Biosynthesis of mycobacterial phosphatidylinositol mannosides

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All mycobacterial species, including pathogenic *Mycobacterium tuberculosis*, synthesize an abundant class of phosphatidylinositol mannosides (PIMs) that are essential for normal growth and viability. These glycolipids are important cell-wall and/or plasma-membrane components in their own right and can also be hyperglycosylated to form other wall components, such as lipomannan and lipoarabinomannan. We have investigated the steps involved in the biosynthesis of the major PIM species in a new *M. smegmatis* cell-free system. A number of apolar and polar PIM intermediates were labelled when this system was continuously labelled or pulse-chase-labelled with GDP-[³H]Man, and the glycan head groups and the acylation states of these species were determined by chemical and enzymic treatments and octyl-Sepharose chromatography respectively. These analyses showed that (1) the major apolar PIM species, acyl-PIM2, can be synthesized by at least two pathways that differ in the timing of the first

acylation step, (2) early PIM intermediates containing a single mannose residue can be modified with two fatty acid residues, (3) formation of polar PIM species from acyl-PIM2 is amphotycin-sensitive, indicating that polyphosphatidylphosphate-Man, rather than GDP-Man, is the donor for these reactions, (4) modification of acylated PIM4 with α 1-2- or α 1-6-linked mannose residues is probably the branch point in the biosyntheses of polar PIM and lipoarabinomannan respectively and (5) GDP strongly inhibits the synthesis of early PIM intermediates and increases the turnover of polyphosphatidylphosphate-Man. These findings are incorporated into a revised pathway for mycobacterial PIM biosynthesis.

Key words: cell wall, lipoarabinomannan, mycobacteria, phosphatidylinositol, phosphatidylinositol mannoside, polyphosphatidylphosphate.

INTRODUCTION

The genus *Mycobacterium* contains a number of medically important pathogens, such as *Mycobacterium tuberculosis* and *M. leprae*, the causative agents of tuberculosis and leprosy respectively. *M. tuberculosis* may infect approx. one-third of the world's population and claims more than 2 million lives per year [1,2]. All mycobacteria synthesize a unique, lipid-rich cell envelope that is a major reason for the intrinsic resistance of these pathogens to common chemical sterilization procedures, as well as many antibiotics and host microbicidal processes [2]. Metabolic pathways involved in the assembly of the mycobacterial cell wall have been shown to be the primary targets of several first- and second-line anti-tuberculosis drugs [2], and the delineation of these pathways has been the subject of intense research.

The mycobacterial cell envelope has a multilaminar structure composed of covalently linked peptidoglycan, arabinogalactan and mycolic acids and an outer layer of non-covalently linked (glyco)lipids [3–5]. One class of glycolipids synthesized by all species of mycobacteria is the phosphatidylinositol mannosides (PIMs) [6,7]. PIMs exist as free glycolipids in either the plasma membrane and/or the cell wall and may also be hyperglycosylated to form other cell-wall components such as lipomannan (LM) and lipoarabinomannan (LAM) [7,8]. Accumulating evidence suggests that the PIMs and/or the LM and LAM are important virulence factors in *M. tuberculosis* and may modulate host immune responses [9–14]. However, the ubiquitous distribution of PIMs and LM/LAM in both pathogenic and non-pathogenic

species of mycobacteria suggests that these glycolipids may also be important for membrane or cell-wall biogenesis. This is supported by recent studies indicating that biosynthesis of Ptd (phosphatidylinositol) [15], PIM [16] or upstream Man donors [17] is essential for the growth of the non-pathogenic species *M. smegmatis*.

The predominant PIM species in mycobacteria are mono- and diacylated PIM2 and PIM6 (Figure 1) [6,7,18]. AcPIM2 (acyl-PIM) is generally considered to be both a metabolic end-product and an intermediate in AcPIM6 and LM/LAM synthesis [19]. This species is synthesized by the elaboration of Ptd with two Man residues and a fatty acyl chain. The first and second Man additions are catalysed by PimA and PimB mannosyltransferases respectively [16,19–22], whereas the acylation of the first Man residue has been recently shown to be catalysed by the product of the *M. tuberculosis* gene Rv2611c [23]. PimA and PimB utilize the soluble sugar nucleotide GDP-Man and lack a recognizable signal sequence, suggesting that these initial steps occur on the cytoplasmic face of the plasma membrane. A third GDP-Man-dependent mannosyltransferase, termed PimC, catalyses the conversion of AcPIM2 into AcPIM3 [24]. However, the gene encoding PimC is absent from many clinical strains of *M. tuberculosis* and from the fast-growing mycobacterial species *M. smegmatis*, suggesting that another mannosyltransferase may be involved in this step [24]. Nothing is known about the donor requirements for this and subsequent mannosylation steps involved in polar PIM biosynthesis, nor the point at which PIM biosynthesis bifurcates to form the cell-wall LM and LAM. In

Abbreviations used: ASAM, *Aspergillus saitoi* α 1-2-mannosidase; PIM, phosphatidylinositol mannoside; Ac_xPIM_y, PIM species with x (1 or 2) fatty acyl chains, linked to either the core α 1-2-linked Man or the myo-inositol head group, and y Man residues; ESI-MS, electrospray ionization MS; HPTLC, high-performance TLC; JBAM, Jack-bean α -mannosidase; LM, lipomannan; LAM, lipoarabinomannan; MALDI-TOF-MS, matrix-assisted laser-desorption ionization-time-of-flight MS; PPM, polyphosphatidylphosphate (C₃₅/C₅₀) phosphate-Man; TFA, trifluoroacetic acid.

