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Dissecting the membrane lipid binding properties and lipase activity of the *Mycobacterium tuberculosis* LipY domains

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ABSTRACT

The *Mycobacterium tuberculosis* LipY protein, a prototype of the proline-glutamic acid (PE) family, exhibits a triacylglycerol (TAG) hydrolase activity that contributes to host cell-lipid degradation and persistence of the bacilli. LipY is found either as a full-length intracytosolic form or as a mature extracellular form lacking the N-terminal PE domain. Even though the contribution of the extracellular form in TAG consumption has been partly elucidated, very little information is available regarding the potential interactions of each form of LipY with either the cytoplasmic membrane for the full-length protein or with the outer membrane for the matured form. Herein, several LipY variants truncated in their N-terminal domain were produced and biochemically characterized in lipid-protein interaction assays using the monomolecular film technique and Fourier transform infrared spectroscopy. Comparison of the catalytic activities of these recombinant proteins showed that LipYΔ149, corresponding to the extracellular form of LipY lacking the PE domain, is more active than the full-length protein. This confirms previous studies reporting that the PE domain negatively modulates the TAG hydrolase activity of LipY. Lipid-protein interaction studies indicate that the PE domain anchors LipY onto membrane lipids. Consistent with these findings, LipYΔ149 was loosely-associated with the mycobacterial cell wall and that this interaction is mediated by the sole lipase domain. Altogether, our results bring new information regarding the molecular mechanisms by which LipY either binds and hydrolyzes host cell lipids or degrades TAG, the major source of lipids within mycobacterial intracytosolic lipid inclusions.
INTRODUCTION

Tuberculosis (TB), which is caused by the highly versatile pathogenic agent *Mycobacterium tuberculosis* (*Mtbt*), remains an important global health issue with more than 10 million new cases and approximately 1.6 million deaths in 2017 [1]. Upon infection, *Mtbt* uses several strategies to avoid and/or resist a wide range of microbicidal processes of immune cells. It can also persist for extensive periods of time within granulomas, resulting in a clinically asymptomatic latent tuberculosis infection (LTBI) [2, 3]. It is estimated that around two billion individuals are latently infected worldwide, providing a major reservoir for *Mtbt* [1]. Our understanding of the processes leading to LTBI establishment and reactivation at the molecular and cellular levels, remains an outstanding challenge for the scientific community and a crucial step for a better control of the disease [4]. It is assumed that *Mtbt*’s survival processes rely mainly on a very dynamic metabolic realignment within the granuloma microenvironment, where *Mtbt* preferentially uses fatty acids (FFA) as a carbon source during long-term infections [5-9]. Several studies demonstrated that pathogenic mycobacteria can utilize host-derived FFA to build up their own intracytosolic lipid inclusions (ILI), which will be further used as a source of nutrient [10-14]. The exact role of these neutral lipid-rich structures in mycobacterial pathogenesis remains elusive, but it has been proposed that ILI may promote *Mtbt* survival and persistence *in vivo* [8, 10, 11, 15]. However, the molecular bases of the dynamics of FFA acquisition and storage remain poorly understood [16].

*Mtbt* contains two specific families of proteins, designated PE and PPE proteins [17, 18], and while the PE proteins possess a conserved N-terminal domain of about 100 amino acids typified by a Pro-Glu signature motif, the PPE proteins possess a 180 amino acids N-terminal domain characterized by a Pro-Pro-Glu signature [19-21]. Due to their abundance in pathogenic mycobacteria, it has been postulated that PE and PPE proteins play important functions in mycobacterial survival and pathogenesis. Nevertheless, the real function of these proteins remains to be elucidated [21-23]. Several studies emphasized the participation of the PE domain in protein translocation, an event very likely to be mediated by the type VII secretion system ESX-5 [24-27]. Among the PE members,
Rv3097c, also known as LipY, is a 437 amino acid protein belonging to the Hormone-Sensitive Lipase family (HSL) possessing a C-terminal lipase domain [28-30]. Deb et al., (2006) demonstrated that LipY is a true triacylglycerol (TAG) lipase involved in intracellular TAG hydrolysis in *Mtb* upon carbon deprivation [29]. Moreover, a *Mtb* lipY-deficient mutant failed to escape from dormancy in an *in vitro* granuloma model [31]. Together, these results point out to LipY as an essential factor required for intracytosolic lipid catabolism and exit from a dormancy state. Subsequent work described that the dual location of LipY in mycobacteria and its secretion rely both on a well-defined sequence of events by the ESX-5 pathway [25]. The consensus YxxxxD/E motif within the PE domain allows the recognition/translocation by the ESX-5 machinery. The protein is subsequently cleaved by the MycP5 protease within the linker region between Gly^{149} and Ala^{150}, leading to the formation of an N-terminal truncated form associated with the mycobacterial cell surface [25]. In *M. marinum* LipY, the PE domain is substituted by a PPE domain [28] and the presence of this surface-exposed mature LipY strongly increases its TAG hydrolase activity [25]. Recently, we have demonstrated that a *M. bovis* BCG ΔlipY mutant is impaired in ILI formation within foamy macrophages, suggesting that LipY is an essential factor involved in host-derived TAG consumption [14]. Additional biochemical data indicated that LipY lacking its PE domain expresses increased TAG-hydrolase activity *in vitro* [28], suggesting that the PE domain acts as a modulator of the catalytic activity [14, 28]. Collectively, these findings imply that LipY plays a central role in TAG metabolism during the *Mtb* life cycle by participating in the hydrolytic processes of both extracellular and intracellular lipids. Nevertheless, little is known about the biochemical properties at the molecular level of the various forms of LipY as well as their respective contribution in hydrolysis of extracellular TAG contained in LB and/or intracellular TAG contained in ILI.

In this study, the shortest domain of LipY exerting hydrolytic activity has been defined and several recombinant variants of LipY have been characterized. We thus examined the ability of these proteins to interact with membranes using Langmuir monolayers as an *in vitro* model of cell membranes along with Fourier Transform InfraRed (FTIR) spectroscopy. Langmuir monolayers consisting of
supramolecular lipid films formed at an air-buffer interface are mimicking biological membranes and represent attractive membrane models [32] particularly suited to study membrane-protein interactions [33-37]. Fourier Transform InfraRed (FTIR) spectroscopy, performed in parallel to Langmuir monolayers, is a suitable technique to investigate the lipid membrane physical states (i.e., chain ordering, phase transition) occurring in presence of a protein [38-40].

Phosphatidylylycerol being the most abundant glycerophospholipid found in mycobacteria [41-43], 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) is often chosen as model phospholipid interfacial experiments. Therefore, combining both techniques can provide crucial information regarding interactions of protein with either the cytoplasmic or the outer membrane. In addition, in order to link their potential physiological roles in vivo, a set of LipY variants were either overproduced in lipid-rich persistent-like M. smegmatis, or in M. marinum which allows a constitutive translocation of these effectors through the ESX-5 machinery. Both mycobacteria were used as tools for establishing the respective role of the PE, linker and lipase domains in the translocation and maturation processes, thereby yielding essential information regarding the hydrolytic mechanisms of intra- as well as extracellular TAG.
RESULTS AND DISCUSSION

Expression and biochemical characterization of LipY and its truncated versions.

