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Phosphatidylinositol synthesis in mycobacteria

Michael Salman [a,*], John T. Lonsdale [a], Gurdyal S. Besra [c], Patrick J. Brennan [b]

[a] *SmithKline Beecham Pharmaceuticals, 1250 S Collegeville Rd., PO Box 5089, Collegeville, PA 19426, USA*
[b] *Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA*
[c] *School of Microbiological, Immunological and Virological Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HN, UK*

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Abstract

The metabolism and synthesis of an important mycobacterial lipid component, phosphatidylinositol (PI), and its metabolites, was studied in *Mycobacterium smegmatis* and *M. smegmatis* subcellular fractions. Little is known about the synthesis of PI in prokaryotic cells. Only a cell wall fraction (P60) in *M. smegmatis* was shown to possess PI synthase activity. Product was identified as PI by migration on TLC, treatment with phospholipase C and ion exchange chromatography. PI was the only major product (92.3%) when both cells and P60 fraction were labeled with [^{3}H]inositol. Also, a neutral lipid inositol-containing product (4.1% of the total label) was identified in the P60 preparations. Strangely, PI synthase substrates, CDP-dipalmitoyl-DAG and CDP-NBD-DAG, added to the assay did not stimulate [^{3}H]PI and NBD-PI yield by *M. smegmatis*. At the same time, addition of both substrates to rat liver and *Saccharomyces cerevisiae* PI synthase assays resulted in an increase in the product yield. Upon addition of CHAPS to the mycobacterial PI synthase assay, both substrates were utilized in a dose-dependent manner for the synthesis of NBD-PI and [^{3}H]PI. These results demonstrate a strict substrate specificity of mycobacterial PI synthase toward endogenous substrates. K_m of the enzyme toward inositol was shown to be 25 μM; Mg^{2+} stimulated the enzyme to a greater degree than Mn^{2+}. Structural analogs of *myo*-inositol, *epi*-inositol and *scyllo*-inositol and Zn^{2+} were shown to be more potent inhibitors of mycobacterial PI synthase than of mammalian analogs. Lack of sequence homology with mammalian PI synthases, different kinetic characteristics, existence of selective inhibitors and an important physiological role in mycobacteria, suggest that PI synthase may be a good potential target for anti-tuberculosis therapy.

Keywords: Mycobacteria; Phosphatidylinositol; Phosphatidylinositol synthase; Phosphatidylinositol mannoside; Fluorescent lipid

Abbreviations: C_{35}, heptaprenol; C_{50}, decaprenol; CDP-NBD-DAG, cytidine diphosphate-1,2-[oleyl,(*N*-(nitrobenzo-2-oxa-1,3-diazole)aminocaproyl)]diacylglycerol; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DAG, diacylglycerol; DAP, diaminopimelic acid; PGP, phosphatidylglycerophosphate; DPG, diphosphatidylglycerol (cardiolipin); GDP-Man, GDP-mannose; GPI, glycosylphosphatidylinositol; LAM, lipoarabinomannan; LM, lipomannan; MOPS, 4-morpholinepropanesulfonic acid; NBD-PI, 1,2-[oleyl,(*N*-(nitrobenzo-2-oxa-1,3-diazole)aminocaproyl)]phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM_x, phosphatidylinositol mannosides; PIM_2, phosphatidylinositol dimannoside; PIM_5, phosphatidylinositol pentamannoside; PLC, phospholipase C; PS, phosphatidylserine

* Corresponding author. Fax: (610) 9177901; E-mail: michael_salman-1@sbphrd.com

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1. Introduction

The cell wall of mycobacteria is a very complex and poorly understood structure and is quite different from that of other Gram-positive bacteria. Many unique lipids (such as mycolic acids, phosphatidylinositol mannosides (PIMs), peptidoglycolipids, etc.) are synthesized almost exclusively by mycobacteria, which allocate for the fatty acids and lipid biosynthesis a larger part of their genome, comparatively to the other prokaryotes [1]. Comprehensive models of the mycobacterial cell wall were produced recently [2,3].

