The plasma membrane of Mycobacterium sp. is the site of synthesis of several distinct classes of lipids that are either retained in the membrane or exported to the overlying cell envelope. Here, we provide evidence that enzymes involved in the biosynthesis of two major lipid classes, the phosphatidylinositol mannosides (PIMs) and aminophospholipids, are compartmentalized within the plasma membrane. Enzymes involved in the synthesis of early PIM intermediates were localized to a membrane subdomain termed PMf, that was clearly resolved from the cell wall by isopyknic density centrifugation and amplified in rapidly dividing Mycobacterium smegmatis. In contrast, the major pool of apolar PIMs and enzymes involved in polar PIM biosynthesis were localized to a denser fraction that contained both plasma membrane and cell wall markers (PM-CW). Based on the resistance of the PIMs to solvent extraction in live but not lysed cells, we propose that polar PIM biosynthesis occurs in the plasma membrane rather than the cell wall component of the PM-CW. Enzymes involved in the biosynthesis of these lipids also displayed a highly polarized distribution between the PMf and PM-CW fractions. The PMf was greatly reduced in non-dividing cells, concomitant with a reduction in the synthesis and steady-state levels of PIMs and amino-phospholipids and the redistribution of PMf marker enzymes to non-PM-CW fractions. The formation of the PMf and recruitment of enzymes to this domain may thus play a role in regulating growth-specific changes in the biosynthesis of membrane and cell wall lipids.

The Gram-positive bacteria belonging to the genus mycobacteria are the causative agents of several important diseases in humans, the most important being tuberculosis (Mycobacterium tuberculosis) (1). All species of mycobacteria contain a highly distinctive cell wall that is thought to account for the resistance of these organisms to many antibiotics and to contribute to their ability to persist outside their host and within the endosomal network of human macrophages. Unlike most other Gram-positive bacteria, mycobacteria contain an asymmetric outer membrane composed of a layer of tightly packed, long chain (C70-C90) mycolic acids (inner leaflet) and a diverse array of free lipids (outer leaflet) (2, 3). This outer membrane is covalently linked to an arabinogalactan polysaccharide, which in turn, is covalently linked to the underlying layer of peptidoglycan (4). Considerable progress has been made in identifying enzymes involved in the synthesis of individual cell wall components, particularly in the fast growing saprophytic species, Mycobacterium smegmatis (4). However, relatively little is known about the precise localization of these enzymes; specifically whether they are present in the underlying plasma membrane or the cell wall and to what extent each of these fractions may be further compartmentalized. Information on the localization of cell wall biosynthetic enzymes is crucial for understanding how these pathways are regulated and how intermediates or fully synthesized wall components are transported to their final cellular sites.

A major class of mycobacterial glycolipids are the phosphatidylinositol mannosides (PIMs) and hypermannosylated derivatives, lipomannan (LM) and lipoarabinomannan (LAM) (2, 5, 6). These lipids are thought to be localized in both the plasma membrane and the overlying cell envelope (7, 8). Both PIM and LAM appear to be important virulence factors in pathogenic species of mycobacteria (6, 9), whereas recent gene disruption studies have shown that phosphatidylinositol (PtdIns), the immediate lipid precursor for PIM biosynthesis, and early PIM intermediates are essential for the growth of M. smegmatis (10, 11). Most of the steps involved in PIM biosynthesis have now been reconstituted in M. smegmatis cell-free systems, and several of the genes encoding enzymes for these steps have been characterized (5, 6, 12–14). PtdIns is synthesized by the condensation of inositol with CDP-diacylglycerol, and subsequently modified with two mannose (Man) residues and one fatty acyl chain to form the apolar PIM species, AcPIM2 (Fig. 1). The two mannosylation steps are catalyzed by PimA and PimB, which utilize GDP-Man and are most likely localized to the cytoplasmic leaflet of the plasma membrane (10, 15). AcPIM2 can be an end-product, or further modified with a second acyl chain and/or additional Man residues to form polar PIM species (having 4–6 Man residues) or LM/LAM (13, 14).

