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A revised biosynthetic pathway for phosphatidylinositol in Mycobacteria

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Hiroyuki Morii[1,*], Midori Ogawa[2], Kazumasa Fukuda[2], Hatsumi Taniguchi[2] and Yosuke Koga[1]

[1]Department of Chemistry and [2]Department of Microbiology, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu, 807-8555, Japan

*Hiroyuki Morii, Department of Chemistry, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu, 807-8555, Japan.
Tel: +81 93 603 1611, Fax: +81 93 693 9921,
email: h-morii@health.uoeh-u.ac.jp

For the last decade, it has been believed that phosphatidylinositol (PI) in mycobacteria is synthesized from free inositol and CDP-diacylglycerol by PI synthase in the presence of ATP. The role of ATP in this process, however, is not understood. Additionally, the PI synthase activity is extremely low compared with the PI synthase activity of yeast. When CDP-diacylglycerol and [^{14}C]1L-*myo*-inositol 1-phosphate were incubated with the cell wall components of *Mycobacterium smegmatis*, both phosphatidylinositol phosphate (PIP) and PI were formed, as identified by fast atom bombardment-mass spectrometry and thin-layer chromatography. PI was formed from PIP by incubation with the cell wall components. Thus, mycobacterial PI was synthesized from CDP-diacylglycerol and *myo*-inositol 1-phosphate via PIP, which was dephosphorylated to PI. The gene-encoding PIP synthase from four species of mycobacteria was cloned and expressed in *Escherichia coli*, and PIP synthase activity was confirmed. A very low, but significant level of free [^{3}H]inositol was incorporated into PI in mycobacterial cell wall preparations, but not in recombinant *E. coli* cell homogenates. This activity could be explained by the presence of two minor PI metabolic pathways: PI/inositol exchange reaction and phosphorylation of inositol by ATP prior to entering the PIP synthase pathway.

Keywords: inositol/phosphatidylinositol phosphate synthase/phospholipid/*Mycobacterium smegmatis*/tuberculosis.

Abbreviations: AI, archaetidylinositol; AIP, archaetidylinositol phosphate; CDP-DAG, CDP-diacylglycerol; FAB-MS, fast atom bombardment-mass spectrometry; PI, phosphatidylinositol: PIP, phosphatidylinositol phosphate; TLC, thin-layer chromatography.

Mycobacterium tuberculosis is a major causative agent of mortality worldwide. *M. tuberculosis* has developed resistance to every new drug used to treat tuberculosis, resulting in the recent global emergence of drug-resistant tuberculosis (*1*).

All mycobacterial cells have lipid-rich cell envelopes, which are the basis of resistance to macrophages or anti-bacterial drugs (*2*). Recent studies revealed that the cell wall constituent lipoarabinomannan and its precursors, phosphatidylinositol (PI) mannosides, have an important role in the growth of mycobacterial cells and their infectivity (*2–9*). Therefore, the biosynthesis of cell wall lipids is a promising target of anti-mycobacterial drugs.

The first step of lipoarabinomannan biosynthesis is the formation of the starting material PI. Salman *et al.* (*10*) reported that PI is synthesized by mycobacterial cell wall constituents from CDP-diacylglycerol (CDP-DAG) and free inositol in the presence of ATP. The role of ATP in the reaction, however, is not understood (*10*). The proposed pathway for mycobacteria was the same as that of eukaryotes. Eukaryotic PI is synthesized from CDP-DAG and *myo*-inositol (*11*), which is generated from D-glucose 6-phosphate (*12*, *13*).

$$\text{D-glucose-6-phosphate} \rightarrow \text{1L-}myo\text{-inositol 1-phosphate} \quad (1)$$

$$\text{1L-}myo\text{-inositol 1-phosphate} \rightarrow myo\text{-inositol} + \text{Pi} \quad (2)$$

$$myo\text{-inositol} + \text{CDP-diacylglycerol} \rightarrow \text{phosphatidyl-}myo\text{-inositol} + \text{CMP} \quad (3)$$

Reactions (1) and (3) are catalysed by 1L-*myo*-inositol 1-phosphate synthase and PI synthase, respectively.

Over the last several years, we have investigated the *in vitro* biosynthesis of an ether-type inositol phospholipid (archaetidyl-*myo*-inositol) in methanoarchaea. We determined that little inositol is incorporated into lipids, whereas inositol 1-phosphate is a good substrate. Based on the archaeal-enzyme study, we re-examined the PI synthase reaction in mycobacteria because of the reported low-specific activity and the presence of ATP in the reaction mixture. ATP is not required for the eukaryotic PI synthase reaction. The

specific activity of [^{3}H] inositol incorporation reported by Jackson *et al.* (*14*) was 2.5 pmol/h/mg protein in mycobacterial cell walls. This corresponds to 0.014% of the specific activity (18 nmol/h/mg protein) in *Saccharomyces cerevisiae* cell homogenates (*15*).

We recently reported a novel biosynthetic pathway of archaetidylinositol (AI) in the cells of the methanoarchaeon *Methanothermobacter thermautotrophicus* (*16*). The reaction sequence in the archaeon after reaction (1) is as follows:

$$\text{CDP-archaeol} + 1\text{L-}myo\text{-inositol 1-phosphate} \rightarrow \text{archaetidyl-}myo\text{-inositol phosphate} + \text{CMP} \quad (4)$$

$$\text{archaetidyl-}myo\text{-inositol phosphate} \rightarrow \text{archaetidyl-}myo\text{-inositol} + \text{Pi} \quad (5)$$

In a mycobacterial cell-free system with the reaction mixture containing inositol 1-phosphate and CDP-DAG without ATP, we detected activity comparable to that of yeast homogenates. The use of a different substrate for PI synthesis led to the discovery of a novel PI-biosynthetic pathway. The present article describes a novel-biosynthetic pathway of PI in mycobacteria via phosphatidylinositol phosphate (PIP) as in AI synthesis in methanoarchaea. In addition to this main *de novo* synthesis of PI, we also detected a PI/inositol exchange reaction and inositol phosphorylation activity. These reactions complicate the experimental results obtained using crude enzyme preparations of the PI metabolism in mycobacteria.

Because of the difference in the PI biosynthetic mechanism in mycobacteria compared to that of Eucarya, this pathway might be a promising target for drug discovery.