Phosphatidylinositol (PI) and PIMs are the prominent and most distinguishable phospholipids of mycobacterial membranes and cell wall. They also provide the lipid anchor for lipomannan (LM) and lipoarabinomannan (LAM), important ligands and immunodeterminants on the mycobacterial envelope. It was suggested that PI and PIMs combined constituted about 56% of all phospholipids in the cell wall and 37% of those in the cytoplasmic membrane of mycobacteria [4]. PI, PIMs, LM and LAM all share in common the presence of tuberculostearic (D-10-methylstearic) acid esterified to the 1-position of the glycerol, and palmitic acid esterified to the 2-position [2], pointing to a metabolic relationship. Indeed, recent evidence suggests the biosynthetic sequence: PI → PIM → LM → LAM [5]. The weight of evidence from this study indicates that PI is the acceptor of two mannose units in succession donated by GDP-Man to give rise to $PIMan_2$. However, the bulk of the Man units that comprise the linear mannan backbone of LM arise from decaprenol-phosphomannose/heptaprenol-phosphomannose (C_{35}-/C_{50}-P-Man).

Paulus and Kennedy elucidated the pathway for PI biosynthesis in mammalian cells [6] by demonstrating that CDP-DAG reacts with free inositol to give rise to de novo synthesized PI. The key enzyme responsible for the transformation is cytidinediphosphate-diacylglycerol:*myo*-inositol transferase, or PI synthase (EC 2.7.8.11). An alternative pathway involving a nucleotide diphosphate-inositol on a par with CDP-choline and CDP-ethanolamine is possible. In this study we concentrated on obtaining some fundamental information on the biosynthesis of PI in mycobacteria prior to a more extensive study of the role and enzymology of the pathway in *Mycobacterium tuberculosis* as a possible site for the development of new anti-tuberculosis drugs.

PI has been seldom found in prokaryotic cells; apparently, the distribution is confined to *Mycobacterium* [4], *Treponema* [7] and *Myxobacteria* [8], although this assumption must be questioned with reports of small amounts (0.1–1% of the total phospholipids) in *Escherichia coli*, *Pseudomonas* and *Erwinia* species [9].

2. Experimental procedures

2.1. Materials

Cytidine 5′-diphospho-*sn*-glycerol 1,2-dipalmitoyl (CDP-DAG), cytidine 5′-monophosphomorpholidate, PI-specific phospholipase C (PI-PLC from *Bacillus cereus*), PLC (*B. cereus*), *scyllo*-inositol, *epi*-inositol and *myo*-inositol, GDP-mannose (GDP-Man), deoxyribonuclease, ribonuclease were purchased from Sigma (St. Louis, MO). 1,2-Diacyl-*sn*-glycero-3-phospho(1-*myo*-inositol) (PI) and 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) and all fluorescent lipids and fluorescent synthetic precursors (NBD-PG, NBD-PA, NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). *myo*-[2-^{3}H]Inositol (80 Ci/mmol) and [^{14}C]acetate (60 mCi/mmol) were from Amersham (Arlington Heights, IL). GDP-[U-^{14}C]Man (321.4 mCi/mmol) was obtained from DuPont NEN (Boston, MA).

2.2. Methods

2.2.1. Strains

Mycobacterium smegmatis, mc^{2}155 [10] was grown in Bacto nutrient broth (Difco Labs, Detroit, MI) for about 24 h and harvested at mid-log phase. When growing cells were labeled with [^{14}C]acetate, acetate was added to the growth medium for 24 h (1 μCi/ml medium). After centrifugation at 4°C, cells were washed in a buffer containing 50 mM MOPS adjusted to pH 7.9 with KOH, 5 μM β-mercaptoethanol and 10 mM $MgCl_2$ (buffer A).

2.2.2. Synthesis of fluorescent CDP-(1-2-acyl, C_6-NBD)-DAG (CDP-NBD-DAG), NBD-PI and NBD-free fatty acid (FA)

CDP-NBD-DAG was synthesized as described previously from cytidine 5′-monophosphomorpholidate and (1-2, acyl, C_6-NBD)-PA [11]. The product was a mixture of isomers. Approx. 80% of the product contained the fluorescent fatty acid in the *sn*-2 position while approx. 20% was in the *sn*-1 position [11,12]. NBD-PI was synthesized from CDP-NBD-DAG as described [11,13], using rat liver homogenate as a source of PI synthase. NBD-PI and NBD-FA were the main products in the reaction. Lipids were stored at −20°C and repurified when necessary.

2.2.3. Preparation of enzymatically active subcellular fractions

Whole rat liver homogenate and rat liver microsomal fractions were prepared as described before [14]. Whole homogenate was used for mass production of NBD-PI and microsomal fraction for kinetic and inhibitory studies. Yeast microsomal fraction from *Saccharomyces cerevisiae* was prepared as described [15].