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The mannosyltransferases involved in polar PIM and LM/LAM biosynthesis utilize the lipid-linked sugar donor, polyenolphosphate-Man (PPM) (13, 14), which is commonly utilized for reactions that occur in the periplasmic space and/or the cell wall compartments (16). The biosynthesis of PIMs is thus likely to be topologically complex, minimally requiring the transport of intermediates from the cytoplasmic to the periplasmic side of the plasma membrane.

In the present study, we have investigated the subcellular localization of the enzymes involved in PIM synthesis. Unexpectedly, we found that early steps in PIM biosynthesis were localized to a distinct subfraction of the plasma membrane, termed PM, that was resolved from cell wall markers after isopycnic centrifugation in sucrose gradients. In contrast, enzymes involved in polar PIM biosynthesis were localized to a fraction containing both plasma membrane and cell wall markers. Enzymes involved in the biosynthesis of other cytoplasmic membrane phospholipids, such as phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtN) were also differentially distributed between these fractions. Remarkably, entrance into stationary growth was associated with loss of the PM, fraction, the redistribution of key enzymes in PIM and phospholipid biosynthesis, and concomitant decrease in PIM and phospholipid biosynthesis. These data suggest that compartmentalization of lipid biosynthetic enzymes in the cytoplasmic membrane may provide a mechanism for regulating the rate of synthesis of essential plasma membrane and cell wall lipids during exponential and stationary growth.

**EXPERIMENTAL PROCEDURES**

Preparation of Cell Lysate— *M. smegmatis* strain mc^155 (17) was grown in Middlebrook 7H9 broth. Mid-log and stationary phase mycobacteria were obtained by 1:100 dilution of a starter culture in fresh medium and incubation at 37 °C for 14–16 h or 150 h, respectively. The cell pellet (typically 1–3 g from 1.5 liters of culture for mid-log phase cells) was washed twice in 50 mM HEPES/NaOH (pH 7.4) and resuspended at 0.2 g of wet pellet/ml in the buffer A (25 mM HEPES/NaOH (pH 7.4), 25% sucrose, and 2 mM EDTA) supplemented with protease inhibitor mixture (Roche Diagnostics). The cell suspension was then lysed by three rounds of nitrogen cavitation at 15,000 kPa with 30 min of equilibration prior to the release of the pressure. Intact cells were removed by centrifugation (2,500 × g, 10 min), and the supernatant (2.5 ml) was loaded onto a continuous sucrose gradient (25–60% in 25 mM HEPES/NaOH (pH 7.4) 9 ml) layered on top of an 80% sucrose cushion (5 mM final), 10% Triton X-100 (0.2% final), and 500 μM CdCl₂-diacetyl-glycerol (10 μM final), prewarmed at 37 °C for 5 min, and supplemented with 0.7 μl of 1 M KCN (25 C/min/ml, 20 μCi/ml final) for a further 30-min incubation. Radiolabeled lipids were purified and analyzed as described above. For PtdSer decarboxylase assay, [3H]PtdSer (generated in the PtdSer assay) was purified by HPTLC and added to gradient fractions, adjusted to 32% sucrose, 5 mM MgCl₂, and 0.2% Triton X-100, as described above for the PtdSer assay, and incubated for 5 min at 37 °C. The PSM synthesis assay was carried out in 22.5 μl of Buffer A containing 4.8 Ci/ml of each fraction, 100 μM geranylergystaric phosphate, 20 mM Triton X-100, and 5 mM MgCl₂. GDP-[3H]Man (9 μCi/ml) was added to initiate the reaction after 5-min prewarming at 37 °C. The reaction was terminated after 40 min for analysis. PtdSer and amphotericin-sensitive mannosyltransferase identical to PIM biosynthetic assay except that each fraction was supplemented with 1 μl octyl β-mannopyranosyl-(1→6)-mannopyranoside, (20), and the reaction was terminated after 40 min for analysis. For PtdSer and amphotericin-sensitive mannosyltransferase assay, radiolabeled lipids were purified and analyzed as described above except that the HPTLC sheet was exposed to a tritium phosphor screen, and an autoradiograph was obtained using Typhoon 8600 (Molecular Dynamics Inc) to quantify relative changes in radioactivity.

