1) or pBH10::LipYtub (lane 2) isolated from THP-1 macrophages at day 2 after infection, or from cultures (lanes 3 and 4). A cell lysate showing the processing of LipYtub in M. marinum (M. mar) strain E11 is included in lane 5. LipYtub was detected with the HA antibody, GroEL2 was analyzed as control for bacterial lysis in A and as a loading control in B, and the chromosomally encoded ESX-5 substrate PPE41 was detected as a secretion control in A.
Interestingly, these samples showed the same processing pattern after X-100 treatment and centrifuged to collect mycobacteria. BCG Copenhagen expressing HA-tagged LipYtub.A further expansion of the original hypothesis. Additional proteins required for LipY secretion are missing. To test this possibility, we infected human THP-1 cells, differentiated to macrophage-like cells in the presence of phorbol 12-myristate 13-acetate, with M. tuberculosis mc6020 and M. bovis BCG Copenhagen expressing HA-tagged LipYtub. At 1, 2, 3, and 6 days after infection, the THP-1 cells were lysed by Triton X-100 treatment and centrifuged to collect mycobacteria. Interestingly, these samples showed the same processing pattern for LipYtub as was observed for this protein upon expression in M. marinum (see Fig. 6B, lane 2, for results from day 2 after infection with M. tuberculosis mc6020; data for M. bovis BCG are not shown). Together these results indicate that processing of LipYtub occurs both in M. marinum and in members of the M. tuberculosis complex, albeit indicating that in the latter species, the secretion and/or processing of LipY is induced during infection.

**DISCUSSION**

Comparative genomics and phylogenetic analyses indicated that the evolution of M. tuberculosis lipY is highly induced upon infection of host cells (46), we hypothesized that the lack of secretion in culture could be due to the fact that additional proteins required for LipY secretion are missing. To test this possibility, we infected human THP-1 cells, differentiated to macrophage-like cells in the presence of phorbol 12-myristate 13-acetate, with M. tuberculosis mc6020 and M. bovis BCG Copenhagen expressing HA-tagged LipYtub. At 1, 2, 3, and 6 days after infection, the THP-1 cells were lysed by Triton X-100 treatment and centrifuged to collect mycobacteria. Interestingly, these samples showed the same processing pattern for LipYtub as was observed for this protein upon expression in M. marinum (see Fig. 6B, lane 2, for results from day 2 after infection with M. tuberculosis mc6020; data for M. bovis BCG are not shown). Together these results indicate that processing of LipYtub occurs both in M. marinum and in members of the M. tuberculosis complex, albeit indicating that in the latter species, the secretion and/or processing of LipY is induced during infection.

Because the expression of M. tuberculosis lipY is highly induced upon infection of host cells (46), we hypothesized that the lack of secretion in culture could be due to the fact that additional proteins required for LipY secretion are missing. To test this possibility, we infected human THP-1 cells, differentiated to macrophage-like cells in the presence of phorbol 12-myristate 13-acetate, with M. tuberculosis mc6020 and M. bovis BCG Copenhagen expressing HA-tagged LipYtub. At 1, 2, 3, and 6 days after infection, the THP-1 cells were lysed by Triton X-100 treatment and centrifuged to collect mycobacteria. Interestingly, these samples showed the same processing pattern for LipYtub as was observed for this protein upon expression in M. marinum (see Fig. 6B, lane 2, for results from day 2 after infection with M. tuberculosis mc6020; data for M. bovis BCG are not shown). Together these results indicate that processing of LipYtub occurs both in M. marinum and in members of the M. tuberculosis complex, albeit indicating that in the latter species, the secretion and/or processing of LipY is induced during infection.

**DISCUSSION**

Comparative genomics and phylogenetic analyses indicated that the evolution of the type VII secretion system ESX-5 predicted the expansion of the PE and PPE gene families in slow-growing mycobacteria (18). These analyses also showed that the recently evolved PE_PGRS and PPE_MPTR (major polymorphic tandem repeat subfamily of PPE proteins) subfamilies have probably originated from PE and PPE genes within the ESX-5 cluster, which resulted in the hypothesis that ESX-5 has a functional link with the recent expansion of PE and PPE proteins. Recently, we showed that ESX-5 is indeed responsible for the secretion of various members of the PE_PGRS and PPE_MPTR protein subfamilies to the cell surface and culture supernatant of M. marinum (3, 29). In this work, we report the identification of two novel substrates of the M. marinum ESX-5 system, the LipY homologues of M. tuberculosis and M. marinum, which contain a PE and PPE domain, respectively. Interestingly, the analysis of LipY allows a further expansion of the original hypothesis. Additional lipY orthologues were identified in fast-growing mycobacterial species, namely M. gilvum and M. vanbaalenii. These lipY homologues have putative N-terminal signal sequences instead of a PE or PPE domain. LipYgiv was not transported to the cell surface, indicating that the PE or PPE domain is required for surface localization. However, it must be acknowledged that the localization of LipYgiv could not be studied in its native host due to problems with transformation. We would like to propose that LipY homologues in slow-growing mycobacteria have evolved to secreted proteins by substituting the original signal peptide for PE or PPE domains and thereby have turned into substrates of the ESX-5 system (Fig. 7). A remarkable snapshot of this evolution process can be observed in the genome of M. kansasi, which has two copies of lipY, one containing a PE domain and one with a PPE domain.