2.2.4. Preparation of mycobacterial cytosolic, membrane and cell wall fractions

M. smegmatis (10 g, wet weight) was washed and resuspended at 4°C in buffer A. Cell suspension was subjected to probe sonication (1 cm probe; Soniprep 150; MSE, Crawley, Sussex, UK) for 10 min in the form of 10×60 s pulses with 90 s cooling intervals between pulses. The whole sonicate was centrifuged at 27 000×*g* for 15 min at 4°C, and the membrane fraction was obtained by centrifugation of the supernatant at 100 000×*g* for 1 h at 4°C. Membranes were resuspended in 1.0 ml of buffer A to yield a total of approx. 10 mg protein which was frozen in small aliquots at −70°C. The supernatant from the 100 000×*g* centrifugation yielded a pale yellow cytosolic enzyme fraction with a protein concentration of 8–10 mg/ml.

The particulate cell wall fraction was obtained as follows. The pellet from the 27 000×*g* centrifugation was resuspended in 20 ml of buffer A and divided between 40 ml centrifuges tubes, to which 15 ml of Percoll (Pharmacia, Sweden) were added and centrifuged at 27 000×*g* for 60 min at 4°C [16]. The upper diffuse band, containing the cell wall fraction, was removed, washed 3 times in buffer A and resuspended in the same buffer to give a concentration of protein of 8–10 mg/ml.

Alternatively, a variation of the protocol was used [17,18]. Briefly, bacteria (3 g, dry weight) were pelleted by centrifugation at 2000×*g* for 10 min and resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted by intermittent probe sonication for 30 min (10×90 s bursts with 90 s of cooling; ten pulses). The unbroken cells were removed by three low-speed centrifugation steps (325, 1310 and 2940×*g*) for 5 min each. The supernatant was digested with 10 μg/ml deoxyribonuclease and ribonuclease for 1 h at 4°C. The crude cell wall preparation was obtained by centrifugation at 5000×*g* for 40 min. The supernatant was centrifuged at 27 000×*g* for 30 min and the resulting supernatant recentrifuged at 100 000×*g* for 2 h. The resulting pellet represented the membrane fraction, and the resulting supernatant was considered the soluble cytosolic fraction. The crude cell wall fraction was washed 3 times with PBS by centrifugation at 27 000×*g* for 30 min. The final pellet was resuspended in PBS and layered onto a sucrose gradient consisting of steps of 15, 30, 40, 50 and 70% (w/v) sucrose and the tubes centrifuged at 100 000×*g* for 2 h. The gradients were collected and monitored for absorption at 280 nm. Fractions were pooled accordingly, diluted with water, centrifuged at 27 000×*g* for 30 min, and washed repeatedly to remove the sucrose. This procedure was originally designed for the fractionation of *Mycobacterium leprae* which yielded four cell wall particulate fractions [18]. In case of *M. smegmatis*, subfractionation of cell wall yielded three fractions: a minor white fraction at the 30–40% interface; a major yellow fraction at the 40–50% interface; a minor white fraction at the 50–70% interface.

2.2.5. Assay for PI synthase activity using [^{3}H]inositol as a substrate

Whole cells, cell wall, membrane and cytosol preparations were assayed for PI synthase activity in a final volume of 200 μl. Each reaction mixture contained: 100 μl of cell fraction (approx. 1 mg of protein), 2 μl of 10 mM ATP, 2 μl of 500 mM glucose,

2 μl of 10 mM GDP-Man (where indicated), 30–800 μM CDP-DAG (where indicated), CHAPS (0–1%), 20 μCi (final concentration 1 μM) of [^{3}H]inositol (specific activity 80 Ci/mmol) and buffer A up to 200 μl. The mixture was incubated at 37°C for the indicated time. In most cases, endogenous CDP-DAG (phospholipid co-purified with cell fragments) was utilized in the reaction. Sometimes, when no TLC separation was required, only scintillation counting of the products was done (as in inhibition studies), reactions were run on a small scale with higher concentrations of total inositol (slightly above K_m) in a final volume of 50 μl (25 μl of cell wall fraction, 5 μCi of 50 μM [^{3}H]inositol (specific activity 1.6 Ci/mmol) and buffer A up to 50 μl. In some experiments, varying amounts of dipalmitoyl-CDP-DAG (30–800 μM) were added. CDP-DAG was sonicated in buffer A in a sonication bath for 10 min at room temperature. In some experiments rat liver or yeast microsomes (50 μg protein) were used in inhibition studies; in this case the final concentration of [^{3}H]inositol was adjusted with unlabeled inositol to be in the vicinity of the K_m value reported in literature: 90 μM for the *S. cerevisiae* [15] and 1.3 mM for the rat liver PI synthase [19].