**Analytical Methods**—Unlabeled phospholipids and glycolipids were extracted from cells in chloroform/methanol (10:10:3, v/v), purified by butanol/water (21.1, v/v) phase partition, and analyzed by HPTLC developed in solvent system. Glycoesphosphatidylsyls (GPLs) were recovered from lysate in 0.1M NaCl/NAOH, 40% 1-butanol/water (2:1, v/v) phase partition, and analyzed by HPTLC developed in solvent system 1. Lipids were also analyzed by gas chromatography-mass spectrometry (21).

**Electron Microscopy**—Gradient fractions or washed membranes were placed on carbon formvar grids (2 min) and stained with 1% uranyl acetate (1 min), washed with water in a Microdialyzer System 100 (Pierce, MWCO 8,000) (3 days, 4 °C), and then dyehydrated in 2× trifluoroacetic acid (100 °C, 2 h). Released monosaccharides were converted to their corresponding alditol acetates and identified by gas chromatography-mass spectrometry (21).

**Expression of *PimB*-hemagglutinin (HA)**—An expression vector was constructed from pJAM2 (22) and pHAX3U, an engineered vector containing three consecutive HA peptide epitope tags (23). A spacer sequence with flanking BamHI sites and an XbaI site near the 5′-end was cloned into the BamHI site of pJAX3U. The spacer was removed by BamHI digestion of the recombinant plasmid followed by self-ligation. The resulting plasmid, pJAM-HA, is a mycobacterial shuttle vector with the inducible acetamidase promoter, a BamHI cloning site, and an HA epitope tag sequence. The *PimB* gene of *M. smegmatis* was cloned into pJAM-HA as follows. The open reading frame was PCR-amplified from genomic DNA using primers with BglII sites at the termini (5′-AGTCTGGCCTGCTGCATCTGCT and 5′-AGATCTGCCAGCAGAGGCTGACG). The PCR product was cloned into pGEM-T Easy, and then subcloned as a BglII fragment into the BamHI site of pJAX3U. The resulting plasmid, pJAX-HA, was expressed in *Mycobacterium smegmatis* mc^155 by electroporation (24). The transformant was selected by kanamycin resistance and was grown in 7H9 broth containing 20 μg/ml kanamycin. Starter culture was grown in the absence of acetamide and 0.2% (w/v) acetamide was added at the time of 100× dilution of the starter culture. Cultures expressing *PimB*-HA were fractionated as described. The resulting plasmid, pJAX-HA, was expressed in *Mycobacterium smegmatis* mc^155 by electroporation (24). The transformant was selected by kanamycin resistance and was grown in 7H9 broth containing 20 μg/ml kanamycin. Starter culture was grown in the absence of acetamide and 0.2% (w/v) acetamide was added at the time of 100× dilution of the starter culture. Cultures expressing *PimB*-HA were fractionated as described.
RESULTS

Identification of Two Plasma Membrane Fractions in Actively Dividing M. smegmatis—M. smegmatis were harvested in exponential phase, lysed by nitrogen cavitation, and the cell-free lysate fractionated by isopyknic centrifugation on a continuous sucrose density gradient. Although most of the cytoplasmic proteins remained at the top of the gradient (Fig. 2A, fraction C), two distinct membrane fractions (corresponding to fractions 4–6 and 7–9) were observed within the gradient. The upper fraction contained a relatively heterogeneous population of membrane vesicles, 20–50 nm in diameter (Fig. 2G, left panel), and 39, 31, and 29% of the major plasma membrane phospholipids, cardiolipin, PtdIns, and PtdEtn, respectively (Fig. 2C). This fraction was essentially free of cell wall components, such as arabinogalactan (Fig. 2F) or the outer layer GPLs (Fig. 2E) and is referred to as the free plasma membrane fraction (PMf). In contrast, the lower fraction was rich in cell wall fragments (Fig. 2G, right panel) and contained the majority of the cell wall arabinogalactan (Fig. 2F). However, this fraction also appeared to contain a tightly linked plasma membrane component, as evidenced by the presence of plasma membrane phospholipids, cardiolipin, PtdIns, and PtdEtn (43, 58, and 36% of the cellular pool) (Fig. 2C) and several plasma membrane enzyme markers (see below). Based on these analyses, this fraction was termed the plasma membrane-cell wall (PM-CW) fraction. It is notable that although the relative abundance of cardiolipin and PtdIns in the two fractions remained constant, the PM-CW fraction was enriched in PtdEtn, suggesting that the two PM components have distinct phospholipid compositions. Some membrane and cell wall markers (Fig. 2, C and E) were recovered from the top of the gradient (fraction C). However, this fraction lacked any lipid biosynthetic activities (see below) and was not investigated further.