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Figure 1 Structures of the major PIM and LM species of mycobacteria

LAM has the same core structure as LM, but is modified with an arabinan and inositol phosphate or Man-rich capping structures (results not shown). The 3-OH of the inositol ring in the PIMs may be modified further with an additional acyl chain to form Ac₂PIM2 and Ac₂PIM6 species. P, phosphate.

contrast with the early steps in PIM biosynthesis, the formation of the long mannan chains in the LM and LAM is supposed to occur in periplasmic space/cell wall and requires the lipid-linked Man donor PPM [polyprenol (C₃₅/C₅₀) phosphate-Man] [19].

M. smegmatis is a fast-growing species of mycobacteria that has proved to be an excellent experimental system for analysing mycobacterial cell-wall biosynthesis [16,19,22,23]. In the present study, we have reconstituted most of the steps in PIM biosynthesis in a new *M. smegmatis* cell-free system. We have used this system to identify putative intermediates in the biosynthesis of polar PIM and LAM and to show that the synthesis of the former requires the presence of PPM. The potential role of GDP in regulating both the early and late steps in this pathway was also investigated and these findings were incorporated into a new model for PIM biosynthesis.

EXPERIMENTAL

Characterization of unlabelled *M. smegmatis* PIMs

M. smegmatis strain mc²155 [25] was grown to mid-exponential phase at 37 °C in 7H9 broth (Difco, Detroit, MI, U.S.A.), supplemented with 0.2% (w/v) glucose, 0.2% (v/v) glycerol and 15 mM NaCl. Bacteria were harvested by centrifugation (2500 g) and sequentially extracted in chloroform/methanol (2:1, v/v; 20 vol. and then with 10 vol.) and chloroform/methanol/water (5:10:4, by vol.; 10 vol.). After centrifugation, to remove insoluble material, methanol and water were added to the pooled supernatants to achieve a chloroform/methanol/water ratio of 5:10:7 (by vol.). The upper and lower phases were separated, back-extracted with the theoretical lower and upper phases respectively and dried

under N₂ gas. The upper phase was resuspended in 5% (v/v) 1-propanol in 0.1 M ammonium acetate and loaded on to a column of octyl-Sepharose (1 ml; Amersham Biosciences, Sydney, Australia), pre-equilibrated in the same buffer. After washing with 5% 1-propanol and 0.1 M ammonium acetate (4 ml), PIMs were eluted with 30, 40 and 50% 1-propanol in water (1 ml each). The eluted fractions were dried in a Speedvac concentrator and combined with the lower organic phase. Individual PIM species were purified on aluminium-backed Silica Gel 60 HPTLC (high-performance TLC) sheets (Merck, Gibbstown, NJ, U.S.A.) developed in chloroform/methanol/1 M ammonium acetate/13 M ammonia/water (180:140:9:9:23, by vol.; solvent 1). Silica scrapings were extracted in chloroform/methanol/water (10:10:3, by vol.), dried under N₂ and stored in 30% 1-propanol at –20 °C. Purified PIMs were re-analysed by HPTLC in solvent 1 and detected with orcinol/H₂SO₄ reagent. The monosaccharide and fatty acid composition of the purified PIM species was determined by GC-MS after solvolysis in 0.5 M methanolic HCl and derivatization of the released methyl glycosides and fatty acid methyl esters with trimethylchlorosilane/hexamethyl-disilazane/pyridine (1:3:9, by vol.) [26]. For molecular species analysis, purified PIMs were analysed by MALDI-TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS) and ESI-MS (electrospray ionization MS) without derivatization. Purified glycolipids in 30% 1-propanol were mixed with saturated α -cyano-4-hydroxycinnamic acid and analysed in a PerkinElmer Biosystems Voyager 4180 MALDI-TOF mass spectrometer in linear negative-ion mode. An accelerating voltage of 20000 V was used, with grid and guide wire voltages of 94–96 and 0.05% respectively. Alternatively, samples in 30% 1-propanol were infused into a Bruker ESI mass spectrometer via a nanospray source and the data were obtained in negative-ion mode. Methylation analysis of purified native PIM species was performed by the method of Ciucanu and Kerek [27] with modifications [26] or by the method of Prehm [28] to preserve base-labile constituents. Methylated PIMs were dephosphorylated in 48% (v/v) HF (at 0 °C for 50 h) and hydrolysed in 2 M TFA (trifluoroacetic acid) (at 100 °C for 2 h), and partially methylated sugars were reduced, acetylated and analysed by GC-MS [26].