Experimental Procedures

Materials

β-D-Glucose 6-phosphate was purchased from Sigma-Aldrich (Tokyo, Japan). [^{14}C(U)]Glucose 6-phosphate (3.7 MBq/ml) was obtained from Moravek Biochemicals, Inc. (Brea, CA, USA). [2-^{3}H]*myo*-Inositol (37 MBq/ml) was obtained from MP Biomedicals, LLC. (Santa Ana, CA, USA). [^{14}C]Inositol 1-phosphate, which was not commercially available, was prepared from [^{14}C(U)]glucose 6-phosphate using *myo*-inositol phosphate synthase from *M. thermautotrophicus* and filtered with an Ultrafilter (USY-1 M = 10,000, Advantec), as described earlier (*16*). The filtrate contained [^{14}C]inositol 1-phosphate, unreacted [^{14}C]glucose 6-phosphate, and other low-molecular weight compounds. This filtrate (hereafter referred to as [^{14}C]inositol 1-phosphate) was used without further purification as a substrate for the PIP synthase reaction. CDP-dipalmitoylglycerol [CDP-DAG(dipalmitoyl)] and CDP-dioleoylglycerol [CDP-DAG(dioleoyl)] were chemically synthesized from 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (Avanti Polar Lipids Inc., Alabaster, AL, USA) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (Avanti Polar Lipids Inc.), respectively, as described earlier (*17*). PI (soybean) was obtained from DOOSAN Serdary Research Laboratories (Toronto, ON, USA). Percoll was obtained from GE Healthcare (Piscataway, NJ, USA). Dowex AG1 (X8; 100–200 mesh, chloride form; BIO RAD, Berkeley, CA, USA) was converted to the formate form. *Mycobacterium smegmatis* mc^{2}155, *M. bovis* BCG, *M. marinum* (clinical isolate) and *M. chelonae* subsp. *abscessus* (JATA63-1) were used in this study.

Preparation of standard PIP

The recombinant AI phosphate (AIP) synthase expressed in *Escherichia coli* cells (*16*) had significant activity (~20%) with CDP-DAG(dioleoyl) was used as the substrate instead of CDP-archaeol. The products of this reaction were confirmed as PIP and a small amount of PI. The product was used as standard PIP.

Growth of microorganisms

Mycobacterium smegmatis mc^{2}155 cells were grown in Difco Nutrient Broth (Becton Dickinson, Franklin Lakes, NJ, USA) for ~2 days while shaking at 37°C and harvested by centrifugation. Cells were washed with buffer A (50 mM MOPS [pH 7.9] containing 10 mM $MgCl_2$ and 5 μM 2-mercaptoethanol). The pelleted cells were stored at −20°C until use.

Preparation of crude cell wall, cytosolic, membrane and cell wall fractions

Frozen cells (~4 g wet weight) of *M. smegmatis* were thawed in 20 ml buffer A (see above) and disrupted by sonication using a SONIFIER 250 (1 cm probe; Branson, Danbury, CT, USA) for 10 min (10 × 60 s pulses with 90-s cooling intervals between pulses) as described by Salman *et al.* (*10*). Crude cell walls were obtained by centrifugation of the above whole sonicate as described by Jackson *et al.* (*14*). Cytosolic and membrane fractions were separated by centrifugation of the 27,000*g*-supernatant at 100,000*g* for 1 h (*10*). The cell wall fraction was purified from the re-suspended 27,000*g*-pellet in buffer A by centrifugation in 60% Percoll at 27,000*g* for 1 h (*10*, *18*). The upper one-third layer of the centrifuge tube was saved as the purified cell wall fraction (hereafter called cell walls). The crude cell wall, cytosolic, membrane and purified cell wall fractions were stored at −20°C until use.

Measurement of PIP synthase activity

The complete assay mixture (final volume, 0.2 ml) contained 50 μl of [^{14}C]inositol 1-phosphate (19 nmol, 2800 Bq), 40 nmol CDP-DAG, 50 mM MOPS buffer (pH 7.9), 10 mM $MgCl_2$, 5 μM 2-mercaptoethanol, 0.4% (w/v) CHAPS and the cell wall fraction (200 μg protein) of *M. smegmatis* cell homogenate. In some experiments, the homogenates of *E. coli* pET21a-PIPS, in which the gene-encoding-PIP synthase of one of *Mycobacterium* species (see below) had been expressed, was used instead of the cell walls. CDP-DAG was dispersed with the aqueous components of the reaction mixture except for CHAPS, [^{14}C]inositol 1-phosphate, and the enzyme preparation in a 1.5-ml microtube by continuous sonication at room temperature for 15 min using a Bransonic 1210 (Branson) bath. After the addition of CHAPS, [^{14}C]inositol 1-phosphate, and the enzyme preparation, the reaction mixture was incubated at 37°C for 1 h. The reaction was stopped by adding 1 ml of 0.1 M HCl in methanol and the mixture was transferred to a 10-ml Teflon-lined, screw-capped glass tube with 1.5 ml of 0.1 M HCl in methanol and 2.5 ml $CHCl_3$. Finally, 2.15 ml of 1 M $MgCl_2$ (pH 2) was added to the mixture to partition the reactants and the products into aqueous and organic layers. After washing twice with 0.1 M HCl/methanol-1 M $MgCl_2$ (pH 2) (1 : 0.8, v/v), the organic layer was evaporated to dryness and the radioactivity in the fraction was counted. PIP-synthase activity was expressed as nano mol of (PIP + PI)/h. Because PI is derived from PIP as described below, (PIP + PI) represents the total PIP synthesized.

Measurement of free inositol incorporation into lipid

Incorporation of free inositol into lipid was measured by essentially the same method used in Salman *et al.* (*10*). The complete reaction mixture (final volume, 0.2 ml) comprising 2.5 μM [^{3}H]inositol (0.5 nmol, 74 kBq/Assay), 40 nmol CDP-DAG, 50 mM MOPS buffer (pH 7.9), 10 mM $MgCl_2$, 5 μM 2-mercaptoethanol, 0.1 mM ATP, 5 mM glucose, 0.4% (w/v) CHAPS and the cell wall fraction (200 μg protein) of *M. smegmatis* cell homogenates, was incubated at 37°C for 1 h and extracted with the same procedure described to measure the PIP-synthase activity.

Acid extraction of lipid from enzyme reaction mixtures or silica gel scraped from a TLC plate

Lipids were extracted from the enzyme reaction mixtures or silica gel scraped from a preparative-TLC plate by the acidic Bligh and Dyer

method (*19*) using $CHCl_3$-0.1 M HCl in methanol-1 M $MgCl_2$ (pH 2) (1 : 2 : 0.8, v/v) as a solvent.

Neutral-acidic fractional extraction of PIP

[^{14}C]PIP was sufficiently extracted using the Bligh and Dyer method (*19*) with an acidic solvent from PIP-synthase reaction mixture, but not extracted using a neutral solvent (neutral extraction; Supplementary Table SI). Therefore, PIP could be separated from the other lipids containing PI by this newly devised fractional-extraction method. The reaction mixture was first extracted using the neutral extraction. PIP was recovered from the acidified remainder (the upper aqueous layer and fluff) into a chloroform fraction.

Thin-layer chromatography

Thin-layer chromatography (TLC) of lipids was performed on a Silica Gel 60 plate (Merck, Tokyo, Japan) with the following solvent: chloroform, methanol, acetic acid and water (80 : 30 : 20 : 10). Phospholipid spots were visualized by spraying acid molybdate reagent. Radioactive spots on the TLC plate were recorded using a Fujifilm FLA-5000 fluor-image analyser with an imaging plate (Fujifilm type BAS-MS for ^{14}C material or BAS-TR for ^{3}H material, Fujifilm, Japan).