2.2.6. Assay for PI synthase using fluorescent NBD lipid analogs as substrate

Cell wall, membrane and cytosol preparations were assayed for PI synthase activity in a final volume of 300 μl. Each reaction contained: 100 μl of cell fraction (approx. 1 mg of protein), 10 μl of 500 mM *myo*-inositol, CHAPS (0–1.0%), 150 μl of 50 μM CDP-NBD-DAG (or NBD-phosphatidic acid, or NBD-phosphatidylglycerol) solution in buffer A, and buffer A up to 300 μl. The final concentration of fluorescent lipid in each case was 25 μM. In some reactions, where we were interested in the formation of both the fluorescent and radioactive forms of PI, 'cold' *myo*-inositol was omitted, and 20 μCi of [^{3}H]inositol (final concentration 0.67 μM, specific activity 80 Ci/mmol) was added to the reaction mixture.

2.2.7. Examination of the reaction products

Reactions were stopped with the addition of $CHCl_3$:CH_3OH (2:1; 2.5 ml per 100 μl reaction mixture), followed by centrifugation to separate the pellet [20,21]. Pellets were extracted one more time with one half the volume of $CHCl_3$:CH_3OH (2:1). The combined $CHCl_3$:CH_3OH (2:1) extracts were washed with $CHCl_3$:CH_3OH:H_2O (4:2:1), and dried. The resulting insoluble pellet was washed with 0.9% NaCl:CH_3OH (1:1), then with H_2O:CH_3OH (1:1) before extracting with $CHCl_3$:CH_3OH:H_2O (10:10:3). Alternatively, when highly polar polyglycosylated products were not of interest, lipid extraction was performed according to Bligh and Dyer [22] with slight modifications. Reactions were stopped with 1 M HCl (up to 1 ml), and lipids were extracted with 3.75 vols. of $CHCl_3$:CH_3OH (1:2), and 1.25 vols. of $CHCl_3$ followed by the addition of 1.25 vols. of 1 M HCl and centrifugation. The upper phase was discarded and the lower phase, containing the lipid products, was washed with 2 M KCl saturated with chloroform.

2.2.8. Analytical procedures

TLC was conducted in one or two dimensions on aluminum backed plates of silicagel 60 F_{254} (E. Merck, Darmstadt, Germany). $CHCl_3$:CH_3OH (2:1) or $CHCl_3$:CH_3OH (1:2) extracts were separated using one of the following systems: (a) $CHCl_3$:CH_3OH:NH_4OH:H_2O (65:25:0.5:3.6); (b) $CHCl_3$:CH_3OH:NH_4OH (65:25:5); (c) $CHCl_3$:acetone:CH_3OH: CH_3COOH:H_2O (3:4:1:1:0.5). $CHCl_3$:CH_3OH:H_2O (10:10:3) extracts were separated in $CHCl_3$:CH_3OH:1 M NH_4acetate:NH_4OH:H_2O (180: 140:9:9:23). For two-dimensional analysis, the TLC plate was developed in $CHCl_3$:CH_3OH:H_2O (60:30:6), then dried for at least 30 min and developed in the second dimension using solvent system $CHCl_3$:CH_3COOH:CH_3OH:H_2O (40:25:3:6). Autoradiograms were obtained by exposing TLC plates to Kodak X-omat AR films at −70°C. To locate ^{3}H-labeled products, EN^{3}HANCE (NEN, Boston, MA) was used according to the manufacturer's instructions. Alternatively, radioactive and fluorescent spots were scanned and quantified using the phosphoimager and ImageQuant image analysis software (Molecular Dynamics, Sunnyvale, CA).

2.2.9. Phospholipase treatment

PI-PLC and PLC (1 unit each) were added to the assay mixture for different periods of time (10–120

min) and lipids were extracted and separated as described above. Alternatively, suspected [^{3}H]PI bands from TLC separation of reaction products were eluted from the silica gel with $CHCl_3$:CH_3OH (1:2), dried under nitrogen, resuspended in buffer A, sonicated for 2 min and then PI-PLC (1 unit per reaction) and PLC (1 unit per reaction) were added. Reactions were run for 10–120 min, then stopped by 1 M HCl and lipids extracted and separated as described above.