LipY is either found in the mycobacterial cytoplasm where it hydrolyzes ILI [29] or translocated via ESX-5 to the bacterial surface, where, upon cleavage of the N-terminal PE domain, the mature enzyme can interact and hydrolyze the host cell lipids [14, 25]. The contribution of the various LipY domains in its interfacial activity has, however, not yet been explored. To provide insights into their specific biological functions, we first generated LipYΔPE, LipYΔ149 and LipYΔ170 truncated forms, by i) removing the PE domain (LipYΔPE; lacking the first 97 residues), ii) deleting the first 149 residues (LipYΔ149; the mature form exposed to the mycobacterial cell wall surface) and iii) by further shortening the protein towards the C-terminus (LipYΔ170; consisting essentially of the C-terminal catalytic domain), respectively (Figure 1A). All genes, including full-length lipY, were cloned into the pSD26 and fused to a 6×His-tag encoding sequence and used to transform the M. smegmatis mc²155 groELΔC strain [44, 45]. Following induction with acetamide, cultures were harvested and the recombinant proteins were purified using Ni²⁺-charged immobilized metal affinity chromatography, yielding 15-20 mg of protein per L of culture. Following size exclusion chromatography, the purity of each protein was subsequently analyzed by SDS-PAGE gel (Figure 1B) and the nature of each protein was further confirmed by MALDI-TOF and N-terminal sequencing analyses (data not shown).

TAG with short, medium and long fatty acyl chains were used as substrates and assayed in the presence of each protein using the pH-stat technique [14, 46, 47]. As shown in Figure 1C-D-E, for each protein, the specific activity (SA) decreased gradually as a function of the lipid chain length. The removal of the first 170 residues did not affect the enzyme type selectivity. LipY, LipYΔPE, LipYΔ149 and LipYΔ170 hydrolyzed preferentially short-chain TAG (tributyrin) with a SA of around 119.0 ± 11.1, 185.0 ± 13.0, 215.0 ± 24.0 and 75.0 ± 6.0 U/mg, respectively. Regardless of the substrate used, LipYΔPE and LipYΔ149 were up to 1.8 times more active than full-length LipY (Figure 1F). LipYΔ170, the shortest LipY variant, exhibited SA of 75.0 ± 6.0, 16.0 ± 2.0 and 2.8
± 0.1 U/mg on tributyrin, trioctanoin and olive oil, respectively; and expressed the lowest activity as compared to the other proteins (Figure 1C-D-E). The SA of LipYΔPE and LipYΔ149 were comparable for tributyrin (185.0 ± 13.0 and 215.0 ± 24.0 U/mg, respectively), trioctanoin (63.0 ± 1.0 and 55.0 ± 5.0 U/mg, respectively) and for long-chain triolein (5.2 ± 0.2 and 6.3 ± 0.1 U/mg, respectively) used as substrates, suggesting that the first 53 residues of the linker motif had only a slight impact on LipY catalytic activity.

Moreover, since some lipases can also act as phospholipases, each LipY mutant form was also tested for their potential phospholipase A1 and A2 activities using a highly sensitive fluorescent-labeled phospholipid assay [43]. As anticipated and previously showed for LipY [43], none of the LipY truncated versions exhibited phospholipase activity.

Overexpression of LipY variants increased TAG consumption in M. smegmatis under lipid-rich persistent-like conditions.

It is well recognized that TAGs are major lipid storage molecules in bacteria belonging to the Actinobacteria phylum [48], including Mycobacterium [15, 49-52]. We recently showed that TAG accumulation under the form of ILI during infection in foamy macrophages was impaired when constitutively overproducing the cytoplasmic LipYΔPE in M. bovis BCG. These effects were neither observed with the full length protein nor with the catalytically-inactive mutant (LipYΔPES309A), supporting that the PE domain is directly influencing the activity of LipY towards both mycobacterial and host-derived TAGs [14, 28]. To gain additional insight and confirm our previous biochemical results, we investigated here the effect of the PE domain and the linker region with respect to lipase activity in vivo. M. smegmatis recombinant strains harboring either the empty pSD26 vector, pSD26::lipY, pSD26::lipYΔPE, pSD26::lipYΔ149 or pSD26::lipYΔ170 were grown for 48 h in a well-defined carbon rich/nitrogen limiting medium that promotes the induction of heavily lipid-loaded mycobacteria [53]. Cultures were harvested and re-suspended in a mineral salt medium devoid of glycerol but supplemented with 0.2% (w/v) acetamide to trigger catabolic reprogramming [53] and
the production of the respective LipY recombinant forms. Bacteria were collected at two distinct time
points (i.e. 6 h and 12 h), and lyophilized prior to apolar lipid extractions and thin layer
chromatography (TLC) for lipid profile analysis. As shown in Figure 2A, the overproduction of LipY
and its variants significantly reduced the intracellular pool of TAG by 37-51% after 6 h and up to
69% after 12 h, in comparison with the control strain carrying the empty vector. In each case,
LipYΔPE and LipYΔ149 were the most active forms in vivo, leading to respectively 26.4% and 19.0%
relative TAG levels, vs. a mean value of 36.0 ± 3.2% for LipY and LipYΔ170 after 12 h of induction.
These results are consistent with our biochemical data and with previous studies using either
genetically-modified M. smegmatis pSD26::lipY and M. smegmatis pSD26::lipYΔPE [28] or M. bovis
BCG pMV261::lipY and M. bovis BCG pMV261::lipYΔPE strains [14, 28].
To gain further insight into this molecular mechanism, 3D structural models were generated using
the I-TASSER server. As proposed previously [14, 54], the N-terminal PE domain could be easily
distinguished from the C-terminal domain and is composed of four α-helices (α1 to α4) (Figure 2B
– highlighted in blue) [14, 54]. Comparison of the LipY and LipYΔPE models [14] suggests that the
four α-helices of the PE domain cover the active site of the enzyme. This steric hindrance may be
responsible for a reduced substrate accessibility and for the lower catalytic activity of the full-length
protein, as previously proposed [14, 28, 55]. Concerning the linker motif, this 53 amino acid region
comprises three α-helices (Figure 2B-C – highlighted in green). From its location, this region is very
likely to be extremely flexible, hence unable to induce a steric barrier masking the catalytic site. The
3D model of LipYΔ149 displays a similar “open” active site (Figure 2D). These observations are in
agreement with the biochemical data. In contrast, amputation of the next 21 amino acids in
LipYΔ149, yielding LipYΔ170 (Figure 2D – highlighted in orange; and Figure 2E) triggers a sharp
drop in the lipase activity (Figure 1F). Since this deletion occurs directly in a α-helix, one can
speculate it would lead to severe conformational alterations affecting catalysis.
From these results, it could be inferred that the increased activity shared by LipYΔPE and
LipYΔ149, as compared to LipY, would result from a better accessibility and/or recognition of the
lipid to the active site, due to the absence of the PE domain. In addition, the 21 first amino acid
residues of the linker region in LipYΔ149 (Figure 2D-E) seem to be essential for the enzyme activity,
possibly by stabilizing this extracellular mature form in a suitable conformation within the cell wall
and during TAG hydrolysis, as previously proposed [14, 28, 55].

**Interactions and binding capacity of LipY and its truncated forms with model membrane lipids.**

To get additional elements regarding the anchoring process of LipY within biological membranes,
we investigated how the LipY domains influence the adsorption capacity of the protein using the non-
hydrolysable 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) monomolecular films.