Fast atom bombardment-mass spectrometry of the PIP-synthase reaction products (PIP and PI)

To obtain the PIP-synthase reaction products in an amount large enough for structural analysis, the volume of the reaction mixture was increased 20 times (concentration of the reactants was not changed) and the incubation time was prolonged to 4 h. PIP (dioleoyl) was obtained by neutral–acidic fractional extraction because PIP (dioleoyl) was unstable during the recovery from silica gel scraped from the TLC plate. The purified PIP was converted to PI (dioleoyl) by incubating with *M. smegmatis* cell walls containing PIP dephosphorylation activity and purified by TLC. The PI (dioleoyl) scraped from TLC plates was extracted using a neutral solvent. The thus obtained PIP and PI were analysed by fast atom bombardment-mass spectrometry (FAB-MS) using a mass spectrometer (JEOL JMS DX-303) with a glycerol matrix in negative mode.

Analytical methods

Phosphate (*20*) and protein (*21*) were determined as described earlier. Radioactivity was counted using a liquid scintillation spectrometer (Aloka LSC-3500E, Japan) with Aquasol-2 (Packard, Meriden, CT, USA) as the scintillation cocktail.

Construction of the expression plasmids for the PIP synthase genes of mycobacteria

Nucleic acid sequences of genes coding for the putative PIP synthases of *Mycobacterium* species (protein id: *M. smegmatis* mc^2 155, ABK73364.1; *M. bovis* BCG, CAL72625.1; *M. marinum* M, ACC40540.1; and *M. abscessus*, CAM62975.1) were obtained from Genome Information Broker (http://gib.genes.nig.ac.jp/). The putative PIP synthases were searched by using FASTA3 search tool and the amino acid sequence of AIP synthase (protein id: AAB86163.1) of *M. thermautotrophicus* as a query. The putative PIP-synthase genes of the mycobacteria were amplified by polymerase chain reaction (PCR) directly from the genomic DNAs of each species. The genomic DNAs of mycobacteria were extracted from the cells suspended in distilled water by boiling for 5 min. After centrifugation (15,000 r.p.m. for 5 min), the supernatants were used as template for PCR. The following primer sequences were used: *M. smegmatis* mc^2 155 forward, 5′-GACGGAAAAGCGCCCACCATATGAGCAATG-3′; reverse, 5′-CTGCCCGCCGAGCGGGATGCGTCGACAAAG-3′; *M. bovis* BCG forward, 5′-ACCGAGTGGGCTCGGCACATATGAGCA AGC-3′; reverse, 5′-AGCTTCAAGCCCTTAAGGTCGACAATC ACC-3′; *M. marinum* forward, 5′-GCACATATGAGCAAGGCGC CCTTCTTGTCC-3′; reverse, 5′-GCCGGGGTCGACATCACC GCTGCGTCTTTC-3′; *M. abscessus* forward, 5′-CACCGCCTGG GAGCAGTTCATATGAG CGGC-3′; reverse, 5′-GGCGCGCTCT CCGTCGACACTCATGGCTGA-3′. The restriction enzyme (*Nde*I for forward primer and *Sal*I for reverse primer) recognition sites are underlined. The amplified genes were cloned with a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transformation was performed with competent *E. coli* TOP10 cells provided by the manufacturer. The genes inserted into the pCR2.1-TOPO vector were digested by restriction enzymes *Nde*I and *Sal*I. After confirming the fragment sizes by gel electrophoresis with a 1.5% agarose gel, the fragments were extracted and purified using GenElute^{TM} Minus EtBr Spin Columns (Sigma-Aldrich, St Louis, MO, USA). The purified fragments were inserted into the corresponding sites of the expression vector, pET21a (Merck Ltd). The presence of the appropriate inserts was confirmed by DNA sequencing. Expression vectors were designated pET21a-smeg-PIPS (PIP synthase of *M. smegmatis* mc^2 155), pET21a-BCG-PIPS (PIP synthase of *M. bovis* BCG), pET21a-mari-PIPS (PIP synthase of *M. marinum*) and pET21a-abs-PIPS (PIP synthase of *M. abscessus*), respectively. The expression vectors were chemically inserted into the expression host cell line BL21(DE3).

Expression of PIP synthase in E. coli

To confirm that the cloned genes from *Mycobacterium* species really encoded PIP synthase, *E. coli* BL21-(Merck Ltd) carrying pET21a-PIPS were grown in Luria Bertani medium containing 50 μg/ml ampicillin. PIPS gene expression was induced by adding isopropyl β-D-thiogalactopyranoside (1 mM) for 3 h. The membrane fraction of the sonicator-disrupted cells in 0.1 M Bicine buffer (pH 8.0) containing 10 mM 2-mercaptoethanol were used for PIP synthase measurements.

Anion-exchange chromatography

To detect inositol and inositol phosphate in the reaction mixture of free inositol incorporation into lipid, the aqueous fraction was fractionated with a 1-ml column of Dowex-1 (X8; formate form). Inositol and inositol phosphate were eluted with water and 0.1 M formic acid/0.2 M ammonium formate, respectively (*22*).

Results

Activity of PIP synthase and PIP phosphatase

When CDP-DAG(dioleoyl) was incubated with [^{14}C]inositol 1-phosphate in the presence of the cell wall fraction of *M. smegmatis*, a significant amount of radioactivity was incorporated into chloroform-soluble materials. Two spots were detected by TLC of the chloroform-soluble products (Rf = 0.25 and 0.44, Fig. 1A, lane 3). The slower spot comigrated with standard PIP (dioleoyl) (Rf = 0.25) prepared as described earlier (Fig. 1A, lane 2). The other radioactive spot comigrated with standard PI (Rf = 0.44). The FAB-MS of the putative PIP gave signals of m/z 941 $(M\text{-}H)^-$ and 699 (phosphatidic acid) (Fig. 1C), which were consistent with the molecular weight and structure of dioleoylglycerophosphoinositol phosphate [PI(dioleoyl) + HPO_3 (PIP(dioleoyl))]. The faster moving spot had a signal of m/z 861 $(M\text{-}H)^-$ (Supplementary Fig. S1) on FAB-MS, identical to PI(dioleoyl). Thus, the two products were chromatographically and spectrometrically identified as PIP and PI, respectively.

The time course of synthesis of PIP and PI from CDP-DAG and [^{14}C]inositol 1-phosphate is shown in Fig. 1B. The incorporation of the radiolabel into PIP preceded that into PI. The apparent specific activity of this enzyme was 5.3 ± 0.83 nmol/h mg/protein ($n = 4$), ~2000 times higher than the specific activity of free [^{3}H]inositol incorporation into PI (2.5 pmol/h/mg) by *Mycobacterium* crude cell walls reported by Jackson *et al.* (*14*).