The PMf and PM-CW fractions also contained the PIM species, AcPIM2 and AcPIM6. These glycolipids had the same distribution as PtdEtn, being enriched in the PM-CW fraction (69 and 58% of total, respectively) relative to the PMf (21 and 23% of total, respectively) (Fig. 2D). To investigate whether the polarized distribution of these glycolipids reflected the compartmentalization of their biosynthesis, individual sucrose density gradient fractions were incubated with GDP-[3H]Man. GDP-[3H]Man is used directly by early mannosyltransferases in PIM synthesis, whereas incorporation of [3H]Man into polar PIMs requires the conversion of GDP-[3H]Man to [3H]polyprenol-phosphate-Man ([3H]PPM) (Fig. 1) (13). As shown in Fig. 2B, the PMf fraction was highly enriched in enzymes and/or precursors required for AcPIM2 synthesis but was unable to sustain biosynthesis of polar PIM species. In contrast, AcPIM2 biosynthesis was low in the PM-CW fraction, whereas synthesis of polar PIM species (AcPIM4–6) was efficiently reconstituted (Fig. 2B) (13). PPM synthesis, catalyzed by the plasma membrane enzyme PPM synthase (19), was evenly distributed in both fractions (Fig. 2B). The lack of synthesis of polar PIMs in the PMf fraction is thus unlikely to be because of the absence of PPM in this fraction.

To confirm that the enzyme activities in the PMf and PM-CW fractions were associated with membrane or particulate fractions and not contaminating cytosolic proteins, both fractions were subjected to two more centrifugation and wash steps. As shown in Fig. 3A, the washed PMf and PM-CW fractions retained their capacity to synthesize predominantly apolar and polar PIMs, respectively. Although an additional low (0.15 M NaCl) or high (1 M NaCl) salt wash of the PMf fraction was effective at removing abundant ribosomes from this fraction (determined by SDS-PAGE and peptide mass fingerprinting, data not shown), neither treatment had any affect on the biosynthesis of AcPIM2 or recovery of major membrane phospholipids (Fig. 3B). Furthermore, vesicular morphology of PMf by negative staining electron microscopy remained unchanged after salt extractions (Fig. 3, C–E). Interestingly, both salt washes did lead to significant reduction in PIM1 synthesis (Fig. 3A) suggesting that attachment of PimA to this fraction was salt-sensitive. Collectively, these results provide strong evidence that PMf and PM-CW contain distinct plasma membrane domains that differ in both their lipid composition and capacity to synthesize apolar and polar PIMs.

To further investigate the compartmentalization of specific enzymes in these pathways, we examined the subcellular localization of a HA-tagged version of PimB, the second GDP-Man-dependent mannosyltransferase in the PIM pathway (Fig. 1). After cell lysis and subcellular fractionation, PimB-HA was primarily localized to the PMf fraction (Fig. 4A, lower panel), consistent with the biosynthetic assays described above, and 15-μl aliquots of the sucrose density fractions were analyzed by 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were incubated with 1% skim milk, then probed with mouse anti-HA monoclonal antibody 262K (Cell Signaling, 1:3000 dilution) followed by sheep anti-mouse IgG antibody (Chemicon, horse radish peroxidase-conjugated, 1:3000 dilution). The bound probe was visualized by ECL (Invitrogen).
Although some of the PimB-HA was detected at the bottom of the gradient, possibly reflecting association with inclusion bodies, this protein was absent from the PM-CW fractions (Fig. 4A). The localization of two other enzymes involved in PIM/LAM synthesis was assessed using specific assays. In contrast to the results obtained using endogenous acceptors (Fig. 2B), most of the PPM synthase activity measured using exogenous substrates localized to the PM fraction (Fig. 4A, upper panel). This discrepancy may reflect differences in the concentration of endogenous polyprenol-phosphate acceptors in the two membrane fractions. In contrast, the activity of an amphotericin-sensitive (PPM-dependent) α1–6-mannosyltransferase (20) was predominantly localized to the PM-CW fraction (Fig. 4A, upper panel). This activity is likely to be required for polar PIM and/or LM/LAM biosynthesis (20), consistent with the notion
that the PM-CW is enriched for enzymes catalyzing these steps.