Preparation of cell lysate and cell-free radiolabelling

M. smegmatis mc²155 was harvested by centrifugation in mid-exponential growth and washed twice with 50 mM Hepes/NaOH (pH 7.4). Pellets were resuspended at 0.2 g of wet pellet/ml using a lysis buffer containing 25 mM Hepes/NaOH (pH 7.4), 25% (w/v) sucrose, 2 mM EGTA and a cocktail of protease inhibitors (Roche). The cells were subjected to three rounds of nitrogen cavitation (Kontes, Vineland, NJ, U.S.A.) at 15000 kPa with a 30 min equilibration period before the release of pressure. Unlysed cells were removed by centrifugation (2500 g, 10 min) and the supernatant was stored at –80 °C. The lysates were supplemented with 5 mM MgCl₂ before incubation at 37 °C and addition of GDP-[³H]Man (6.3 Ci/mmol, final concentration 9 μ Ci/ml) to initiate labelling. The labelling reaction was terminated at the indicated time points by adding chloroform/methanol (1:1, v/v, 6.7 vol.). After sonication, the insoluble material was removed by centrifugation (15000 g, 2 min) and the supernatant was dried under N₂. Labelled lipids were recovered by biphasic partitioning between water and 1-butanol (1:2, v/v) and analysed by HPTLC in solvent 1. Radiolabelled lipids were detected by treating the HPTLC sheets with EA Wax (EA Biotech, Flemington, Scotland, U.K.) or En³Hance (NEN Life Science Products, Boston, MA, U.S.A.) and exposing them to a BioMax MR film (Kodak, Rochester, NY, U.S.A.) at –80 °C.

Figure 2 Characterization of *M. smegmatis* PIMs

(A) Total lipids were extracted from exponential-phase *M. smegmatis* and analysed by HPTLC (in solvent 1) before or after mild base treatment (0.2 M methanolic NaOH, 2 h, 40 °C). PIMs were detected with orcinol/H₂SO₄ reagent. (B) HPTLC-purified PIMs were analysed by ESI-MS in negative-ion mode. The region of the mass spectra containing [M-H]⁻ molecular ions of AcPIM2 and Ac₂PIM2 is shown. (C) AcPIM2 was methylated under conditions that preserve base-labile acyl groups and then dephosphorylated by HF and hydrolysed in 2 M TFA. The released permethylated sugars were reduced, acetylated and analysed by GC-MS. The presence of 2,3,4,6-tetra-methyl-1,5-diacetylmannitol (t-Man), 2,3,4-trimethyl-1,5,6-triacetylmannitol (6-Man) and 3,4,5-methyl-1,2,6-triacetyl-*myo*-inositol (1,2,6-Ino) showed that a Man residue in AcPIM2 was substituted at position 6. Hexa-acetylated *scyllo*-inositol (*scyllo*-Ino) was used as an internal standard. Unmarked peaks correspond to reagent peaks.

Structural analysis of ³H-Man-labelled lipids

Ester-linked fatty acids were cleaved by O–N transacylation using monomethylamine [29]. JBAM (Jack-bean α -mannosidase) digestions (48 units/ml; Sigma) were performed in 0.1 M sodium acetate (pH 5.0), containing 0.1 % taurodeoxycholate (10 μ l) for 18 h at 37 °C [30,31]. ASAM (*Aspergillus saitoi* α 1-2-mannosidase) digestions (0.1 m-units/ml; Glyko, Novato, CA, U.S.A.) were performed in 0.1 M sodium acetate (pH 5.0), containing 0.1 % taurodeoxycholate (10 μ l) for 18 h at 37 °C. In some cases (Figure 5B), a second aliquot of ASAM (2 μ -units) was added after 16 h and the digestion was continued for a further 22 h. For determining the size of the glycan head group, labelled PIM species were purified by HPTLC as described above and an aliquot (3300 d.p.m., except fraction B, 33 000 d.p.m.) of each fraction was deacylated with monomethylamine. The delipidated glycan head groups were recovered by biphasic partitioning in water/1-butanol (1:2, v/v) and dephosphorylated in 48 % aqueous HF (at 0 °C for 50 h) [26]. The resultant neutral glycan was desalted and separated by HPTLC with five developments using 1-propanol/acetone/water (9:6:5, by vol.; solvent 2). For determining the acylation state, labelled PIM intermediates were loaded on to a column (13 mm $\times$ 45 mm) of octyl-Sepharose pre-equilibrated in 25 % 1-propanol in 0.1 M ammonium acetate and eluted with a linear gradient of 1-propanol (25–50 % 1-propanol in 0.1 M ammonium acetate) at a flow rate of 0.20 ml/min. 1-Propanol concentration was calculated from the refractive index, whereas radioactivity was measured by scintillation counting. The acylation state of labelled PIMs was defined by their elution position relative to the following radioactive standards: palmitic acid (one acyl chain), *Leishmania mexicana* glycosyl-phosphatidylinositols (two acyl chains) [32], authentic AcPIM2 (three acyl chains) and authentic Ac₂PIM2 (four acyl chains).

RESULTS

Structural analysis of *M. smegmatis* PIMs

To facilitate subsequent analyses of different PIM molecular species labelled in the *in vitro* assays, we first investigated the

Table 1 MALDI-TOF-MS analysis of purified *M. smegmatis* PIMs

The fatty acid composition deduced from GC-MS analysis of the fatty acid methyl esters released after solvolysis in 0.5 M methanolic HCl.