Cellular localization of PIP synthase activity was examined. Only the cell wall fraction had appreciable total and specific PIP synthase activity (Table I). The activity was almost completely dependent on the

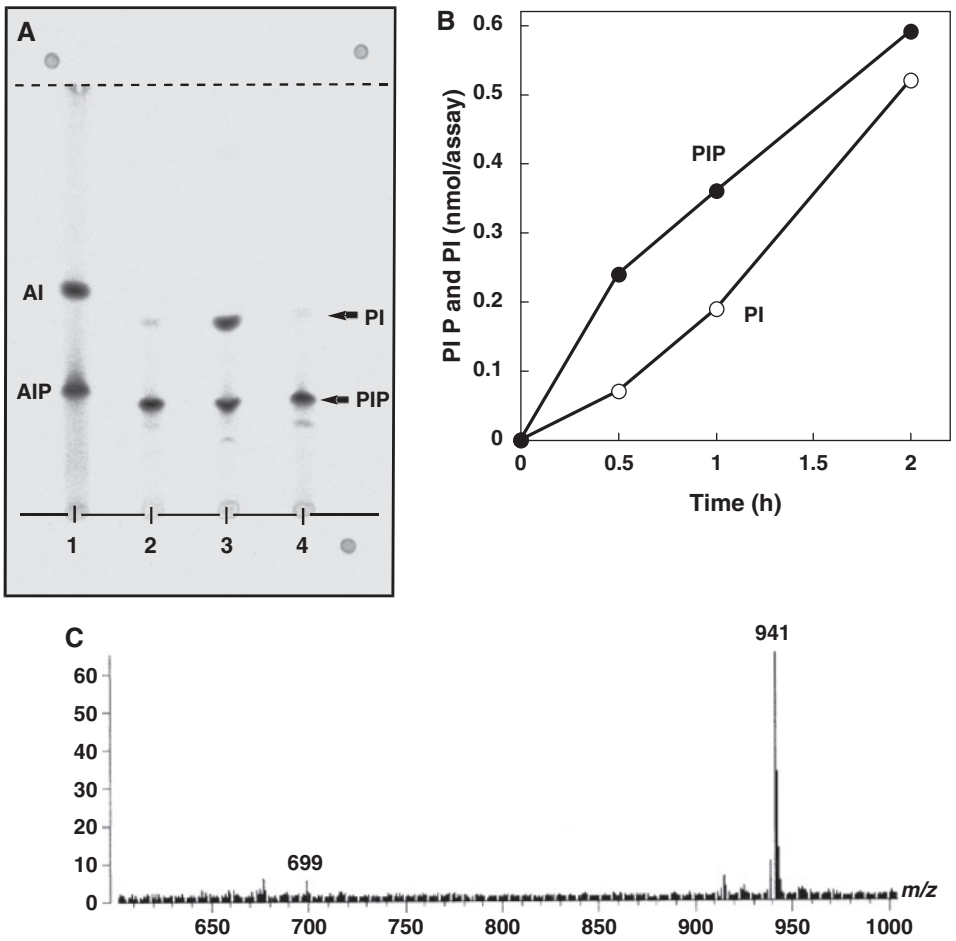

Fig. 1 Identification of the reaction products of PIP synthase. (A) Thin-layer chromatogram of ^{14}C-labelled products of the PIP (AIP)-synthase reaction. The source of the AIP synthase was cell homogenates of *E. coli* pET21a-MTH1691 (lanes 1 and 2), the purified cell wall fraction of *M. smegmatis* cell homogenates (lane 3) or cell homogenates of *E.coli* pET21a-abs-PIPS (CAM62975.1, PIP synthase of *M. abscessus*; lane 4). Lipid substrate in the enzyme reaction was CDP-archaeol (lane 1) or CDP-DAG (dioleoyl) (lanes 2–4). The products were extracted and developed by TLC. Radioactive spots were detected by autoradiography. s.f., solvent front; AI, archaetidylinositol; AIP, archaetidylinositol phosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate. (B) Time-course of PIP synthase reaction. PIP and PI were formed from [^{14}C]inositol 1-phosphate and CDP-DAG (dioleoyl) catalysed by the purified cell wall fraction of *M. smegmatis* cell homogenates. The reaction was stopped at the indicated time points by the addition of 0.1 M HCl/methanol, radioactivity of chloroform-soluble products was counted. The ratio of PIP and PI was determined by autoradiography after TLC development of the chloroform-soluble products. (C) Negative ion FAB-mass spectra of PIP enzymatically synthesized from CDP-DAG (dioleoyl) and *myo*-inositol 1-phosphate (non-radiolabelled).

Table I. Incorporation of [^{14}C]inositol 1-phosphate (PIP synthase activity) and free [^{3}H]inositol into lipids (PIP and PI) using *M. smegmatis* cell wall, membrane and cytosol fractions or crude cell walls.

	PIP synthase activity					
	[^{14}C]Inositol 1-phosphate			[^{3}H]Inositol		
Fraction	Total activity (nmol/h)	Percent	Specific activity (nmol/h/mg)	Total activity (nmol/h)	Percent	Specific activity (nmol/h/mg)
Purified cell wall[a]	42	85	6.3	0.11	90	0.017
Membrane	4.4	9	0.6	0.0080	7	0.001
Cytosol	3.3	7	0.1	0.0049	4	0.000
Crude cell wall[b]	–	–	2.7	–	–	0.078

Assay was performed as described in 'Experimental Procedures' section with CDP-DAG(dioleoyl) and various enzyme preparations. [a]*Mycobacterium smegmatis* purified cell walls were obtained by centrifugation through a 60% Percoll layer. [b]*Mycobacterium smegmatis* crude cell walls were obtained from pellets upon centrifugation between 3000 r.p.m. for 10 min and 27,000*g* and resuspended in buffer.

presence of CDP-DAG and *myo*-inositol 1-phosphate (Table II). Differences in the fatty acyl group composition of CDP-DAG significantly affected PIP synthase activity (~50%).

To determine the reaction sequence of synthesis of PIP and PI, [^{14}C]PIP and [^{14}C]PI synthesized from the incubation of CDP-DAG (dipalmitoyl) and [^{14}C]inositol 1-phosphate for 4 h were isolated by

Table II. Requirements for PIP synthase.

[^{14}C]Inositol 1-phosphate	Source of enzyme	CDP-DAG	Relative incorporation, %
+[a]	*Mycobacterium smegmatis* purified cell walls	CDP-DAG (dioleoyl)	100
+	*Mycobacterium smegmatis* purified cell walls	CDP-DAG (dipalmitoyl)	54
+	*Mycobacterium smegmatis* purified cell walls	None	1
−[b]	*Mycobacterium smegmatis* purified cell walls	CDP-DAG (dioleoyl)	1
+	None	CDP-DAG (dioleoyl)	0
+	*Escherichia coli* pET21a-abs-PIPS[c]	CDP-DAG (dioleoyl)	292
+	*Escherichia coli* pET21a[d]	CDP-DAG (dioleoyl)	1

The reaction mixture of the PIP synthase reaction is described in 'Experimental Procedures' section. After incubation at 37°C for 1 h, radioactivity in the chloroform soluble materials was counted. [a][^{14}C]Inositol 1-phosphate was synthesized from [^{14}C]glucose 6-phosphate in a complete inositol phosphate synthase reaction mixture with the supernatant fraction of the *Methanothermobacter thermautotrophicus* homogenate. [b]The solution included only [^{14}C]glucose 6-phosphate as the radiolabelled material. [c]Homogenate of *E. coli* pET21a-abs-PIPS (CAM62975.1, PIP synthase of *Mycobacterium abscessus*). [d]Homogenate of *E. coli* pET21a.