This was achieved by measuring the interfacial binding properties of each LipY variant onto DOPG
monolayers, first by determining the effect of initial surface pressure ($\Pi_i$) on the interaction of the
different protein forms with the monolayer. For this purpose, the increase in surface pressure ($\Delta\Pi_{\text{max}}$),
directly reflecting the variation of DOPG molecular area (i.e. lipid packing) upon protein adsorption,
was monitored immediately following protein injection at various $\Pi_i$ values, ranging from 5 to around
20 mN/m. The plot $\Delta\Pi_{\text{max}} = f(\Pi_i)$ depicted in Figure 3A allowed to evaluate the adsorption
parameters of LipY and its derivatives onto DOPG monolayer. In all cases, the $\Delta\Pi_{\text{max}}$ was found to
decrease linearly with the increase of $\Pi_i$. Linear extrapolation to zero surface pressure increase
($\Delta\Pi_{\text{max}} = 0$) allowed to estimate the critical surface pressure ($\Pi_c$) [56] (also called “maximum
insertion pressure” [57]) as being equal to 22.5 ± 1.2, 17.2 ± 0.83, 16.4 ± 0.49 and 14.3 ± 0.25 mN.m$^{-1}$
for LipY, LipYΔPE, LipYΔ149 and LipYΔ170 adsorbed onto DOPG film, respectively. Above
these $\Pi_c$ value, specifically related to the protein and the lipid forming the monolayer, no increase in
the surface pressure occurred [56]. The full-length protein appeared as the most tensioactive form
binding to DOPG monolayers, followed by LipYΔPE and LipYΔ149, while LipYΔ170 poorly
adsorbed onto the phospholipid membrane. It appears that the ranking in the penetration capacity
(i.e., $\Pi_c$ values) of the four proteins is directly related to the size of the deletions.
The plots depicted in Figure 3A can also provide additional information regarding the binding parameters of these enzymes (Table 2), such as $\Delta \Pi_0$ ($y$-intercept of the curves corresponding to $\Pi_c = 0$) and the synergy factor noted “$a$” (slope of the linear regression + 1) introduced by Salesse’s group [58-60], where a positive “$a$” value is linked to favorable binding of the protein and the $\Pi_c$ represents an insertion surface pressure. In contrast, a negative synergy factor correlates with unfavorable binding of a protein to a phospholipid monolayer whereas the associated $\Pi_c$ corresponds to an exclusion surface pressure. Finally, an “$a$” value close to zero corresponds to a stationary state where the binding of the protein is neither favored nor disfavored by the lipid monolayer.

Based on these rules, the positive value of the synergy factor (+0.113 ± 0.010) and the occurrence of a $\Delta \Pi_0$ (20.0 ± 1.1 mN.m$^{-1}$) lower than the related $\Pi_c$ (22.5 ± 1.2 mN.m$^{-1}$) observed for LipY was consistent with a high penetration capacity of LipY onto phospholipid films. In contrast, with LipYΔ170 ($a = -0.177 ± 0.005$; $\Delta \Pi_0 = 16.9 ± 0.3$ mN.m$^{-1}$) and LipYΔPE ($a = -0.564 ± 0.043$; $\Delta \Pi_0 = 26.9 ± 1.3$ mN.m$^{-1}$), the negative $a$ values and a $\Delta \Pi_0$ larger than the corresponding $\Pi_c$ were correlated with a repulsion of both enzymes as a function of the compactness of the monolayer.

Regarding LipYΔ149, a negative but almost close to zero synergy factor ($-0.023 ± 0.001$) was determined and the $\Delta \Pi_0$ (16.8 ± 0.5 mN.m$^{-1}$) was not significantly different from the $\Pi_c$ value (16.4 ± 0.49 mN.m$^{-1}$). These results reflect that the binding of LipYΔ149 onto DOPG monolayer is neither favored nor disfavored. As a consequence, the decrease in the adsorption capacity of this protein may only be related to the reduction of the “free” area due to an increase in the lipid packing with the surface pressure [58, 60].

It can be inferred that, upon deletion of the PE domain and/or the linker unit, the proteins are excluded from the DOPG monolayer, in contrast to the full-length protein which retains the capacity to bind to phospholipid films. Thus, the PE domain may, presumably, favor the adsorption of LipY onto DOPG monolayers, thereby playing a key role in the penetration/binding of LipY to membranes. Conversely, cleavage of the N-terminus results in a mature protein that remains loosely attached to the cell wall, as proposed by Daleke et al. [25].
IR spectroscopy was subsequently used to study the protein to lipid interaction from a molecular point of view. Since lipid molecules are active in infrared (IR) through their hydrophobic [61], interfacial and polar head group [62], the molecular characterization of phospholipid assembly and phenomena affecting the behavior of the hydrophobic core of the lipid molecule [63-65] was followed in the presence or absence of the different proteins. Here, multilamellar liposomes of DOPG were used as a simple model for biomembranes and the conformational changes of lipids induced by the interaction of the acyl chain region with LipY and its mutants was monitored by analysis of the methylene stretching band vibration (Figure 3B). This region of IR spectra is indeed dominated by two main bands namely symmetric ($\nu_s$(CH$_2$)) and antisymmetric ($\nu_{as}$(CH$_2$)) methylene stretching located near 2850 and 2920 cm$^{-1}$, respectively (data not shown). The thermotropism of lipid is characterized by a shift of the wavenumber of these stretching vibration bands, which are sensitive to the presence of gauche conformers [66-68], making them useful probes for following lipid phase transition and membrane fluidity. The Figure 3B displays the temperature dependence of $\nu_{as}$(CH$_2$) vibration of the DOPG acyl chain in the absence and the presence of recombinant proteins. According to the literature, the transition temperature (Tm) for pure DOPG is -18°C [69, 70]; therefore all experiments were carried out in the DOPG liquid-crystalline phase. All the wavenumbers of $\nu_{as}$(CH$_2$) mode from 27 to 40°C were lower in the presence of each protein form in comparison to the pure lipid, indicating that all the proteins studied altered the acyl chain conformation. These changes have been attributed to the presence of hydrophobic protein segments within the hydrophobic core of lipid membranes increasing the steric hindrance and therefore decreasing the membrane flexibility [71, 72]. However, while the differences of the $\nu_{as}$(CH$_2$) of the lipid alone and in the presence of each protein was significant, the impact of each protein on the conformational change of the acyl chains was difficult to quantify. Above 40°C, the wavenumber of the $\nu_{as}$(CH$_2$) mode in the presence of LipY was still below the wavenumber of the lipid alone, meaning that the protein was still interacting with the acyl chains of lipid membranes, while the effect of the LipY mutant forms (LipYΔPE, LipYΔ149 and LipYΔ170) was less obvious. This suggests that LipY strongly interacts with the lipid chains.
while this interaction is weaker for the mutants. Thus, regardless of the temperature, LipY induces a stronger conformational change in the lipid acyl chains, than the different mutants, suggesting that the presence of the PE domain enhanced the insertion within the hydrophobic core of the bilayers while the extracellular LipYΔ149 is weakly anchored to DOPG liposome. Overall, these results are in agreement with those obtained using DOPG monomolecular films.

Translocation through ESX-5 and anchoring investigations of LipY and its truncated forms \textit{in vivo}.