PIM species	[M-H] ⁻ (m/z)	Predicted structure	Predicted [M-H] ⁻ (m/z)
Ac ₂ PIM2	1649	Hex(C _{16:0} -Hex)(C _{16:0})PI _{16:0-19:0}	1650
	1677	Hex(C _{16:0} -Hex)(C _{18:0})PI _{16:0-19:0}	1678
	1691	Hex(C _{16:0} -Hex)(C _{19:0})PI _{16:0-19:0}	1692
AcPIM2	1413	Hex(C _{16:0} -Hex)PI _{16:0-19:0}	1412
Ac ₂ PIM6	2297	Hex ₅ (C _{16:0} -Hex)(C _{16:0})PI _{16:0-19:0}	2298
	2325	Hex ₅ (C _{16:0} -Hex)(C _{18:0})PI _{16:0-19:0}	2326
	2341	Hex ₅ (C _{16:0} -Hex)(C _{19:0})PI _{16:0-19:0}	2340
AcPIM6	2060	Hex ₅ (C _{16:0} -Hex)PI _{16:0-19:0}	2060

structures of the major steady-state PIMs. These glycolipids were purified by HPTLC and subjected to compositional and MS analyses. Four glycolipids with the expected HPTLC migration of mono- and diacylated forms of PIM2 and PIM6 were resolved (Figures 1 and 2A). These glycolipids were hydrolysed by mild base treatment (Figure 2A) and contained Man and *myo*-inositol as the only sugars in GC-MS compositional analysis (results not shown). The negative-ion ESI and MALDI MS of each species contained [M-H]⁻ molecular ions, consistent with the presence of mono- and diacylated PIM2 and PIM6 (Figure 2B and Table 1). The negative ESI spectrum of AcPIM2 and AcPIM6 contained one major [M-H]⁻ molecular ion. Based on the fatty acid composition of these functions, these molecular species contain two palmitic (C_{16:0}) and one tuberculostearic (C_{19:0}) acids. In contrast, the negative ESI spectrum of Ac₂PIM2 and Ac₂PIM6 contained three molecular ions of approximately equal intensity that differed from each other by either 14 or 28 a.m.u. (Figure 2B and Table 1). As *M. smegmatis* Ptd contains predominantly C_{16:0} and C_{19:0} fatty acids (results not shown), these analyses suggest that AcPIM2 and AcPIM6 are substituted predominantly with C_{16:0}, whereas Ac₂PIM2 and Ac₂PIM6 are substituted with C_{16:0} and either C_{16:0},

Figure 3 Cell-free synthesis of PIMs

(A) *M. smegmatis* cell lysate was continuously labelled with GDP-[^{3} H]Man and the extracted lipids were analysed by HPTLC in solvent 1. (B) Labelled PIM species (40 min incubation period in A) were mock-treated (lane 1) or treated (lane 2) with monomethylamine and analysed by HPTLC (solvent 1). (C) HPTLC-purified [^{3} H]PIMs (120 min as in A) were deacylated and dephosphorylated, and the resultant neutral glycan moiety was analysed on HPTLC sheets developed in solvent 2. Lane S represents a standard glycan ladder prepared from a PIM extract of *M. smegmatis* metabolically labelled with [^{3} H]Man.

C_{18:0} or C_{19:0}. To define the site of the first acyl substitution, AcPIM2 was methylated under conditions that preserve alkali-labile groups. GC-MS analysis of the permethylated alditol acetates generated after HF dephosphorylation, 2 M TFA hydrolysis, reduction and acetylation revealed the presence of 1,2,6-tri-substituted *myo*-inositol, terminal Man and 6-substituted Man (Figure 2C). Only 2,6-di-substituted *myo*-inositol and terminal Man were detected when AcPIM2 was deacylated and HF-dephosphorylated before methylation (results not shown), demonstrating that position 6 of a core Man residue was modified with a base-labile substituent. Collectively, these results suggest that *M. smegmatis* synthesizes a similar range of PIM molecular species as pathogenic species of mycobacteria [7].

Reconstitution of PIM biosynthesis in a cell-free system

Cell-free lysates of *M. smegmatis* were prepared by nitrogen cavitation. Addition of GDP-[^{3} H]Man and MgCl₂ to this lysate resulted in the time-dependent accumulation of several labelled lipid species (designated fractions A–G) that had an *R_f* value similar to mature PIMs on HPTLC (Figure 3A). These lipids were not labelled when the lysate was incubated with [^{3} H]Man, indicating that the cell-free system was free of metabolically active bacteria (results not shown). Most of the lipids labelled in the cell-free system were sensitive to monomethylamine treatment, indicating the presence of ester-linked fatty acids (Figure 3B).

Table 2 Structures of lipids labelled with GDP-[^{3} H]Man in *M. smegmatis* cell-free system

The glycan head groups of HPTLC-purified glycolipids were released with HF and re-analysed by HPTLC in solvent 2. Where fractions contained more than one glycan head group, the abundance is indicated by +++ (major species), ++ (minor) or + (trace). The presence of fatty acyl chains was based on sensitivity to monomethylamine. The degree of acylation of the major species in each fraction was based on their elution position on octyl-Sepharose. Fraction F was shown to contain two isomers of AcPIM5; one species contained two terminal α 1-2-linked Man residues, whereas the second was resistant to JBAM and was probably capped with α 1-6-linked Man. N/A, not applicable; n.d., not determined.