To examine whether the compartmentalization of PIM biosynthesis occurred in vivo, exponentially growing *M. smegmatis* was metabolically labeled with [3H]Man for 10 min, and the distribution of in vivo labeled precursors assessed after cell lysis and sucrose density centrifugation. As shown in Fig. 4B, a significant proportion of newly synthesized AcPIM2 was present in PMf, whereas this fraction completely lacked polar PIMs. In contrast, AcPIM2 and polar PIMs were present in the PM-CW fraction. The presence of significant levels of AcPIM2 in the PM-CW fraction may indicate rapid transport from the PMf and/or some synthesis of AcPIM2 in this fraction. Collectively, these data support the notion that the first two mannosyltransferases in PIM synthesis are primarily localized to PMf, whereas the PPM-dependent mannosyltransferases involved in polar PIM (and LAM) biosynthesis appear to be localized to the PM-CW fraction.

Localization of Steady-state Pools of PIM—The previous analyses suggested that enzymes involved in polar PIM biosynthesis were primarily localized in the PM-CW fraction. Although PPM, the Man donor for these reactions, is made in the plasma membrane, it is possible that subsequent mannosyltransferase reactions utilizing PPM occur in the cell wall fraction comprising the mycolyl-arabinogalactan and outer layer lipids. To address the likely site of synthesis of the PIMs, we investigated the extent to which the major PIM species are transported to externally disposed layers of the cell wall. Live *M. smegmatis* cells were extracted with various aqueous-organic solvent or detergent mixtures to identify conditions that resulted in the selective extraction of non-covalently linked cell wall glycolipids but not plasma membrane phospholipids (7, 8). As shown in Fig. 5, water-saturated 1-butanol was effective at extracting outer wall GPLs glycolipids but not plasma membrane phospholipids from live cells (Fig. 5, A and B). In contrast, GPL and membrane phospholipids were extracted from disrupted cells (Fig. 5, A and B), confirming that lack of extraction from live cells was not because of insolubility but rather accessibility to the solvent. The major PIM species displayed similar extraction susceptibilities to the membrane phospholipids (Fig. 5C). These data indicate that the abundant PIM species in the PM-CW fraction are largely associated with the plasma membrane rather than the outer lipid layer of the cell wall, supporting the notion that PIM biosynthesis is compartmentalized within the plasma membrane.

PtdSer and PtdEtn Synthesis Is Also Compartmentalized in PMf and PM-CW Fractions—We next examined whether other unrelated plasma membrane enzymes are similarly compartmentalized. The cyanide-sensitive, respiratory chain NADH oxidase is considered a general marker for the plasma membrane (25). This marker was largely recovered in the PM-CW fraction (Fig. 6, upper panel) demonstrating that the PMf is depleted of some plasma membrane markers. Enzymes involved in PtdEtn biosynthesis are also expected to be good markers for the plasma membrane, as the intermediates in this pathway (CDP-diacylglycerol and PtdSer) are plasma membrane components (see Discussion). Remarkably, PtdSer synthase activity was predominantly located in the PM-CW fraction (Fig. 6, lower panel), whereas PtdSer decarboxylase activity was predominantly located in PMf (Fig. 6, lower panel). These data suggest that PtdSer is initially synthesized in the PM-CW fraction and then transported to PMf, where it is decarboxylated to PtdEtn.