N-terminally truncated forms of LipYtub and LipYmar were not transported across the cell envelope, showing that the presence of a PE or PPE domain is required for secretion via ESX-5. Unfortunately, our attempt to investigate whether PE and PPE domains are sufficient for secretion failed because PE_LipYtub and PPE_LipYmar were unstable when expressed alone. However, in another study, it was shown that the PE domain of PE_PGRS33 can be secreted on its own by ESX-5.3

In M. marinum, surface-exposed LipYtub and LipYmar were processed, which resulted for LipYtub in the removal of the PE domain and first half of the linker domain. No further processing or degradation was observed of LipYtub after extended incubation of M. marinum lysates, indicating that LipY cleavage results from a specific maturation process, linked to ESX-5 translocation (Fig. 7). Similar patterns of N-terminal processing have previously been observed for PE_PGRS and PPE_MPTR proteins secreted by ESX-5 (29), although the responsible protease was not identified. This could mean that processing of ESX-5 substrates is a common process, similar to the removal of a signal sequence. Interestingly, processing has also been observed for the ESX-1 substrate EspB (16), which was recently shown to be cleaved by the ESX-1-encoded protease MycP1 (47). To further investigate the nature of the LipYtub cleavage site, we altered the neighboring residues using site-directed mutagenesis. Although introducing point mutations could block processing at the original cleavage site, the mutations always resulted in an alternative processing pattern, as is also observed for signal sequence removal (48, 49). Besides the removal of the secretion domain, processing could also have a functional implication. M. tuberculosis utilizes fatty acids as the principal energy source during the latent stage of infection (50–52), and it has been shown that it stores fatty acids in the form of TAGs (51). Dormancy leads to high up-regulation of LipYtub, whereas a ΔlipYtub mutant has a severely compromised ability to degrade stored TAGs upon starvation (32). This indicates that LipY is involved in degradation of intracellular TAGs. In this study, we show that surface-exposed LipYtub can also hydrolyze extracellular lipids. LipY may therefore also be involved in fatty acid acquisition by hydrolyzing host lipids during infection. Mishra et al. (33) showed that the lipase activity of LipY is involved in the degradation of intracellular lipids during infection.

both LipY_{tub} and LipY_{mar} is significantly higher in the absence of the PE or PPE domains. Therefore, processing of surface-exposed LipY will result in increased lipase activity and more efficient hydrolysis of host lipids.

Intriguingly, LipY_{tub} was found to be neither surface-exposed nor processed when expressed in cultures of M. tuberculosis and M. bovis BCG. However, infection of THP-1 macrophages with M. tuberculosis or M. bovis BCG did result in LipY_{tub} processing, suggesting that the pathway required for secretion and/or processing in M. tuberculosis complex members is activated upon infection of host cells. Endogenous M. tuberculosis lipY is induced upon infection of macrophages (46), and it is conceivable that additional proteins required for LipY secretion and processing are also up-regulated during an infection. These proteins are not the ESX-5 system itself, which seems to be constitutively produced. Instead, it could for instance be a specific chaperone required for secretion or because proteins secreted via type VII secretion systems are often secreted as dimers, another ESX-5 substrate (3, 53).

Our data showing that the PE/PPE domain of LipY is essential for secretion conflict with the results presented in Ref. 33, where it was shown that localization of LipY_{tub} and LipY_{mar} in the cell wall of M. smegmatis and M. bovis BCG and on the bacterial surface of M. bovis BCG is independent of these domains. Moreover, our analysis in M. tuberculosis and M. bovis BCG showed that LipY is not surface-exposed when grown in culture. Although we cannot explain these differences, we have analyzed the LipY secretion pathway in more detail using ESX-5 mutants. Immunoblotting and whole cell immunogold labeling EM showed a clear lack of surface localization of both LipY_{tub} and LipY_{mar} in the ESX-5 mutant, demonstrating that these are indeed ESX-5 substrates. This was again confirmed in our whole cell lipase assay, in which the p-nitrophenyl stearate hydrolysis observed with LipY_{tub} in wild-type M. marinum was absent in the ESX-5 mutant strain. Some PE and PPE proteins are encoded in operons (18). One such couple, consisting of PE25 and PPE41, forms a complex (54) and is secreted by ESX-5 as a heterodimer. However, computational predictions suggest that many other PE and PPE proteins, including several that are encoded alone in the genome, also have cognate interaction partners (55). This raises the hypothesis that PE and PPE proteins are targeted to ESX-5 in pairs. Experiments are underway in our laboratory to investigate how PE and PPE domains may interact in targeting to ESX-5 and to identify the residues within the PE and PPE domains that constitute the ESX-5 secretion signal.

Acknowledgments—We are grateful to Edith Houben and Ben Appelmelk for helpful discussions and to Aniek van der Woude and Eveline Weerdenburg for technical assistance. We also thank Ida Rosenkraiz for kindly providing the ESAT-6 antibody.

REFERENCES