Fraction	Proposed species	Glycan head group	Monomethylamine sensitivity	No. of acyl chains
A	Ac ₂ PIM1	Man ₁ Ino	+	4
B	PPM (+++)	N/A	–	N/A
	Ac ₂ PIM2 (++)	Man ₂ Ino	+	n.d.
	AcPIM1 (+)	Man ₁ Ino	+	n.d.
C	AcPIM2	Man ₂ Ino	+	3
D	PIM1 (+++)	Man ₁ Ino	+	2
	AcPIM2 (++)	Man ₂ Ino	+	n.d.
E	AcPIM4 (+++)	Man ₂ Ino	+	3
	PIM2 (+)	Man ₂ Ino	+	n.d.
F	AcPIM5	Man ₂ Ino	+	3
	AcPIM5'	Man ₂ Ino	+	3
G	AcPIM6	Man ₂ Ino	+	3

Only lipids in fraction B and a faint band located between fractions C and D were insensitive to monomethylamine treatment. The major doublet in fraction B is probably PPM, on the basis of its characteristic HPTLC mobility [19], its sensitivity to mild acid hydrolysis (results not shown) and the finding that synthesis of these bands was inhibited by amphotycin (see below). The faint band between fractions C and D possibly represents a minor PPM species with a shorter prenyl group, but it was not characterized further.

To characterize further the structure of the putative PIM species, the glycan head groups of each fraction were released by deacylation and dephosphorylation and re-analysed by HPTLC. These analyses showed that the major lipids in fractions A, C, D, E, F and G contain 1, 2, 1, 4, 5 and 6 Man residues respectively attached to inositol (Figure 3C and Table 2). Species containing three Man residues were not identified. This analysis also revealed the presence of two monomethylamine-sensitive species co-migrating with PPM in fraction B, one containing inositol modified by two Man residues and another containing inositol modified by a single Man residue. In addition, trace amounts of inositol linked to two Man residues were detected in fractions D and E. The degree of acylation of the purified PIM species was determined by octyl-Sepharose column chromatography (Figure 4). Species containing 1–4 aliphatic chains can be clearly resolved on this matrix using a linear gradient of 1-propanol. The major labelled lipids in fractions C and E–G contained three acyl chains, whereas those in fractions A and D contained four and two acyl chains respectively (Figure 4 and Table 2).

The polar PIM species AcPIM5 and AcPIM6 are expected to differ from similarly sized LAM intermediates since they contain either one or two terminal α 1-2-linked Man residues respectively, instead of α 1-6-linked Man residues [8]. To examine whether the putative AcPIM5 and AcPIM6 species in fractions F and G contained α 1-2-linked Man residues, these fractions were exhaustively digested with either JBAM (digests α 1-2 and α 1-6 Man) or ASAM (only digests α 1-2 Man). As expected, the putative AcPIM4 species in fraction E was sensitive to JBAM, but resistant to ASAM (Figure 5A). Even after more extensive incubation with ASAM, this species remained undigested

Figure 4 Acylation state of labelled PIM

Lipids in fractions A and C–G were chromatographed on octyl-Sepharose eluted with a linear gradient of 1-propanol (25–50%). Bars at the top of each graph indicate elution positions of standard lipids containing one ([³H]palmitate), two (*L. mexicana* glycosyl-Ptds), three (authentic *M. smegmatis* AcPIM2) and four (authentic *M. smegmatis* AcPIM2) fatty chains. —, radioactivity; ---, 1-propanol gradient.

(Figure 5B). Taken together with other structural analyses (Table 2), we conclude that the labelled lipid in fraction E is predominantly AcPIM4 (Man α 1-6Man α 1-6Man α 1-6[acyl-Man α

Figure 5 Mannosidase digestion of PIM species

(A) [³H]-Man-labelled lipids in fractions E–G were mock-treated (–) or treated (+) with either JBAM for 18 h (JB) or ASAM for 18 h (AS). The asterisk indicates AcPIM5 generated by partial ASAM digestion of AcPIM6. (B) Lipids in fractions E–G were treated more exhaustively with ASAM. Lipids were analysed by HPTLC (solvent 1).

1-2]PI). The lipids in fraction F were partly resistant to ASAM treatment even after extended incubation (Figure 5B). On the basis of this and other structural analyses (Table 2), fraction F contains Man α 1-6Man α 1-6Man α 1-6[acyl-Man α 1-2]Ptd (AcPIM5'), a predicted intermediate in LAM biosynthesis, as well as Man α 1-2Man α 1-6Man α 1-6[acyl-Man α 1-2]Ptd (AcPIM5). In contrast, the labelled lipid in fraction G was completely susceptible to ASAM treatment (Figure 5B), suggesting that it corresponds to a final PIM product, Man α 1-2Man α 1-2Man α 1-6Man α 1-6Man α 1-6[acyl-Man α 1-2]Ptd (AcPIM6).