Mycobacteria belonging to the \textit{M. tuberculosis} complex express LipY in infected cells but are unable to do so when growing \textit{in vitro} under standard laboratory conditions [25, 28]. Furthermore, \textit{M. smegmatis} lacks an ESX-5 secretion system. Thus, to further delineate LipY processing and anchoring to the mycobacterial cell wall, studies were done in \textit{M. marinum}, which is able to translocate and mature LipY through the ESX-5 secretion machinery [25]. To achieve this goal, pVV16-based constructs allowing the constitutive expression of the lipY truncated versions fused to a C-terminal His-tag (Figure 4A) were generated. The corresponding \textit{M. marinum} strains were grown and bacterial pellets subjected to detergent extraction using Genapol\textsuperscript{®}-X080, a powerful non-ionic detergent which allows to solubilize proteins that are localized within the capsule and the mycomembrane without impacting the cytoplasmic membrane (CM), the peptidoglycan (PG) or arabinogalactan (AG) fractions [25, 27, 73-75]. Proteins were then revealed by immunoblotting using a HisProbe\textsuperscript{TM} HRP conjugate [25] (Figure 4B-C). As expected, two immunoreactive bands were detected in the strain harboring pVV16::lipY, corresponding to the mature (black arrow) and cytoplasmic forms (grey arrows) (Figure 4C – upper panel). When cells were treated with Genapol\textsuperscript{®}-X080, the mature protein was only detected in the surface-exposed fraction, demonstrating that translocation and maturation occurred. Two other variants, LipYΔ149 and LipY\textsuperscript{E92A} (carrying a point mutation within the YxxxD/E consensus secretion signal abrogated maturation and secretion of the protein) were also included as cytoplasmic controls [25, 26] (Figure 4B). LipY maturation catalyzed
by the MycP5 protease occurred at a specific site between Ser\textsuperscript{148}, Gly\textsuperscript{149} and Ala\textsuperscript{150} [25]. However, that point mutations at each of these three residues failed to abolish maturation and secretion of the protein suggested alternative cleavage sites [25]. Our results, using a LipY\textsuperscript{G149D} mutant, support the findings by Daleke \textit{et al.} [25] (Figure 4C).

Overall, these data prompted us to investigate the contribution of the linker domain in both translocation and anchoring processes and to define whether the S\textsuperscript{148}GA\textsuperscript{150} motif and the linker domain were required for maturation and surface localization of the protein in \textit{M. marinum}.

Therefore, two additional LipY mutated proteins were constructed by fusion of the PE domain to the S\textsuperscript{148}GA\textsuperscript{150} motif to retain the putative proteolytic site and by deleting: \textit{i}) either the 51 residues of the linker motif to generate a PE-SGA-LipYΔ149 recombinant protein; or \textit{ii}) the entire linker motif thus producing the PE-SGA-LipYΔ170 chimera (Figure 4A). In both cases, the chimeric proteins PE-SGA-LipYΔ149 and PE-SGA-LipYΔ170 were translocated and found anchored to the mycobacterial surface (Figure 4C). Interestingly, a single band of approximately 30 kDa was detectable for the PE-SGA-LipYΔ149 variant, suggesting that the cleavage may have occurred, as for a WT protein, at the G\textsuperscript{149}A\textsuperscript{150} position. In contrast, concerning the PE-SGA-LipYΔ170-expressing strain, two distinct bands of lower molecular weights (~ 26-28 kDa) were detected, suggesting two different cleavage sites (Figure 4C). To precisely define these cleavage sites, \textit{M. marinum} displaying the pVV16::PE-SGA-lipYΔ170 was treated with Genapol\textsuperscript{®}-X080. The supernatant containing the cell wall-associated proteins was then loaded onto a Ni\textsuperscript{2+} NTA affinity column to purify the 6×His-tagged proteins. Using this approach, a single and pure band of approximately 28 kDa, highly reactive with the 6×HisProbe, was detected (Figure 4D). Despite several attempts, we, however, failed to co-purify the second band with a lower molecular weight. N-terminal sequencing by using Edman degradation on pure fractions containing the 28 kDa band identified a protein cleaved just after the S\textsuperscript{148}GA\textsuperscript{150} motif, thus leading to the sole lipase domain starting at the sequence E\textsuperscript{171}THFA. Interestingly, this mature LipYΔ170 form lacking the linker region was able to bind the mycobacterial cell wall, demonstrating, for the
first time, that if the linker domain does not seem to be essential for anchoring the protein to the mycomembrane, it remains crucial for full lipase activity (Figure 1F).

Concluding remarks

Understanding the physiological properties of lipid-rich persistent-like bacilli at both cellular and molecular levels, and more precisely how mycobacteria utilize host-derived lipids for building-up their own ILI is crucial. We show here that the secreted LipYΔ149 protein was not only more active than its full-length cytoplasmic form (Figure 5), but was also essential for intraphagosomal-TAG breakdown, thus leading to lipid-rich persistent-like mycobacterial phenotype within foamy macrophages. Our biochemical characterization of several truncated forms of LipY confirmed that the N-terminal PE domain negatively affects the TAG hydrolase activity of the protein by generating a steric hindrance in the vicinity of the active site. By combining biochemical and biophysical approaches, we also demonstrate that the PE domain affects also the lipid binding activity onto phospholipid monolayers and liposomes (Figure 5). Both FTIR spectroscopy and monomolecular film experiments emphasized the PE-mediated anchoring capacity of LipY within phospholipids. Mycobacteria cell fractionation followed by immunoblotting strongly suggests that the linker region is dispensable for proper maturation and localization, but remains crucial for the enzymatic activity. Altogether, we provide compelling evidence that the PE domain as well as the linker region impact on the enzymatic properties of LipY by distinct molecular mechanisms, which are directly linked to its physiological substrates either host-derived or intracellular TAG in the form of ILIs (Figure 5).
EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

_E. coli_ DH10B cells (_Life technologies_, Saint Aubin, France) were grown at 37°C in Luria Bertani (LB) broth (_Euromedex_, Souffelweyersheim, France) or onto LB agar plates. Culture media were supplemented with 200 µg/mL hygromycin B or 50 µg/mL kanamycin (_Euromedex_, Souffelweyersheim, France). The _M. smegmatis_ mc^2155 groELAC strain [44] was usually grown at 37°C under shaking (220 rpm) in complete Middlebrook 7H9 medium (_BD-Difco_) supplemented with 0.05% Tween 80 (v/v), 0.2% glycerol (v/v), 0.5% bovine serum albumin (BSA) (w/v), 0.2% glucose (w/v). The _M. marinum_ M strain [76] was grown at 32°C under shaking (220 rpm) in Middlebrook 7H9 medium supplemented with 0.05% Tween 80 (v/v), 0.2% glycerol (v/v) and 10% oleic acid, albumin, dextrose, catalase (OADC enrichment; _BD-Difco_). Transformants were selected on Middlebrook 7H9 agar containing either 50 µg/mL hygromycin B or 50 µg/mL kanamycin. Plates were incubated at 37°C for 3-5 days for _M. smegmatis_ mc^2155 groELAC and at 32°C for 10-15 days for _M. marinum_.

Construction of plasmids

For construction of pSD26::lipY and pSD26::lipY^ΔPE, _M. tuberculosis_ H37Rv genomic DNA was used as template as previously described [28]. The _lipY_ gene was amplified by PCR using primers pSDlipY-F and pSDlipY-R and _lipY^ΔPE_ was PCR-amplified using primers lipY^ΔPE^-F and pSDlipY-R (Table 1). The corresponding amplicons harboring specific restrictions sites were digested with BamHI and cloned into pSD26 [77] under the control of the acetamidase inducible promoter and used to express and purify the recombinant proteins in _M. smegmatis_.

To construct pSD26::lipY^Δ149 and pSD26::lipY^Δ170, the _lipY^Δ149_ and _lipY^Δ170_ genes were amplified from pSD26::lipY using the primers pSDlipY^Δ149^-F/pSDlipY^ΔPE^-R and pSDlipY^Δ170^-F/pSDlipY-R respectively (Table 1). The pSD26::lipY^{G146D} and pSD26::lipY^{E92A} constructs were...
generated by site directed mutagenesis using primers G149D-F/R and E92A-F/R primers, respectively (Table 1).