PMf Is Down-Regulated in Stationary Phase Mycobacteria—To further investigate the physiological role of lipid biosynthetic enzyme compartmentalization, we examined whether distinct PMf and PM-CW fractions occur in stationary phase mycobacteria where the demand for new membrane lipid and cell wall precursors is expected to be reduced. Analysis of the total lipid pool confirmed that levels of PtdEtn decreased markedly in stationary phase cells (Fig. 7A). Although levels of PIM remained relatively constant or increased in stationary phase cells, the rate of apolar PIM biosynthesis decreased by 10-fold in stationary phase cells (Fig. 7B). Remarkably, stationary phase cells had a greatly reduced PMf fraction, as indicated by in vitro PIM biosynthesis assay (Fig. 7C) and a phospholipid analysis (Fig. 7D) of gradient fractions. The loss of a distinct PMf was associated with the redistribution of PimB-HA to denser fractions in the gradient (Fig. 7E). Equally striking, PtdSer decarboxylase activity, which was highly localized to the PMf in exponentially growing *M. smegmatis*, was distributed throughout the gradient containing stationary cell lysates (Fig. 7F). In contrast, the distribution of PtdSer synthase, a marker for the PM-CW fraction was unchanged in both growth stages. Collectively, these data show that the marked down-regulation in PIM and PtdEtn biosynthesis in non-dividing cells is associated with the loss of the PMf fraction and the redistribution of PimB and PtdSer decarboxylase to other fractions within the gradient. The targeting of lipid biosynthetic enzymes to the PMf may thus be required for efficient lipid biosynthesis during rapid growth.
Man, and the labeled lipidic products analyzed by HPTLC and synthesis was measured by incubation of fractions with GDP-[3H]Man.

PPM and PIM biosynthetic activities in the PM fraction were associated with the membrane component and not due to co-sedimenting cytoplasmic protein complexes. These data demonstrate that the enzyme activities involved in the synthesis of two major PIM end-products, AcPIM2 and AcPIM6, are localized in distinct subcellular compartments. As the PM-CW fraction contained both plasma membrane and cell wall markers, it is possible that enzymes involved in polar PIM synthesis are located in one or both compartments. However, several lines of evidence suggest that these enzymes are in the plasma membrane fraction. Firstly, the steady-state pools of phospholipids and PIMs remain resistant to solvent extraction, under conditions that result in quantitative extraction of outer cell wall glycolipids, suggesting that the major PIM pools remain associated with the cytoplasmic or periplasmic face of the plasma membrane. Second, we have recently shown that newly synthesized PPM remains in the plasma membrane under the in vitro assay conditions used in this study (13). As PPM is utilized by the mannosyltransferases involved in polar PIM biosynthesis, the latter enzymes must also be localized in the plasma membrane. Finally, although AcPIM2 often accumulates to high levels under normal growth conditions, this pool of PIMs is rapidly chased into polar PIM species under inositol starvation conditions (26). Most of the apolar PIMs must therefore remain in the plasma membrane, together with PPM and PPM-dependent mannosyltransferases. These studies suggest that enzymes involved in the synthesis of apolar and polar PIMs are segregated within distinct membrane domains of rapidly dividing M. smegmatis. Besra et al. (14) also found that enzymes involved in apolar PIM and LM biosynthesis were enriched in a crude membrane and cell envelope fraction, respectively, prepared by differential centrifugation. Although it was concluded that LM biosynthesis likely occurs in the cell wall component (14), our results raise the possibility that enzymes involved in LM and LAM biosynthesis are located in a cell wall-associated subdomain of the plasma membrane. Interestingly, a recent and very comprehensive proteomic analysis of different subcellular fractions of M. tuberculosis (27) suggests that similar membrane subdomains could occur in pathogenic mycobacteria. Specifically, considerable overlap was found in the protein composition of a membrane and crude cell wall fraction (prepared by differential centrifugation) of M. tuberculosis with the latter fraction containing many enzymes involved in lipid biosynthesis (27). Although some of these enzymes could be associated with the cell wall components or the outer mycolic acid-containing bilayer, it is likely that many are associated with adherent plasma membrane, highlighting the need to further define these fractions.