Interestingly, partial JBAM digestion of AcPIM4 (fraction E) did not result in the appearance of less polar PIM species (results not shown), suggesting that AcPIM4 was synthesized from endogenous AcPIM3 by the transfer of a single [³H]Man residue. Similarly, the lack of appearance of AcPIM4 after ASAM digestion of the AcPIM5 species in fraction F suggested that these intermediates were synthesized from endogenous (non-radiolabelled) AcPIM4 (Figure 5). In contrast, partial digestion of AcPIM6 by ASAM generated a new PIM species that co-migrated with AcPIM5 (Figure 5A, asterisk). Therefore at least

Figure 6 Effect of amphomycin on PIM biosynthesis

Cell lysate was preincubated for 5 min without (lane 1) or with (lane 2–4) 10 μ g/ml amphomycin (AM) before adding GDP-[^{3} H]Man. Radiolabelling was then continued for 40 min. Lipids from amphomycin-treated samples were extracted and mock-treated (lane 3) or treated (lane 4) with monomethylamine (MA) before HPTLC (solvent 1). The monomethylamine-sensitive band co-migrating with PPM (indicated by the asterisk) is probably AcPIM1 and/or Ac₂PIM2, which are still synthesized in the presence of amphomycin. The faint doublet remaining after monomethylamine treatment (lane 4) is probably the residual amount of PPM species synthesized in the presence of amphomycin. Additional bands below AcPIM6 (**) are candidates for higher-order LAM intermediates and were not characterized further in the present study.

some of [^{3} H]AcPIM6 must have been synthesized by two [^{3} H]Man additions to AcPIM4.

Most of the PIM species identified in these analyses are predicted to be biosynthetic intermediates on the basis of their structure and kinetics of labelling. However, the strongly labelled species Ac₂PIM1 (fraction A) does not fit the predicted pathway. Also, this species was only labelled at later time points (Figure 3A), suggesting that it is an end-product rather than an intermediate in the biosynthetic pathway (see other results below). Final assignments of all identified Man-containing lipids are summarized in Table 2.

PPM is the Man donor for biosynthesis of AcPIM4, AcPIM5 and AcPIM6

We next examined whether any of the steps in PIM biosynthesis were dependent on the lipid-linked Man donor PPM. PPM synthesis can be selectively inhibited *in vitro* with amphomycin, an antibiotic that forms a complex with the polyphenol phosphate precursor of PPM [19]. As shown in Figure 6 (lanes 1 and 2), preincubation with amphomycin effectively inhibited PPM synthesis. Consistent with previous studies [19], synthesis of PIM1 and AcPIM2 was unaffected by this treatment. The residual radioactive species in the PPM fraction (Figure 6, asterisk) was sensitive to monomethylamine O–N transacylation (lanes 3 and 4) and this species is probably Ac₂PIM2 and AcPIM1 (trace) on the basis of the glycan head-group analysis of fraction B (Figure 3C). A second monomethylamine-sensitive species migrated slightly faster than AcPIM4 (Figure 6, arrowhead). On the basis of the glycan head-group analysis of fraction E (Figure 3C), this species is probably PIM2. These results show that monoacylated and diacylated forms of PIM1 and PIM2 were synthesized in the presence of amphomycin, confirming that the first two mannosyltransferases are GDP-Man-dependent.

Figure 7 Pulse-chase labelling of PIM intermediates with GDP-[^{3} H]Man

(A) The cell lysate was radiolabelled with GDP-[^{3} H]Man for 5 min and then chased with 1 mM non-radioactive GDP-Man ($t = 5$). PIM1 is indicated by arrowheads. Note that the faint band migrating slightly slower than AcPIM2 at 120 min is not PIM1, but probably a minor monomethylamine-resistant species as identified in Figure 3(B, lane 2). The upper one-third of the HPTLC plate did not contain radioactive bands and is not shown. (B) An identical experiment to (A) was conducted in the presence of 10 μ g/ml amphomycin. The lysate was preincubated with amphomycin for 5 min before the addition of GDP-[^{3} H]Man ($t = 0$). Lipids were analysed by HPTLC in solvent 1.

In contrast, synthesis of AcPIM4, AcPIM5 and AcPIM6 was effectively stopped in the presence of amphomycin (Figure 6, lanes 1 and 2), suggesting that the direct donor for the syntheses of these intermediates is PPM rather than GDP-Man. To confirm that amphomycin was not directly inhibiting the PIM mannosyltransferases, the cell lysate was incubated with GDP-[^{3} H]Man for 5 min to generate [^{3} H]PPM and then incubated for a further 80 min in the presence of amphomycin. Labelled polar species (AcPIM4–6) were still generated during the 80 min incubation, demonstrating that polar PIM biosynthesis continued in the presence of amphomycin as long as a source of [^{3} H]PPM was available (results not shown).