To generate pSD26::PE-SGA-lipYΔ149 and pSD26::PE-SGA-lipYΔ170, a two-step cloning procedure was applied. Briefly, the PE domains for PE-SGA-lipYΔ149 and PE-SGA-lipYΔ170 genes were amplified from pSD26-lipY using primers pSDlipY-F/PE-149-R and pSDlipY-F/PE-170-R, respectively. In parallel, the lipYΔ149 and lipYΔ170 fragments were PCR-amplified from pSD26::lipY using primers PE-149-F/pSDlipY-R and PE-170-F/pSDlipY-R, respectively. The complete DNA fragments corresponding to PE-SGA-lipYΔ149 and PE-SGA-lipYΔ170 were obtained by overlapping PCR using primers pSDlipY-F and pSDlipY-R along with a mixture of the both DNA template (Table 1). The final PCR products were purified, digested with BamHI and cloned into BamHI-restricted pSD26, yielding pSD26::PE-SGA-lipYΔ149 and the pSD26::PE-SGA-lipYΔ170.

To produce the constitutive expression vectors, lipY, lipY_{E92A}, lipY_{G149D}, PE-SGA-lipYΔ149 and PE-SGA-lipYΔ170 were PCR-amplified from their respective pSD26 derivatives using primers pVV-lipY-F/pVV-lipY-R. The lipYΔ149 gene was amplified using pSD26::lipYΔ149 as template and primers pVV-lipY-F/pVV-lipYΔ149-R. All DNA fragments were further digested with NdeI and HindIII restrictions enzymes (Promega, Charbonnieres, France) and subsequently cloned within pVV16 in frame with a C-terminal 6×Histidine coding sequence. The resulting plasmids were introduced in E. coli DH10B, analyzed by DNA sequencing (GATC Biotech, Germany) and used to transform M. marinum, as previously described [78].

Expression and purification of recombinant proteins

Expression and purification of recombinant proteins were performed as previously reported [45] with some modifications. Briefly, M. smegmatis mc^2155 groELΔC strain carrying pSD26-lipY or truncated forms of lipY or mutated lipY, were used to inoculate 20 mL of complete 7H9 Middlebrook medium containing 50 μg/mL hygromycin B during 3 days at 37°C under shaking (220 rpm). The preparation (OD_{600nm} = 3.0-6.0) were used to inoculate 400 mL of culture medium (OD_{600nm} = 0.1)
for a large-scale production. Bacteria were grown at 37°C with shaking (220 rpm) until an \( \text{OD}_{600\text{nm}} \) value between 2.5 and 3.0 was reached and protein expression was induced by adding acetamide (\textit{Sigma-Aldrich}, Saint-Quentin Fallavier, France) to a final concentration of 0.2% (w/v) for 16 h. Bacteria were harvested, re-suspended in ice-cold buffer A (30 mL 10 mM Tris/HCl pH 8.0, 150 mM NaCl) containing 1% \( N \)-lauroylsarcosine and were broken using a French Pressure cell at 1,100 psi. After centrifugation, the supernatant (S1) was recovered while the resulting pellet was re-suspended in buffer A (30 mL) and sonicated twice during 30 s with 30 s breaks between each cycle and stirred overnight at 4°C. After centrifugation, the new supernatant (S2) was pooled with S1 supernatant and both supernatants were loaded onto a Ni\(^{2+}\)-NTA resin beforehand equilibrated with buffer A. The column was subsequently washed with buffer A without detergent prior to elution with increasing concentrations of imidazole. The eluted fractions were analyzed by performing on 12% SDS/PAGE as described by [79]. Fractions containing pure proteins were pooled, purified by size exclusion chromatography with a Hiload 16/60 Superdex 200 gel filtration column using buffer A; then concentrated by ultrafiltration to a final concentration of 0.6 mg/mL and stored at -80°C. Theoretical physical properties (molecular mass, extinction coefficient at 280 nm and isoelectric point) of all proteins containing the 6×His-tag were obtained from the ProtParam tool (http://ca.expasy.org/tools/protparam.html).

**Lipase activity**

Enzymatic hydrolysis of TAG emulsions, namely tributyrin (TC4), trioctanoin (TC8) or olive oil (TC18), were monitored titrimetrically for 10 min at 37°C using a pH-stat (Metrohm 718 STAT Titrino; \textit{Metrohm Ltd.}, Switzerland). Assays were performed in 2.5 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl and 3 mM NaTDC (Sodium taurodeoxycholate). Free fatty acids (FFA) released were automatically titrated with 0.1 N NaOH (0.01 N NaOH for titration the free fatty acids derived from olive oil) to maintain a fixed end-point pH value of 7.5. The specific activities of
enzymes were expressed in units per mg of pure enzyme. One unit corresponds to the release of one µmole of fatty acid per minute.

**Generation of ILI-positive cells, lipid extraction and analysis**

*M. smegmatis* strains harboring plasmids pSD26, pSD26::lipY, pSD26::lipYΔPE, pSD26::lipYΔ149 and pSD26::lipYΔ170 were grown in 7H9 Middlebrook complete medium containing 50 µg/mL hygromycin B at 37°C under shaking 220 rpm until an OD_{600nm} value between 1-1.5 was reached. After centrifugation for 10 min at 5,000 g, pellets were then washed with sterile Phosphate Buffer Saline (PBS) buffer pH 7.4 containing 0.05% Tween-20 (PBS-T), with classic sterile PBS buffer and finally normalized and re-suspend at OD_{600nm} = 10 in sterile PBS. Subsequently, solution of PBS-containing bacteria was used to inoculate with an initial OD_{600nm} of 0.05 a fresh Minimal Mineral Salt Medium Nitrogen Limiting (MSM NL) (Na_{2}HPO_{4} 2 g/L, KH_{2}PO_{4} 1 g/L, NaCl 0.5 g/L, MgSO_{4} 0.2 g/L and NH_{4}Cl 0.05 g/L) containing 1% glycerol as carbon source as previously described [53]. Cells were grown for 48 h at 37°C and 220 rpm in the presence of 50 µg/mL hygromycin B and 0.02% (v/v) tyloxapol to avoid any clump formation. Induction of recombinant proteins was performed by adding 0.2% (w/v) acetamide. After 6 or 12 h of induction, the cells were collected by centrifugation during 15 min at 5,000 g.

Cells were washed three times in distilled water, lyophilized overnight and weighed to calculate the exact mass of mycobacterial dry extract. Apolar lipids were extracted as previously described [80]. Briefly, 2 mL of MeOH-0.3% NaCl (10:1, v/v) were added per 50 mg dry extract. The saline-MeOH solution containing the bacterial dry extract was mixed with 1 mL of petroleum ether in Pyrex® tube and incubated at RT onto a tube rotator for at least 15 min. After centrifugation at 3,000 g during 5 min, the upper organic layer was transferred to a fresh tube. This step was repeated three times and a final centrifugation was done for 15 min at 3,000 g to remove residues carried over during the extraction. The upper organic layer containing apolar lipids was transferred to a fresh pre-weighted vial, and the solvent was evaporated to dryness under a stream of nitrogen. The obtained apolar lipids.
fraction containing TAG were then re-suspended in 300 µL dichloromethane and analyzed by Thin layer chromatography (TLC) using aluminum TLC plates (Silica Gel 60, Merck) using heptane/diethyl ether/formic acid mixture (55:45:1 v/v/v) as eluent. The spots were visualized by vaporization of a saturated copper acetate-85% orthophosphoric acid (1:1, v/v) and charring. Plates were then scanned using a Chemidoc™ MP Imaging System (Bio-Rad) and densitometric analyses done using the ImageLab™ software version 5.0 (Bio-Rad) to determine relative TAG content in each sample.