TLC. The purified [^{14}C]PIP was converted to [^{14}C]PI in the presence of the cell wall fraction of *M. smegmatis* cell homogenates (Fig. 2, lane 2). Similar results were obtained in the case of [^{14}C]PIP (dioleoyl) without purification synthesized from CDP-DAG (dioleoyl) by the homogenate of *E. coli* carrying pET21a-mari-PIPS plasmid (Supplementary Fig. S2). The PIP phosphatase activity was localized in the cell wall fraction of *M. smegmatis* cells. On the other hand, [^{14}C]PIP was not formed from [^{14}C]PI in the presence of the cell wall fraction of *M. smegmatis* cell homogenates (Fig. 2, lane 4; Supplementary Fig. S2, lane 4).

Cloning of a PIP synthase and characterization of the recombinant enzyme

The nucleic acid sequences of the cloned PIP synthase genes of *M. bovis* BCG, *M. smegmatis* mc^2 155 and *M. abscessus* were completely compatible with the sequences registered in the genome data base (http://gib.genes.nig.ac.jp/). The sequence of the cloned PIP synthase gene of *M. bovis* BCG was the same as that of *M. tuberculosis* (CAE55505.1) (Fig. 3). The sequence of the cloned PIP synthase gene of *M. marinum* was different from that of *M. marinum* M (ACC40540.1). The nucleic acid and amino acid sequence (AB559817) identities between the laboratory strain and *M. marinum* M (ACC40540.1) were 86% (614/710 bases) and 88% (207/234 amino acids), respectively. A typical sequence of a CDP-alcohol phosphatidyltransferase motif (*23*) was found in all of the amino acid sequences (Fig. 3). All of the *E. coli* cell homogenates in which the PIP synthase genes of four *Mycobacterium* species (*M. bovis* BCG, *M. marinum*, *M. smegmatis* mc^2 155 and *M. abscessus*) were expressed, had sufficient PIP synthase activity (9.0–15.5 nmol/h/mg). A main reaction product was PIP, based on TLC (Fig. 1A, lane 4). *Escherichia coli* pET21a cell homogenates carrying an empty vector possessed little PIP-synthesis activity (Table II). PI synthase activity (incorporation of free [^{3}H]inositol into lipid) was, however, not detected in the *E. coli* cell homogenates in which the PIP-synthase gene was expressed. Therefore, we concluded that the putative PIP-synthase genes are the structural genes that encode PIP synthase but not PI synthase, and PIP synthesis from CDP-DAG and *myo*-inositol 1-phosphate was catalysed by a single enzyme.

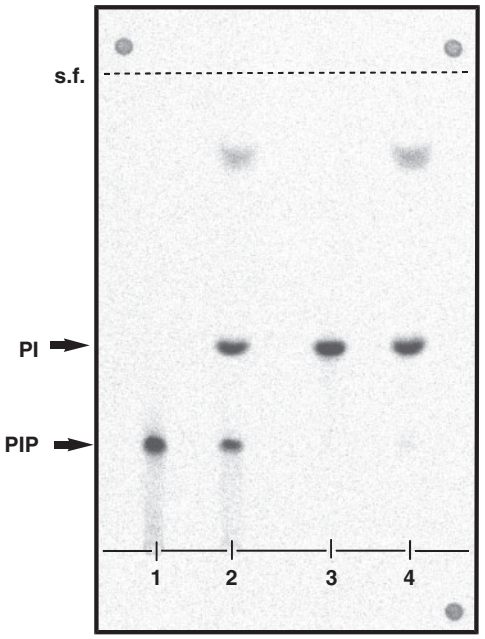

Fig. 2 Conversion of PIP to PI by the purified cell wall fraction of *M. smegmatis* cell homogenates (PIP phosphatase). [^{14}C]PIP and [^{14}C]PI were prepared from CDP-DAG (dipalmitoyl) and [^{14}C]inositol 1-phosphate using the PIP synthase reaction conditions described in the 'Experimental Procedures' section. The purified [^{14}C]PIP (lanes 1 and 2) or the purified [^{14}C]PI (lanes 3 and 4) was incubated at 37°C for 4 h with the cell wall fraction of *M. smegmatis* homogenate (lanes 2 and 4) or without the cell wall fraction (lanes 1 and 3). Other constituents in the reaction mixture were the same as in the PIP synthase reaction mixture without [^{14}C]inositol 1-phosphate. After the reaction, the lipids were extracted, separated by TLC, and recorded by autoradiography.

Inositol kinase activity

Although we demonstrated that PI was synthesized from CDP-DAG and inositol 1-phosphate via PIP, Salman *et al.* (*10*) reported the incorporation of free inositol into PI in the presence of mycobacterial cell walls. We attempted to confirm the reaction and to clarify the physiological significance of these reactions related to PI metabolism. Free [^{3}H]inositol was incorporated into lipids in the presence of *M. smegmatis* purified cell walls under the same conditions described

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TUB     MSKLPFLSRAAFARITT-PIARGLLRVGLTPDVVTILGTTASVAGALTLF
BCG     MSKLPFLSRAAFARITT-PIARGLLRVGLTPDVVTILGTTASVAGALTLF
MAR     MSKAPFLSRAAFARVTN-PLARGLLRIGLTPDAVTIIGTTASVAGALVLF
SME     MSNVYLMTRAAYVKLSR-PVAKAALRAGLTPDIVTLAGTAAAVIGALTLF
ABS     MSG--LLSRETFAKITN-PLASALLRAGFTPDTVTIFGTAASVVAALTLF
MTH     MPDINESMLNQFRPVIRRFIDPIADRIALPADYITLTGFLVACAASAG-Y
PFU     ----MLSNLRPLAKKPLEKIAEPFSKLGITPNQLTMVGFFLSLLASYEYY
                          :     : .:..: :*: *   :   .:  :

TUB     PMGKLFAGACVVWFFVLFDMLDGAMARERGGGTRFGAVLDATCDRISDGA
BCG     PMGKLFAGACVVWFFVLFDMLDGAMARERGGGTRFGAVLDATCDRISDGA
MAR     PMGKLFPGACVVWFFVLFDMLDGAMARERGGGTRFGAVLDAACDRISDGA
SME     PIGQLWWGAVVVSFFVLADMLDGAMAREQGGGTRFGAVLDATCDRLGDGA
ABS     PTGHLFWGGMAVWLFAMFDMLDGAMARARGGGTRFGAVLDATCDRVADGA
MTH     ASGSLITGAALLAASGFIDVLDGAVARRRFRPTAFGGFLDSTLDRLSDGI
PFU     LNNQVFG-SLILLLGAFLDALDGSLARLTGRVTKFGGFLDSTMDRLSDAA
          . :   .  :    : * ***::**     * **..**:: **:.*.