The PM fraction was depleted of enzymes involved in AcPIM2 synthesis but contained enzymes and precursor lipids to form polar PIMs. Control experiments clearly showed that the PIM biosynthetic activities in the PM fraction were associated with the membrane component and not due to co-sedimenting cytoplasmic protein complexes. These data demonstrate that the enzyme activities involved in the synthesis of two major PIM end-products, AcPIM2 and AcPIM6, are localized in distinct subcellular compartments. As the PM-CW fraction contained both plasma membrane and cell wall markers, it is possible that enzymes involved in polar PIM synthesis are located in one or both compartments. However, several lines of evidence suggest that these enzymes are in the plasma membrane fraction. Firstly, the steady-state pools of phospholipids and PIMs remain resistant to solvent extraction, under conditions that result in quantitative extraction of outer cell wall glycolipids, suggesting that the major PIM pools remain associated with the cytoplasmic or periplasmic face of the plasma membrane. Second, we have recently shown that newly synthesized PPM remains in the plasma membrane under the in vitro assay conditions used in this study (13). As PPM is utilized by the mannosyltransferases involved in polar PIM biosynthesis, the latter enzymes must also be localized in the plasma membrane. Finally, although AcPIM2 often accumulates to high levels under normal growth conditions, this pool of PIMs is rapidly chased into polar PIM species under inositol starvation conditions (26). Most of the apolar PIMs must therefore remain in the plasma membrane, together with PPM and PPM-dependent mannosyltransferases. These studies suggest that enzymes involved in the synthesis of apolar and polar PIMs are segregated within distinct membrane domains of rapidly dividing M. smegmatis. Besra et al. (14) also found that enzymes involved in apolar PIM and LM biosynthesis were enriched in a crude membrane and cell envelope fraction, respectively, prepared by differential centrifugation. Although it was concluded that LM biosynthesis likely occurs in the cell wall component (14), our results raise the possibility that enzymes involved in LM and LAM biosynthesis are located in a cell wall-associated subdomain of the plasma membrane. Interestingly, a recent and very comprehensive proteomic analysis of different subcellular fractions of M. tuberculosis (27) suggests that similar membrane subdomains could occur in pathogenic mycobacteria. Specifically, considerable overlap was found in the protein composition of a membrane and crude cell wall fraction (prepared by differential centrifugation) of M. tuberculosis with the latter fraction containing many enzymes involved in lipid biosynthesis (27). Although some of these enzymes could be associated with the cell wall components or the outer mycolic acid-containing bilayer, it is likely that many are associated with adherent plasma membrane, highlighting the need to further define these fractions.

The PM fraction was depleted of enzymes involved in AcPIM2 biosynthesis. Although the first committed step in this pathway was localized to the PM-CW fraction, the second step was almost exclusively present in the PM fraction in exponentially growing cells. M. tuberculosis and M. smegmatis homologues of genes encoding putative PtdSer synthase and PtdSer decarboxylase are predicted to encode integral membrane proteins with one or multiple transmembrane domains (10), indicating a localization in the plasma membrane. Although the PM fraction appears to be the main site of PtdEtn synthesis in rapidly dividing mycobacteria, this membrane fraction was relatively depleted of PtdEtn, compared with the PM-CW fraction. Similarly, the PM fraction was depleted of the apolar PIM species
AcPIM2 despite being the major site of synthesis of this glycolipid. These data strongly suggest that the PM, and PM-CW fractions are directly or indirectly connected and that mechanisms exist to actively transport PtdSer, PtdEtn, and apolar PIMs between these membranes.

The PM could correspond to specific subdomains of the plasma membrane that underlie the cell envelope or alternatively to intracellular inclusions that are in direct or indirect continuity with the plasma membrane. Lateral heterogeneities in the cell envelope and intracellular lipid bodies have both been visualized in M. smegmatis and other mycobacteria using lipophilic dyes (28, 29). However, intracellular lipid inclusions are normally absent from exponentially growing mycobacteria when the PM is most prominent (29). Similarly, FM 4-64, a styryl dye that labels the plasma membrane or intracellular membranes in direct or indirect continuity with the plasma membrane, only labeled the cell envelope and septum of exponentially growing M. smegmatis (data not shown). Collectively, these observations suggest that the PM represents a subdomain of the plasma membrane-envelope complex. There is increasing evidence that specific domains of the prokaryote plasma membrane, such as the cell poles, the mid-region, and the septum, can have distinct protein or lipid compositions (30–34). A striking example is the localization of Sec protein export machinery to the poles, septum, and intermediate positions along the cell axis of the rod-shaped bacterium, Bacillus subtilis (35). Intriguingly, these subdomains were only detected in rapidly dividing cells and were dependent on ongoing phospholipid biosynthesis (35). The possibility that the PM may include or correspond to the septum membrane is suggested by the finding that ongoing mannoglycoconjugate biosynthesis is required for septum formation in M. smegmatis (36). It will be of interest to determine whether the M. smegmatis PM also contains proteins involved in protein export and/or the assembly of new cell wall components.