In silico protein modelling

All three-dimensional model structures were built with the automatic protein structure homology modeling server using the I-Tasser software program [81, 82]. LipY, LipYΔPE were generated using the 4Q3O PDB code [83] as structural template and were already published in [14]. Regarding LipYΔ149 and LipYΔ170 their 3D-model structures were generated using the 4XVC PDB code [84] as template. The final model structures of LipYΔ149 (93% coverage; normalized Z-score = 3.01; C-score=0.09) and LipYΔ170 (97% coverage; normalized Z-score = 3.03; C-score=0.13) were visualized using the PyMOL Molecular Graphics System (version 1.4, Schrödinger, LLC).

Enzyme adsorption kinetics onto DOPG monomolecular films

All experiments were performed at room temperature (RT) using home-made Teflon trough (volume, 9.4 mL; surface area, 8.5 cm²) and the KSV5000 barostat equipment (KSV Nima, Helsinki, Finland) equipped with a Langmuir film balance to measure the surface pressure (Π), and monitored by the KSV Device Server Software v.3.50 as previously described [57, 85]. Before each experiment, the Teflon trough was cleaned with tap water, and then gently brushed in the presence of distilled ethanol, before being washed again with tap water and abundantly rinsed with Milli-Q water. The Teflon trough was filled with 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, prepared with Milli-Q water and filtered through a 0.45 µm Millipore membrane. Residual surface-active impurities
were removed before each experiment by simultaneous sweeping and suction of the surface. The monolayer was prepared by spreading a few microliters of a DOPG solution (1 mg/mL in chloroform) over the clean air/buffer interface of the cylindrical trough using a high precision Hamilton microsyringe until the desired initial surface pressure \( (\Pi_0) \) was reached. The waiting time for the spreading solvent evaporation and for the film to reach equilibrium vary from 10 to 20 min depending on the volume spread and the initial surface pressure. After solvent evaporation and stabilization of the film, the lipase was injected into the aqueous subphase at a final concentration of 40 nM and the surface pressure increase due to the adsorption/penetration of the lipase onto the DOPG monomolecular film was continuously recorded until the equilibrium surface pressure \( (\Pi_e) \) was reached [56, 57]. At this stage, data recording was maintained to ensure that a plateau value in terms of surface pressure had been well reached. The aqueous sub-phase was continuously stirred with a 1-cm magnetic bar stirring at 250 rpm.

**Fourier Transform infrared (FTIR) spectroscopy**

**Sample preparation**

Multilamellar liposomes were obtained by hydrating 2% (w/v) DOPG powder with 100 mM phosphate buffer pH 8 containing 150 mM NaCl. Samples were vortexed extensively above and below the main phase transition temperature \( (T_m = \sim 18^\circ C) \), by using liquid nitrogen (N\(_2\)). Three heating and cooling cycles were carried out. Protein lipid interaction was carried out by adding different protein sample concentrated to 1 mg/mL to the lipid mixture in order to reach a 1:10 (w/w) protein: lipid ratio. Control sample was made by adding the same volume of protein buffer to the lipid mixture.

**FTIR measurement**

IR spectra were recorded with a Jasco FT-IR 6100 equipped with a liquid N\(_2\) refrigerated Mercury-cadmium-telluride detector, the spectrometer was continuously purged with dried air. Spectra were collected using samples solution placed between two CaF\(_2\) windows separated with 5 \( \mu \)m
polyethylene terephthalate film spacers. Then, the FTIR cell was placed in a thermostated cell holder. Temperature was controlled with a pike technologies temperature controller working with the Peltier effect. The sample was equilibrated for 5 min at the required temperature before beginning the recording. The FTIR measurements were recorded between 4,000 and 800 cm\(^{-1}\). Each spectrum was obtained by averaging 88 scans recorded at a resolution of 0.5 cm\(^{-1}\). In order to determine the symmetric \(\nu_s(\text{CH}_2)\) and antisymmetric \(\nu_{as}(\text{CH}_2)\) methylene stretching wave numbers, a polynomial baseline was subtracted (Jasco spectra analysis software) in order to overcome the water stretching vibration contribution. All the data were obtained in duplicate from independent samples.

**Extraction of surface-exposed proteins and immunoblotting**

Approximately 10 OD\(_{600}\) unit of bacterial cultures were harvested for 10 min at 4,000 g and the pellet washed twice in PBS containing 0.05% Tween-20. Surface-exposed proteins were then isolated by incubating the bacteria with PBS containing 0.5% Genapol\textsuperscript{®}-X080 (v/v) (Sigma-Aldrich, Saint-Quentin Fallavier, France) at RT, as previously reported [25]. Control samples were treated with a PBS buffer devoid of Genapol\textsuperscript{®}-X080 detergent. After 30 min, supernatants were collected and precipitated with a final concentration of 12% trichloroacetic acid and proteins separated onto a 12% SDS-PAGE and transferred onto a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (Bio-Rad). Immunoblotting of \(\delta\)His-tagged proteins was performed using a HisProbe\textsuperscript{TM} HRP conjugate (Thermo-Scientific). The exported MMAR\_0427 (which shares 89% of identity with the \textit{M. tuberculosis} monoglyceride lipase Rv0183 protein found to be exported to the mycobacterial cell-wall and involved in mycobacterial cell-wall remodeling [46, 86]) was used as control for subcellular location. Immunoblotting was performed by cross reaction using rabbit polyclonal antibodies and horseradish peroxidase-conjugated anti-rabbit IgG (Sigma Aldrich). Detection was achieved using Pierce\textsuperscript{TM} ECL Western Blotting substrate solution (Thermo-Scientific) and visualized with a ChemiDoc\textsuperscript{TM} MP System (Bio-Rad).
N-terminal sequencing of surface-exposed PE-SGA-LipYΔ170

*M. marinum* harboring pVV16::PE-SGA-lipYΔ170 was grown till the OD reaches 1-1.5 and collected by centrifugation 15 min 5,000 g at 4°C. The pellet was then washed twice with PBS and resuspended in PBS containing 0.5% Genapol®-X080 and stirred for 1 h at RT. The supernatant was recovered and precipitated with a final concentration of 12% trichloroacetic acid. Proteins were solubilized in 10 mM Tris/HCl pH 8.0, 150 mM NaCl buffer containing 8M urea and subjected to Ni²⁺-NTA affinity chromatography. The eluted fractions were analyzed by 12% SDS/PAGE [79] and those containing pure proteins were pooled and transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was stained with Ponceau Red and the corresponding band was excised from the gel, washed 3 times in ethanol/water (90:10) solution, dried and subjected to N-terminal Edman sequencing using a Shimadzu PPSQ 31B protein sequencer.
ACKNOWLEDGMENTS