Pulse-chase labelling of PIMs

When the cell-free system was pulse-labelled with GDP-[^{3} H]Man for 5 min and then incubated with unlabelled GDP-Man, the label in PIM1 and PPM decreased, with a concomitant increase of label in AcPIM2 (Figure 7A). The absence of a strong precursor-product relationship between the different polar PIM species is consistent with the notion that most of these glycolipids were synthesized from pre-existing intermediates rather than synthesized *de novo* from Ptd. The rapid turnover of [^{3} H]PPM during the chase is addressed below (see Figure 8). To demonstrate more clearly the precursor-product relationship between PIM1 and AcPIM2, the same experiment was performed in the presence

Figure 8 Inhibition by GDP of PIM biosynthesis *in vitro*

(A) Cell lysates were pulse-labelled as described in Figure 6(A), and label-chased in the presence of 1 mM GDP-Man with (●) or without (○) the inclusion of 40 units/ml alkaline phosphatase in the chase medium. The HPTLC plate was exposed to a tritium storage phosphor screen and an autoradiograph was obtained using a Typhoon 9410 imager (Amersham Biosciences) to quantify the relative changes in PPM radioactivity. (B) Cell lysates were preincubated for 5 min with the indicated final concentrations of either GDP or alkaline phosphatase (AP) before continuous labelling with GDP-[³H]Man for 60 min. (C) Lysates were labelled continuously with GDP-[³H]Man without chase and 2 mM GDP was added at 5 or 20 min during the time course. In all cases, lipids were analysed on HPTLC sheets developed in solvent 1.

of amphotycin. As expected, neither [³H]PPM nor polar PIMs were significantly labelled under these conditions (Figure 7B). In contrast, PIM1 was strongly labelled under these conditions and the label in this species was subsequently chased into the full complement of other early intermediates, including the mono- and diacylated forms of PIM1 and PIM2 (Figure 7B).

Inhibition of PPM biosynthesis by GDP

The turnover rate of [³H]PPM in the pulse-chase labelling experiments was faster than could be accounted for by the appearance of label in either the PIMs (Figure 7A) or LM/LAM fractions (results not shown), raising the possibility that the newly synthesized PPM could be catabolized. PPM catabolism could be mediated by the enzyme PPM synthase, catalysing the reverse of the synthetic reaction (i.e. the transfer of Man from GDP-Man to polyphosphatide) in the presence of excess GDP [33]. This possibility was supported by the finding that inclusion of alkaline phosphatase in the chase medium to hydrolyse excess GDP decreased the turnover of [³H]PPM by approx. 50% (Figure 8A). Similarly, inclusion of alkaline phosphatase in the continuous labelling experiment increased the amount of label in both the PPM and the polar PIMs (Figure 8B). Addition of GDP to the continuous labelling experiment significantly decreased (0.2 mM) or completely inhibited (2 mM) the synthesis of [³H]PPM as well as PIM intermediates. Finally, addition of GDP at 5 or 20 min time points in the continuous labelling experiment resulted in nearly complete disappearance of labelled PPM (Figure 8C). Collectively, these results suggest that GDP strongly suppresses all GDP-Man-dependent reactions, including the synthesis of PPM and the early PIMs. It also enhances the catabolism of pre-formed [³H]PPM, presumably by allowing PPM synthase to convert [³H]PPM into GDP-[³H]Man and polyphosphatide.

DISCUSSION

PIMs are synthesized by all species of mycobacteria and appear to be essential for normal growth and survival. Using *M. smegmatis*, a fast growing species of mycobacteria that has been used to define

many aspects of cell-wall biosynthesis conserved in pathogenic species, we have characterized all the intermediates synthesized in a cell-free system and used this system to examine the donor requirements and the regulation of this pathway.

Consistent with previous results [16,20,21], a number of apolar PIM species were labelled in the cell-free system, which appear to be intermediates in the synthesis of the major PIM species AcPIM2. On the basis of the characterization of these products, it is proposed that AcPIM2 is synthesized via two pathways (Scheme 1). These pathways differ in the timing of the first acylation step, which may reflect the presence of more than one acyltransferase [23]. Although the precise topology of these reactions has not been experimentally determined, they probably occur on the cytoplasmic leaflet on the basis of the finding that the first two mannosyltransferases in this pathway lack recognizable signal sequences and utilize the soluble sugar nucleotide GDP-Man [16,22]. PPM synthesis also utilizes GDP-Man and is catalysed by a cytoplasmically disposed enzyme [34].

The polar PIM species synthesized in the *M. smegmatis* cell-free system were AcPIM4, two isomers of AcPIM5 and AcPIM6. The two isomers of AcPIM5 only differ in the linkage of their terminal Man residues (Figure 4) and probably represent branch point intermediates in the synthesis of LM/LAM and AcPIM6 [8]. The conversion of *de novo* synthesized AcPIM5 into AcPIM6 was very efficient, as shown by the presence of two [³H]Man residues in AcPIM6 when compared with only one [³H]Man in earlier polar PIM intermediates. It is possible that the addition of the two α 1-2-linked Man residues to AcPIM4 prevents further mannosylation of this species to LM/LAM, leading to the accumulation of AcPIM6 as a metabolic end-product. As the efficiency of labelling of the two AcPIM5 species in this cell-free system was similar to the rate of synthesis of LM and polar PIM species *in vivo* (J. H. Patterson, Y. Morita and M. J. McConville, unpublished work), the activity of the capping α 1-2 mannosyltransferase(s) may play a key role in regulating the flux of PIM intermediates into these different cell-wall constituents.