TUB     VFCGLLWWIAFHMRDRPLVIATLICLVTSQVISYIKARAEASG-LRGDGG
BCG     VFCGLLWWIAFHMRDRPLVIATLICLVTSQVISYIKARAEASG-LRGDGG
MAR     VFGGLLWWVAFGMRDRLLVVATLICLVTSQVISYIKARAEASG-LRGDGG
SME     VFAGLTWWAAFGLDSPSLVVATLICLVTSQVISYIKARAEASG-LRGDGG
ABS     VFAGLVWWAAFGWGSTSLVVATLICMITSQVISYVKARAEASG-LRADGG
MTH     IIIGITAGGFTGLLTG------LLALHSGLMVSYVRARAESLG-IECAVG
PFU     IIFGIALGELVNWKVA------FLALIGSYMVSYTRCRAELAGSGTLAVG
        :: *:                 ::.:  . ::** :.***  *      *

TUB     FIERPERLIIVLTGAGVSDFPFVPWPPALSVGMWLLAVASVITCVQRLHT
BCG     FIERPERLIIVLTGAGVSDFPFVPWPPALSVGMWLLAVASVITCVQRLHT
MAR     IIERPERLIIVLAGAGVSDFPFIAWPPALPVAMWLLAVTSVITCGQRLYT
SME     IIERPERLVIVLIGAGLSDLPFFPLPWTLHVAMWVLAVASVVTLLQRVHA
ABS     LIERPERLIIVLAGAIFSGGFGVQWP--LHTAMWVLAVASLVTVAQRMHA
MTH     IAERAERIIIILAGSLAG---YLIHPWFMDAAIIVLAALGYFTMIQRMIY
PFU     IAERGERLLILVIAGLFG---------IIDIGVYLVAILSWITFLQRVYE
        : ** **::*:: ..  .          :  .: ::*  . .*  **:

TUB     VWTSPGAID-----------------RMAIPGKGDR
BCG     VWTSPGAID-----------------RMAIPGKGDR
MAR     VWTSPGATDLLVPSAPVRDDDAQGHPRSGDPGKTQR
SME     VRTSPGAMEPLHP-----------ANGEKPETSEP
ABS     VRTSPGALDLLLPNS----------DAGQDTAETNQP
MTH     VWQRLK-----------------------------
PFU     AKKRLEK----------------------------
        .
```

Fig. 3 Multiple alignment of PIP Synthase and AIP Synthase. Genes homologous to *Methanothermobacter thermautotrophicus* (MTH1691) gene were identified in mycobacterial genome. The genes were tentatively annotated as PIP synthase. The amino acid sequence data of CAE55505.1, *Mycobacterium tuberculosis* (TUB); CAL72625.1, *M. bovis* (BCG); ACC40540.1, *M. marinum* (MAR); ABK73364.1, *M. smegmatis* (SME); CAM62975.1, *M. abscessus* (ABS); MTH1691, *Methanothermobacter thermautotrophicus* (MTH); PFU0462, *Pyrococcus furiosus* (PFU) were obtained from the NCBI site (www.ncbi.nih.gov). Multiple alignment of the seven sequences was constructed using the alignment software CLUSTAL W 1.83. Underlined sequences are CDP-alcohol phosphatidyltransferase motif.

by Salman *et al.* (*10*). The specific activity of [^{3}H]inositol incorporation, however, was only 0.27% that of [^{14}C]inositol 1-phosphate incorporation (Table I), as low as that reported by Salman *et al.* The products of free [^{3}H]inositol incorporation were identified by TLC as PI (the major spot) and PIP (the minor spot) (Fig. 4A, lane 1). The isolated minor spot (PIP) was converted to PI with *M. smegmatis* cell walls (Fig. 4B, lane 2), namely PIP was a precursor of PI. Although PIP formation requires both CDP-DAG and inositol 1-phosphate (Table II), inositol 1-phosphate was not added to this reaction. This suggests that inositol 1-phosphate is formed in the reaction mixture. Water-soluble inositol metabolites in the free [^{3}H]inositol incorporation reaction mixture were analysed by anion-exchange chromatography (Fig. 4C). Before the reaction started, all [^{3}H]inositol-derived materials were eluted with water and no ^{3}H material was retained on the column. After 2 h incubation, 5% of ^{3}H material was retained on the column by washing with water and eluted in the same fraction as inositol phosphate (0.1 M formic acid/0.2 M ammonium formate) (*22*) (Fig. 4C). This finding suggests that inositol was phosphorylated, probably by ATP in the *M. smegmatis* cell walls (possibly by inositol kinase).

PI/Inositol exchange reaction in Mycobacteria

As described earlier, in the presence of CDP-DAG and ATP, a small amount of [^{3}H]inositol was incorporated

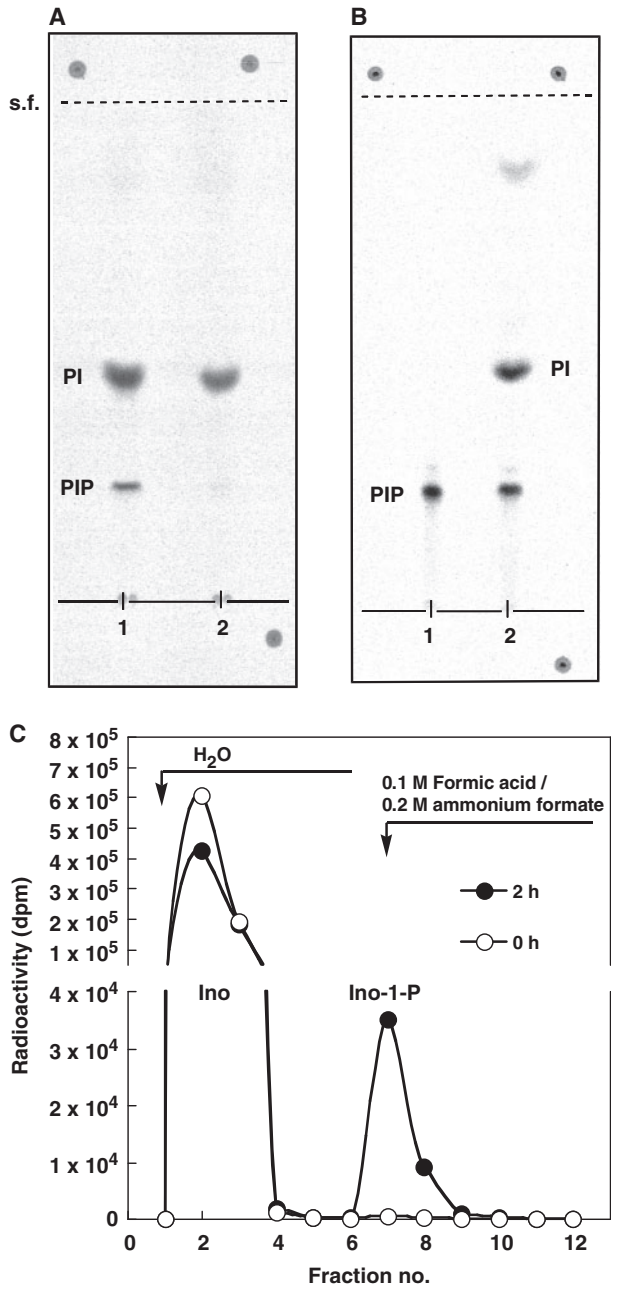

Fig. 4 Analysis of the reaction products from the free [^{3}H]inositol incorporation reaction by the purified *M. smegmatis* cell wall fraction. (A) Thin-layer chromatogram of ^{3}H-labelled $CHCl_3$-soluble products of the free [^{3}H]inositol incorporation reaction with CDP-DAG (dioleoyl) (lane 1) or without CDP-DAG (lane 2). (B) Conversion of [^{3}H]PIP to [^{3}H]PI. [^{3}H]PIP was prepared from [^{3}H]inositol and CDP-DAG (dipalmitoyl) as described in the 'Experimental Procedures' section. The purified [^{3}H]PIP was incubated at 37°C for 4 h with the cell wall fraction of *M. smegmatis* cell homogenate (lane 2) or without the cell wall fraction (lane 1). (C) Elution profiles of ^{3}H-labelled water-soluble products of free [^{3}H]inositol incorporation reaction by an anion-exchange column. The crude cell walls of *M. smegmatis* were incubated with free [^{3}H]inositol in the presence of 1 mM ATP without CDP-DAG for 0 or 2 h. The water-soluble components were applied to a Dowex-1 anion-exchange column and eluted with water and 0.1 M formic acid/0.2 M ammonium formate. Fractions (2 ml) were collected and radioactivity was measured. Authentic inositol phosphate was detected by phosphate determination.

into PI and PIP in the presence of *M. smegmatis* cell walls (Fig. 4A, lane 1). It should be noted that in the absence of CDP-DAG only the PI spot was detected (Fig. 4A, lane 2). The amount of [^{3}H]inositol incorporated into PI was 91% that obtained in the reaction with CDP-DAG (Table III). That is, the addition of CDP-DAG had almost no effect on inositol incorporation into PI. A reaction that proceeds without the addition of CDP-DAG is generally assumed to be a PI/inositol exchange reaction (*24*). Although PI as an acceptor of inositol was not added to the reaction mixture in this case, the cell wall fraction contains sufficient amounts of PI for the exchange reaction (*2*, *10*). Thus, the incorporation reaction of free [^{3}H]inositol into PI without CDP-DAG was concluded to be a PI/inositol exchange reaction.

The addition of PI (40 nmol) had no effect on free [^{3}H]inositol incorporation by the homogenate of *E. coli* pET21a-abs-PIPS. We also concluded that PIP synthase did not carry PI/inositol-exchange activity.

Discussion

These findings clearly demonstrated that mycobacterial PI was synthesized from CDP-DAG and 1L-*myo*-inositol 1-phosphate via PIP, which was dephosphorylated to PI. The time course of [^{14}C]inositol 1-phosphate incorporation into PIP and PI by the purified cell walls also supported this reaction sequence. PIP was not formed from PI by phosphorylation. Two possible enzyme activities, PIP synthase and PIP phosphatase, were detected in the cell wall fraction of *M. smegmatis*. The apparent specific activity of PIP synthesis of the mycobacterial cell wall fraction was comparable to yeast PI synthase activity in the membrane fraction. Consequently, we concluded that the main PI synthesis pathway in mycobacteria proceeded as the following new reaction sequence (Equations 6 and 7; see also Fig. 5).

$$\text{CDP-DAG} + \text{inositol 1-phosphate} \rightarrow \text{PIP} + \text{CMP} \quad (6)$$

$$\text{PIP} \rightarrow \text{PI} + \text{Pi} \quad (7)$$

The stereostructure of the phosphoinositol moiety of mycobacterial PI (*25*) is identical to that of soy bean (*26*) and *M. thermautotrophicus* (1D-*myo*-inositol 1-phosphate) (*27*); therefore, its precursor must be 1L-*myo*-inositol 1-phosphate. Bachhawat and Mande *(28)* identified an inositol 1-phosphate synthase homologue (*INO1*) in *M. tuberculosis* that functionally complements an *INO1*-deletion mutation in *S. cerevisiae*. This finding suggested that the reaction product of mycobacterial inositol 1-phosphate synthase is likely 1L-*myo*-inositol 1-phosphate. On the other hand, the complete structure of the reaction product of inositol 1-phosphate synthase of *M. thermautotrophicus* was determined to be 1L-*myo*-inositol 1-phosphate (*16*). Therefore, methanoarchaeal [^{14}C]inositol 1-phosphate was used as the substrate for the mycobacterial

PIP-synthase reaction. Because significant activity was detected, 1L-*myo*-inositol 1-phosphate is an adequate substrate for mycobacterial PIP synthase.

We reexamined Salman *et al.*'s experiments (*10*) of the PI synthesis reaction in mycobacterial cells from CDP-DAG with free inositol in the presence of ATP and glucose. In our experiments, [^{3}H]inositol was also incorporated into lipids in the presence of the *M. smegmatis* cell wall fraction under the same conditions. The specific activity of [^{3}H]inositol incorporation, however, was quite low (0.27%) compared with [^{14}C]inositol 1-phosphate incorporation (Table I). In contrast to mycobacterial cell walls, the recombinant PIP synthase-containing *E. coli* cell homogenates did not have any PI synthase activity. If PI was synthesized from CDP-DAG and inositol by a PI synthase reaction, another enzyme besides PIP synthase must exist in the mycobacterial cell walls.