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AUTHOR’S CONTRIBUTIONS

Conceived and design the experiments: PS, JFC, LK and SC

Performed the experiments: PS, NS, SD, IP, VP and HG

Analyzed the data: PS, NS, SD, VP, HG, JFC, LK and SC

Contributed reagents/materials/analysis tools: JFC, LK and SC

Wrote the manuscript: PS, HG, JFC, LK and SC with the help of CdC

All this work was supervised by SC

ABBREVIATIONS

Proline-Glutamic acid (PE); Fourier Transform infrared spectroscopy (FTIR); Tuberculosis (TB);
Latent Tuberculosis (LTBI); Intracellular Lipid Inclusion (ILI); Hormone-Sensitive Lipase (hHSL);
Triacylglycerol (TAG); Fatty acids (FFA); Specific Activity (SA); 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG); Polyvinylidene fluoride (PVDF); Luria Bertani (LB); Tributyrin (TC4);
Trioctanoin (TC8); Triolein (TC18); Room temperature (RT); Phosphate Buffered Saline (PBS);
Minimal Mineral Salt Medium Nitrogen Limiting (MSM NL); Thin layer chromatography (TLC).
The authors have no conflict of interest to declare.
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804 Characterization of an exported monoglyceride lipase from Mycobacterium tuberculosis possibly
Table 1. Primers used in this study. Nucleotides of the primers that differ from the wild-type sequence are presented in bold and restriction sites incorporated into the primers are underlined and their name is indicated in parenthesis.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5’ → 3’</th>
<th>Origin</th>
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</thead>
<tbody>
<tr>
<td>pSDlipY-F</td>
<td>AAA<strong>GGATCC</strong>GTGTCCTTATGTTGTTCGTTGG (BamHI)</td>
<td>[28]</td>
</tr>
<tr>
<td>pSDlipY-R</td>
<td>A<strong>AGGATCC</strong>GGCGATACCGAGTTGGCG (BamHI)</td>
<td>[28]</td>
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<tr>
<td>pSDlipYΔPE-F</td>
<td>AAA<strong>GGATCC</strong>TTGCCACGCGGATACCGAGTTGGCG (BamHI)</td>
<td>[28]</td>
</tr>
<tr>
<td>pSDlipYΔPE-R</td>
<td>A<strong>AGGATCC</strong>GGCGATACCGAGTTGGCG (BamHI)</td>
<td>[28]</td>
</tr>
<tr>
<td>pSDlipYΔ149-F</td>
<td>AAA<strong>GGATCC</strong>GTGCCACGCGGATACCGAC (BamHI)</td>
<td>This study</td>
</tr>
<tr>
<td>pSDlipYΔ170-F</td>
<td>AAA<strong>GGATCC</strong>GAAACTCACTTTGCAATCCCAAATCCAC (BamHI)</td>
<td>This study</td>
</tr>
<tr>
<td>E92A-F</td>
<td>GCCGCCGCGCGCTGGCCAAC</td>
<td>This study</td>
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<tr>
<td>E92A-R</td>
<td>G<strong>TGGC</strong>CCGGCAGGCCGCGG</td>
<td>This study</td>
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<tr>
<td>G149D-F</td>
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</tr>
<tr>
<td>G149D-R</td>
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<td>This study</td>
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<tr>
<td>PE-149-F</td>
<td>CTGGCCAATGCTAGTGGGCGGATCCTAGTTGGGCAATCCACTTTGCC</td>
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<td>PE-149-R</td>
<td>G<strong>TGC</strong>AATCCGGCATGCCAATCCACTTTGCC</td>
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<td>PE-170-R</td>
<td>G<strong>C</strong>AAAGTGAGTTCCACGCGCCACTAGTAATGCC</td>
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<tr>
<td>pVV-lipY-F</td>
<td>GGAATCACTTCG<strong>CATATG</strong>GTGTCCTTATGTTGTTCGTTGGCG (NdeI)</td>
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<tr>
<td>pVV-lipY-R</td>
<td>GTGGTGGGTGGTG<strong>AGCTT</strong>GCGCGATCCTGAGTTGGCTGGCTG (HindIII)</td>
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<tr>
<td>pVV-lipYΔ149-F</td>
<td>GGAATCACTTCG<strong>CATATG</strong>GCGCGGCTCGATCCGATCCTGACATCGC (NdeI)</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2. Binding parameters ($\Pi_c$, synergy and $\Delta\Pi_0$) of LipY, LipYΔ149, LipYΔ170 and LipYΔPE in the presence of a DOPG Monolayer $^a$

<table>
<thead>
<tr>
<th></th>
<th>LipY</th>
<th>LipYΔ149</th>
<th>LipYΔ170</th>
<th>LipYΔPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Pi_c$ (mN.m$^{-1}$)</td>
<td>22.5 ± 1.2</td>
<td>16.4 ± 0.49</td>
<td>14.3 ± 0.25</td>
<td>17.2 ± 0.83</td>
</tr>
<tr>
<td>synergy</td>
<td>+0.113 ± 0.010</td>
<td>−0.023 ± 0.001</td>
<td>−0.177 ± 0.0045</td>
<td>−0.564 ± 0.043</td>
</tr>
<tr>
<td>$\Delta\Pi_0$ (mN.m$^{-1}$)</td>
<td>20.0 ± 1.1</td>
<td>16.8 ± 0.50</td>
<td>16.9 ± 0.30</td>
<td>26.9 ± 1.3</td>
</tr>
</tbody>
</table>

$^a$ Data derived from Figure 3A. Experiments were carried out at 25°C in a cylindrical Teflon trough as described in Experimental Procedures. Buffer: 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. Final enzyme concentration, 40 nM. Data are mean values of three independent assays. The uncertainty was calculated as previously described [59, 60].
Figure 1: Biochemical characterization of LipY protein and its truncated forms. A) Schematic representation of LipY and its mutant forms. The PE domain is highlighted in blue, the linker region before the Gly$^{149}$ maturation site is colored in green, the remaining linker region is colored in orange and the lipase domain is highlighted in grey. B) Protein purity assessed onto 12% SDS-PAGE, lane 1 corresponds to the Unstained Protein Molecular Weight Marker (MW) (Euromedex), lanes 2 to 5 correspond to 6 μg of LipY, LipY$\Delta$PE, LipY$\Delta$149 and LipY$\Delta$170, respectively, loaded onto the gel and stained with Coomassie blue. C-D-E). SA determination of LipY and its mutant forms using TC4 (C), TC8 (D) and TC18 (E) as substrates. Experiments were carried out at 37°C in 15 mL of 2.5 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl and 1 or 3 mM NaTDC for TC4 and TC8, respectively. Olive oil (TC18) was assayed in the same conditions without NaTDC and with 10% arabic gum. One international enzymatic unit corresponds to 1 μmole of fatty acid released per min. Values are means of 3 independent experiments ± S.D. F) Representation of the relative activities of the LipY truncated forms using TC4, TC8 and TC18 as substrates in comparison to the full-length LipY. **, p-value < 0.01. *, p-value < 0.05. Statistical analysis was done using one-way ANOVA followed by a post hoc Tukey’s Honest Significant Difference test.