The transition from exponential to stationary growth was associated with a marked decrease in PtdEtn levels and apolar PIM biosynthesis, consistent with the reduced demand for new plasma membrane in stationary phase cells. These growth-specific changes in lipid biosynthesis were closely associated with the loss or significant reduction in the PM fraction, as measured by marker enzyme activities and phospholipid analysis. In particular, both PimB and PtdSer decarboxylase were displaced from fractions normally containing the PM, whereas the distribution of the PM-CW marker, PtdSer synthase, remained unchanged. Strikingly, neither PimB nor PtdSer decarboxylase were redistributed to the PM-CW fraction indicating that intracellular organization of these enzymes changes dramatically when cells reach stationary growth. PimB may form large aggregates in stationary phase cells, accounting for its localization near the bottom of the sucrose density gradient, whereas the broad distribution of PtdSer decarboxylase in the gradient may reflect association of this integral membrane protein with intracellular lipid bodies of variable size and density (28, 29). These data raise the intriguing possibility that the recruitment of lipid biosynthetic enzymes to the PM is essential for efficient PIM and PtdEtn biosynthesis in exponentially growing cells. The regulated recruitment and dissociation of enzymes from this membrane would constitute a new mechanism for regulating lipid biosynthesis in these bacteria.

Nothing is known about the mechanism of membrane targeting of PimB or PtdSer decarboxylase to the PM. PimB lacks a distinct hydrophobic transmembrane sequence (10, 15) suggesting that it may interact with other PM proteins or lipids. By analogy, Baulard et al. (37) have recently shown that the catalytic domain of M. smegmatis Ppm1 is tethered to an integral membrane protein, whereas the amphipathic protein, MinD, which is involved in negatively regulating the formation of the cell division Z-ring in Escherichia coli, is recruited to membrane domains that are enriched in acidic phospholipids, such as cardiolipin (38). Interestingly, PimA, the first mannosyltransferase in the PIM biosynthetic pathway shares a high degree of sequence similarity with PimB and is also targeted to PM in exponentially growing cells (Fig. 3). However, PimA enzymatic activity was largely released from the PM fraction after salt extraction (Fig. 3), whereas PimB enzyme activity remained tightly associated. It is possible that the lower affinity of PimA for PM may provide a mechanism for regulating the initial step in this pathway.

The compartmentalization of enzymes involved in PIM and aminophospholipid biosynthesis in mycobacteria shares some parallels with the situation in eukaryotic cells. Specifically, enzymes involved in catalyzing early and late intermediates in glycosylphosphatidylinositol biosynthesis are compartmentalized in the endoplasmic reticulum of animal cells and the protozoan parasite, Leishmania mexicana (23, 39). Similarly, enzymes involved in the synthesis of PtdSer and its conversion to PtdEtn are compartmentalized in the endoplasmic reticulum, the mitochondria, and junction zones between these organelles (40). Compartmentalization may increase the efficiency of some steps through substrate channeling or the localized concentration of lipid precursors. Alternatively, the segregation of lipid biosynthetic enzymes may prevent the depletion of lipids that are both metabolic end-products and precursors for other lipid biosynthetic reactions. For example, compartmentalization of enzymes involved in PIM biosynthesis may prevent the rapid depletion of PtdIns or apolar PIM, which are both intermediates for polar PIM and LM/LAM biosynthesis. Finally, compartmentalization could introduce spatial heterogeneity into the bulk lipid composition that may influence the recruitment or activity of membrane proteins. In summary, these studies provide strong evidence that the mycobacterial membrane is functionally compartmentalized and that the association/dissociation of lipid biosynthetic enzymes with this domain play a role in regulating the rate of synthesis of essential plasma membrane and cell wall lipids.

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