Unlike the early steps in PIM biosynthesis, polar PIM biosynthesis requires PPM rather than GDP-Man (Scheme 1). This conclusion is supported by the findings that (i) polar PIM biosynthesis was inhibited by amphotycin, a selective inhibitor

Scheme 1 Model for PIM biosynthesis in *M. smegmatis*

The mannose donor requirements for early and late steps in the pathway are indicated, as is the point at which polar PIM and LAM biosynthesis may bifurcate, on the basis of the identification of the two isomers of AcPIM5. The assembly of LM and LAM also requires PPM [19]. —, steps reconstituted in the cell-free assay; ---, predicted steps not detected in the present study. PP, polyphosphatase.

of PPM synthesis (Figures 6 and 7B), (ii) label from GDP-[³H]Man continued to accumulate in the polar PIMs, but not apolar PIMs when lysates were chased in the presence of excess unlabelled GDP-Man (Figure 7A) and (iii) stimulation of PPM synthesis by inclusion of alkaline phosphatase in the assay was associated with increased polar PIM synthesis (Figure 8B). The mannosyltransferases that extend the polar PIMs with long α 1-6-linked Man chains to form LM/LAM are also associated with crude cell-wall fractions and are dependent on PPM [19]. As the utilization of lipid-linked monosaccharides (polyphrenol/dolichol phosphate sugars) is characteristically associated with reactions that occur in the periplasmic space of prokaryotes or in the lumen of the eukaryote secretory pathway [35,36], the polar PIMs and LAM are probably synthesized extra cytoplasmically.

The conversion of AcPIM2 into AcPIM3 is probably an important step in regulating the relative size of PIM2 and PIM6 steady-state pools. Unexpectedly, AcPIM3 was not labelled to detectable levels in the cell-free system despite the fact that AcPIM2 is very abundant in these cells and was the predominant PIM species to be synthesized. It is possible that the mannosyltransferase that catalyses this step is more labile than all the other PIM mannosyltransferases or is present at very low levels. Alternatively, conversion of AcPIM2 into AcPIM3 may involve other steps, such as the transbilayer movement of AcPIM2 species that are not reconstituted in the cell-free system. Interestingly, some strains of *M. tuberculosis* express a mannosyltransferase termed PimC, which catalyses the conversion of AcPIM2 into AcPIM3 in an *M. smegmatis* cell-free system [24]. PimC shares homology with

PimA and PimB and utilizes GDP-Man, suggesting that it may be orientated on the cytoplasmic leaflet of the plasma membrane. However, disruption of PimC in *M. bovis* had no effect on the relative abundance of the steady-state pools of Ac₁₋₂PIM2 or Ac₁₋₂PIM6, suggesting that the conversion of AcPIM2 into AcPIM3 can be catalysed by a second mannosyltransferase. The activity and/or access of AcPIM2 to this second mannosyltransferase may be important in regulating the conversion of AcPIM2 into polar PIMs and LM/LAM.

Mature PIM2 and PIM6 are modified with at least one extra fatty chain, linked to the core Man residue, and in many cases a second extra fatty chain, linked to the inositol head group [37] (Figure 2). Both mono- and diacylated PIM species were labelled in the cell-free system, indicating that sufficient endogenous donors exist to sustain these reactions. Unexpectedly, the most prominent diacylated PIM species to be labelled in this system was Ac₂PIM1. It is possible that the inositol acyltransferase is unable to access newly synthesized AcPIM2 and AcPIM6 and/or that newly synthesized AcPIM2 and AcPIM6 are diluted by the large pools of unlabelled PIMs. Alternatively, the formation of AcPIM1 may be catalysed by a novel acyltransferase that is distinct from the acyltransferases involved in the synthesis of Ac₂PIM2 and Ac₂PIM6. Although the physiological function of PIM acylation remains unknown, a very recent study has shown that disruption of one of the acyltransferases in *M. smegmatis* results in the accumulation of non-acylated PIMs and salt-sensitive growth in Luria-Bertani medium [23].

All of the GDP-Man-dependent reactions in this pathway result in the production of GDP. In the cell-free system, GDP was found to inhibit the synthesis of PIM1 and AcPIM2 and to increase the turnover of labelled PPM. High levels of GDP may drive PPM synthase to catalyse the transfer of Man from PPM to GDP, to form GDP-Man, the reverse of the normal biosynthetic reaction [33,38]. Increased levels of GDP occur when mycobacteria enter the stationary phase (J. H. Patterson, Y. Morita and M. J. McConville, unpublished work), raising the possibility that this nucleotide may be an important regulatory molecule under physiological conditions. Since PPM may also act as a carrier for mycolic acids [39], the down-regulation of PPM would lead to decreased synthesis of polar PIM and LM/LAM and the transfer of mycolic acids to the cell wall. Intriguingly, many of the genes involved in PIM biosynthesis in *M. tuberculosis* are located within a putative transcriptional unit that also contains an open reading frame for a putative GDP-Man hydrolase [16]. Whether this enzyme plays a role in regulating GDP levels and PIM and LAM biosynthesis is currently being investigated.

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