Figure 2: Overexpression of lipY and its mutant forms within lipid-rich mycobacteria. A) *M. smegmatis* recombinant strains harboring empty pSD26, pSD26::lipY, pSD26::lipY$\Delta$PE, pSD26::lipY$\Delta$149 and pSD26::lipY$\Delta$170 were grown in minimal salt medium containing 0.05 g/L of NH$_4$Cl and 1% glycerol as carbon source for 48 h to promote ILI formation. Bacteria were re-suspended in medium devoid of carbon, containing 0.2% (w/v) acetamide and were collected after 6 h or 12 h of induction. Cells were lyophilized and the same amount of dry cell weight was used for apolar lipid extraction. TAG levels from each culture were analyzed by TLC with triolein as standard. The TLC plate is representative of individual experiments performed in duplicate. TLC densitometric analysis of relative TAG levels in each sample was performed by using the empty vector culture at
6h post-induction as a reference. Results are expressed as mean values ± SD of two distinct and independent experiments. B-E) Three dimensional structure modeling of LipY protein and its truncated forms. The PE domain is highlighted in blue and the catalytic triad composed of serine (Ser\textsuperscript{309}), aspartic acid (Asp\textsuperscript{383}) and histidine (His\textsuperscript{414}) residues are shown in yellow. The green part represents the polypeptide linking the catalytic domain to the PE domain before the Gly\textsuperscript{149} maturation site, the remaining linker region is indicated in orange and the lipase domain is shown in grey. B) LipY with its PE domain highlighted in blue. C) LipYΔPE where the removal of the PE domain is triggering a large opening of the active site. D) LipYΔ149 without PE and linker region, allowing a better accommodation of the substrate without the catalytic pocket. E) LipYΔ170 corresponding to the lipase domain only. The three-dimensional model structures of LipY, LipYΔPE were from [14]. LipYΔ149 and LipYΔ170 3D-model structures were generated with the automatic protein structure homology modeling server using the I-Tasser software program [81, 82] and the 4XVC PDB code [84] as template. The final model structures of LipY proteins were visualized using the PyMOL Molecular Graphics System (version 1.4, Schrödinger, LLC).

Figure 3: Interfacial physico-chemical properties of LipY domains. A) Adsorption of LipY, LipYΔPE, LipYΔ149 and LipYΔ170 onto DOPG monomolecular films. Each enzyme (40 nM final concentration) was injected in aqueous phase (10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl) below the lipid film at various initial surface pressures (\(\Pi_i\)). The maximal surface pressure increase (\(\Delta\Pi_{\max}\)) was then recorded and plotted as a function of \(\Pi_i\). The critical surface pressure for penetration (\(\Pi_c\); intercept of the linear regression with the x-axis) and \(\Delta\Pi_0\) (intercept of the linear regression with the y-axis) were determined. Experiments were carried out in a cylindrical Teflon trough as described in the Materials and Methods section. Data are presented as mean values of two independent assays performed in duplicate (CV% < 5.0%). B) Temperature dependence of the wavenumber of the asymmetric methylene stretching \(\nu_{as}(\text{CH}_2)\) for DOPG in the absence and in the presence of LipY, LipYΔPE, LipYΔ149 and LipYΔ170 at protein-to-lipid ratio of 1:10. Average
behavior of DOPG alone or in the presence of LipYΔPE, LipYΔ149 and LipYΔ170 have been highlighted in thick black and compared to DOPG in the presence of LipY (filled blue dots).

Figure 4: Investigation of LipY and its mutant form maturation processes through the Type VII secretion system of M. marinum. A) Schematic representation of LipY and its mutant forms. The PE domain is highlighted in blue, the linker region before the Gly\textsuperscript{149} maturation site is colored in green, the remaining linker region is colored in orange and the lipase domain is highlighted in grey.

Specific point mutation (E92A and G149D) have been highlighted in red. B) Subcellular localization of cytoplasmic LipY\textsuperscript{E92A}, LipYΔ149 proteins and the exported MMAR\textsubscript{0427} protein (which shares 89% of identity with the monoglyceride lipase M. tuberculosis Rv0183 protein) were used as controls.

Recombinant cells expressing 6×His-tagged LipY proteins were treated with buffer containing (+) or not (−) Genapol\textsuperscript{®}-X080 detergent. Pellet (P) and supernatant (S) fractions containing cytoplasmic and surface-exposed proteins, respectively, were separated by centrifugation. Samples were loaded onto 12% SDS-PAGE and immunoblotted using HisProbe reagent. Grey arrows represented unprocessed forms whereas black arrows represented matured forms of the proteins. C) Subcellular localization of LipY, LipY\textsuperscript{G149D}, PE-SGA-LipYΔ149 and PE-SGA-LipYΔ170 proteins in M. marinum by detergent extraction, as described above. D) Purification of the 6×His-tagged mature form of PE-SGA-LipYΔ170 proteins in M. marinum following detergent extraction and Ni-NTA affinity chromatography. Protein molecular weight and purity were assessed on 12% SDS-PAGE stained with Coomassie blue (CB) and further confirmed by western blotting (WB). Protein was loaded onto PVDF membrane, stained with Ponceau Red and N-terminal sequencing was performed. Analysis of LipY maturation process towards distinct recombinant forms of LipY. WT and PE-SGA-LipYΔ170 were confirmed by N-terminal sequencing whereas PE-SGA-LipYΔ149 cleavage site remains putative. The maturation site is underlined and the SGA motif essential for LipY export is highlighted in red.
Figure 5: Schematic representation of the contribution of LipY domains in ILI interaction/hydrolysis, secretion, maturation, and anchoring processes allowing host cell TAG breakdown.

Upon infection the LipY protein is produced within the mycobacterial cytoplasm and possesses a dual localization. We proposed that during FFA acquisition, a weak portion of LipY remains in the cytoplasm in a full-length state, interacting either with the cytoplasmic membrane (1) or with the peripheral phospholipid layer of ILI (2) and that this interaction is mainly mediated by the PE domain (1-2). However, this N-terminal region also reduces the TAG-hydrolase activity of the lipase domain thus resulting in a slow breakdown of the neutral lipids contained within ILI (2). In the same time, the remainder of the protein is targeted to the ESX-5 machinery (3), and further recognized and maturated by the MycP5 protease (4). This results in the formation of a mature truncated form of LipY variant (LipYΔ149) that is anchored within the mycobacterial cell-wall (5). Deletion of the entire linker region did not affect this anchoring process suggesting that the lipase domain is responsible for the interaction within the mycomembrane (5). The truncated form LipYΔ149, devoid of the PE domain and the first 53 amino acids of the linker, possesses a greater activity than the full-length protein (5) and contributes to FFA acquisition by hydrolyzing host cell TAG within the phagosomal lumen of foamy macrophages (5). The red star corresponds to a schematic representation of the catalytic serine within the lipase domain. CM: cytoplasmic membrane; PG: peptidoglycan, AG: arabinogalactan; MA: mycolic acids; DAG: diacylglycerol; MAG: monoacylglycerol; TAG: triacylglycerol; FFA: free fatty acid.
FIGURES

Figure 1

A

B

C

D

E

F

TC4

TC8

TC18

[Graphs and diagrams showing enzyme activity and molecular weight separations for different constructs of LipY and variants such as LipYΔPE, LipYΔ149, and LipYΔ170.]

[Graphs showing the specific activity (U/mg) and relative activity (AU) for TC4, TC8, and TC18 with different constructs.]
Figure 2

A

B

C

D

E

LipY

LipYΔPE

LipYΔ149

LipYΔ170
Figure 4

A

LipY

LipYΔ149

LipY<sup>E92A</sup>

LipY<sup>G149D</sup>

PE-SGA-LipYΔ149

PE-SGA-LipYΔ170

B

B (kD) P- P+ S- S+

LipYΔ149

LipY<sup>E92A</sup>

MMAR_0427

C

C (kD) P- P+ S- S+

LipY

LipY<sup>G149D</sup>

PE-SGA-LipYΔ149

PE-SGA-LipYΔ170

MMAR_0427

D

D

LipY ...<sup>143FTGTP</sup><sup>SGA</sup><sup>AGFDIA</sup><sup>156</sup>...

PE-SGA-LipYΔ149 ...<sup>92ELANASGAAGFDIA</sup>...

PE-SGA-LipYΔ170 ...<sup>92ELANASGAETHFAIP</sup>...