In summary, we propose four possible reactions/pathways concerning inositol phospholipid metabolism:

(i) *De novo* synthesis from CDP-DAG and free inositol (Eucarya);
(ii) *De novo* synthesis from CDP-DAG (CDP-archaeol) and inositol 1-phosphate (Bacteria/Archaea);
(iii) PI/inositol exchange reaction (Eucarya); and
(iv) Phosphorylation of inositol by inositol kinase followed by the above reaction (ii).

Pathway (i) is well-established in eukaryotes. Pathway (ii) was just recently reported (*16*). Here, we discuss pathways (iii) and (iv) in greater detail. There are two results that support pathway (iv). First, during incubation for the free inositol incorporation reaction, inositol was anionically modified (probably phosphorylated because of the presence of ATP as a phosphoryl group donor; Fig. 4C). Second, [^{3}H]PIP was synthesized by an [^{3}H]inositol incorporation reaction with CDP-DAG and was converted to [^{3}H]PI by *M. smegmatis* cell walls (Fig. 4B, lane 2). These two results suggest that free inositol was phosphorylated to inositol 1-phosphate, which then reacted with CDP-DAG to form PIP.

Activity of the PI/inositol exchange reaction (iii) is usually measured in the absence of CDP-DAG (*24*, *29*). Even if CDP-DAG was omitted from the reaction mixture, but a significant, although small amount of free [^{3}H]inositol was incorporated into the lipid (Table III). Salman *et al.* (*10*) obtained a similar

Table III. Effect of components in the reaction mixture on incorporation of free [^{3}H]inositol into lipid by the purified cell walls of *M. smegmatis*.

Reaction mixture	Relative incorporation (%)
Complete	100
−CDP-DAG	91
−ATP	84
−CDP-DAG, −ATP	72
−Glucose	126

The complete reaction mixture contained 2.5 μM [^{3}H]inositol (0.5 nmol, 74 kBq/Assay), 40 nmol CDP-DAG (dioleoyl), 0.1 mM ATP, 50 mM MOPS buffer (pH 7.9), 5 μM 2-mercaptoethanol, 10 mM $MgCl_2$, 5 mM glucose and *M. smegmatis* cell walls. After incubation at 37°C for 1 h, radioactivity in the chloroform soluble materials was counted.

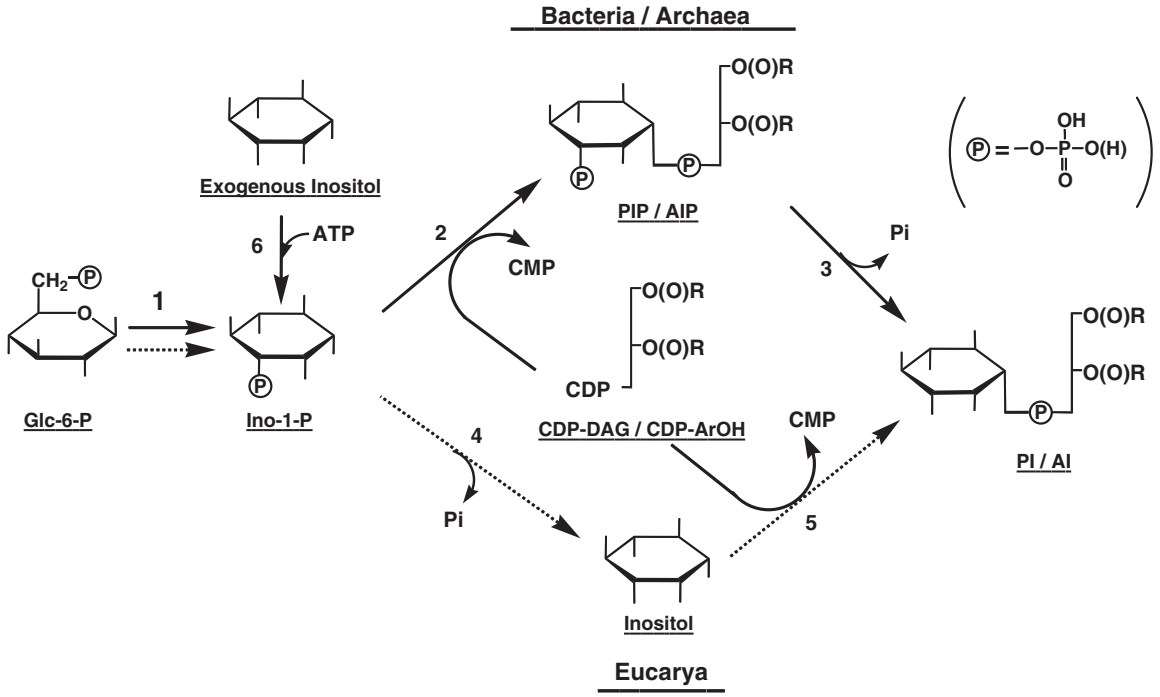

Fig. 5 Proposed biosynthetic pathway of PI and Archaetidyl-*myo*-inositol (AI) in Bacteria and Archaea (solid arrow), and Eucarya (broken arrow). Lane 1: 1L-*myo*-Inositol 1-phosphate synthase; lane 2: Phosphatidyl-*myo*-inositol phosphate (PIP) synthase/Archaetidyl-*myo*-inositol phosphate (AIP) synthase; lane 3: PIP phosphatase/AIP phosphatase; lane 4: 1L-*myo*-Inositol 1-phosphate phosphatase; lane 5: Phosphatidyl-*myo*-inositol (PI) synthase; lane 6: Inositol kinase.

result and explained that endogenous CDP-DAG reacted with the free inositol. However, the cell walls are considered to contain very little CDP-DAG because the cell walls have hardly any PIP synthase activity when exogenous CDP-DAG is not added to the reaction mixture (Table II). Furthermore, CDP-DAG added to the reaction mixture did not stimulate free [^{3}H]inositol incorporation into lipid in the presence of *M. smegmatis* cell walls (Table III). These results exclude the possibility that free inositol directly reacted with CDP-DAG [i.e. pathway (i)]. Therefore, the incorporation reaction of free [^{3}H]inositol into PI without CDP-DAG was concluded to be a PI /inositol exchange reaction [i.e. pathway (iii)]. For the PI/inositol exchange reaction, an acceptor PI must be present in the reaction mixture. Although no exogenous PI was added to the reaction mixture, the cells walls, which contain sufficient PI for an exchange reaction, are a possible source of acceptor PI (*2*, *10*).

It has been reported that PI synthesis and exchange of the inositol head are catalysed by a single PI synthase from yeast (*24*) and *Arabidopsis* (*30*), and that the PI/inositol exchange reaction is not the result of a traditional D-type phospholipase in soybean microsomes (*29*). The mechanism in eukaryotes, however, is not likely applicable to mycobacteria, because mycobacteria do not possess PI synthase and the exchange reaction is not due to PIP synthase. Therefore, we speculate that the PI/inositol exchange reaction in mycobacteria is exceptionally catalysed by phospolipase D. The PI/inositol exchange reaction seems to be an unusual form of phospholipid metabolism that does not necessarily lead to a net synthesis of PI. At this point, the physiologic significance of the exchange reaction is unknown.

The level of free [^{3}H]inositol incorporation into lipid in the reaction was almost the same with or without CDP-DAG (Table III). The major product of the [^{3}H]inositol incorporation reaction with CDP-DAG was PI (Fig. 4A, lane 1). These results support the notion that free [^{3}H]inositol incorporation was due to the PI/inositol exchange reaction (iii), but see below. PIP was also detected as a minor product of the [^{3}H]inositol incorporation reaction with CDP-DAG, but not without CDP-DAG (Fig. 4A). These results indicate that the phosphorylated inositol reacted with the exogenous CDP-DAG.

Salman *et al. (10)* synthesized the fluorescent analogue of CDP-DAG, CDP-C_6-NBD-DAG, When this fluorescent substrate was incubated with the *M. smegmatis* cell wall fraction, NBD-PI was synthesized (*10*). This result does not support the PI/inositol exchange pathway (iii), but rather suggests that reaction (ii) occurred after the inositol phosphorylation reaction (iv).

In conclusion, in *Mycobacterium* species PI is synthesized from CDP-DAG and inositol 1-phosphate via PIP (Fig. 5, reactions 2 and 3). This is the main *de novo* biosynthetic pathway of mycobacterial PI. In addition to the main pathway, a side entrance of free inositol into the main pathway by phosphorylation, which might be an inositol salvage pathway (Fig. 5, reactions 6, 2 and 3), and a PI/inositol exchange reaction occurred in the cell wall fraction of *M. smegmatis*.

Fortunately, we detected PIP as an intermediate of PI synthesis using an acidified solvent system, which is essential for extracting PIP because PIP is partitioned in the aqueous fraction and/or fluff with a neutral solvent due to its high polarity. This property was applied to isolate PIP from the other polar lipids, including PI.

During the course of this study, Morita *et al.* (*31*) independently detected PIP in *M. smegmatis* cells *in vivo*. Although they considered that PIP was formed from PI by phosphorylation, their data, such as the stimulation of PIP accumulation by the addition of a phosphatase inhibitor or the increase in PIP formation by the addition of CTP, suggests that PI is formed from PIP. Thus, the presence of PIP as a precursor of PI was confirmed *in vivo* and *in vitro* by an independent research group.

Because the PI synthetic mechanism involving PIP synthase in *Mycobacterium* species is clearly different from the mechanism of human and animal PI synthesis and PIP synthase and PI synthase discriminate substrates, PIP synthase is a promising target for the development of new anti-mycobacterium drugs, which might have an important impact given the recent emergence of multi-drug resistant strains of *M. tuberculosis*.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

Patent pending (Japan Patent application No. 2009-272247) for the author Hiroyuki Morii.

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