2.2.10. Analysis of phospholipase C treated water-soluble products

Separation of water-soluble radioactive products of phospholipase C treatment was achieved on a Dowex anion exchange (AG 1-X8 resin, 100–200 mesh formate form; Bio-Rad Laboratories, Richmond, CA) column as described [23,24] with free [^{3}H]inositol as a standard.

2.2.11. Miscellaneous

Protein was determined by bicinchoninic acid (BCA) assay reagent (Pierce, Rockford, IL). Mycolic acid synthesis assay on subcellular fractions using [^{14}C]acetate as a substrate was performed as described earlier [16]. NAHD oxidase activity was determined by measuring the absorbance at 340 nm after addition to the cuvette of 0.1 mg protein from each fraction [25]. Basic BLAST search was performed on NCBI server. Sequences were aligned using CLUSTALW version 1.7 software package [26]. Phylogenetic trees were constructed using TreeView algorithm (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) with bootstrap analysis.

3. Results

3.1. Incorporation of [^{3}H]inositol into lipids and characterization of the products

Different subcellular fractions of *M. smegmatis* were examined for the capacity to synthesize PI, using [^{3}H]inositol as a substrate. Only the P60 cell wall fraction was observed to possess appreciable PI synthase activity (Fig. 1); activity in the membrane and cytosolic fractions was shown to be only 1.7 and 0.4%, respectively, of that in the cell wall fraction.

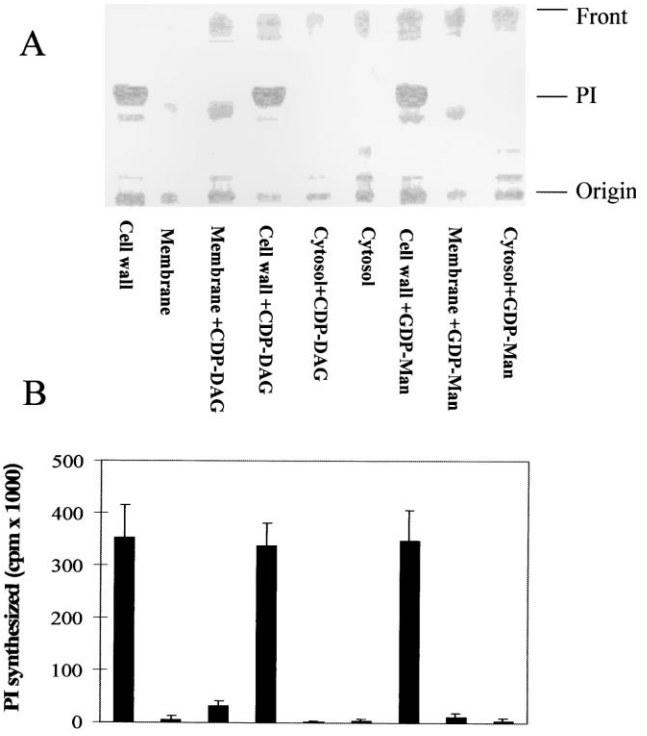

Fig. 1. Synthesis of PI by different subcellular fractions of *M. smegmatis*. (A) Cell wall, membrane and cytosol fractions (1 mg each) were incubated for 1 h at 37°C with 20 μCi of [^{3}H]inositol in a final volume of 200 μl. Some reactions contained CDP-dipalmitoyl-DAG (final concentration 150 μM) or GDP-Man (100 μM). Lipids were extracted, analyzed by TLC using $CHCl_3$:CH_3OH:NH_4OH:H_2O (65:25:0.5:3.6) as a solvent system, and plates exposed to Kodak X-omat AR film. (B) Radioactive bands were extracted from the gel and counted by liquid scintillation counting.

Neither exogenous CDP-DAG nor GDP-Man when added to the assay, stimulated the formation of radiolabeled product. Product was identified as PI based on comigration with commercial standards of PI in three of the TLC systems. When the major radioactive product was extracted from the silica gel, incorporated by sonication into liposomes and treated with PI-PLC and PLC, radioactivity disappeared, in a time-dependent manner, from the lipid fraction and steadily increased in the water-soluble fraction (data not shown). Direct addition of both phospholipases to the assay mixture also resulted, in a time-dependent manner, in the hydrolysis of the radioactive lipid formed; however, the extent of hydrolysis was less than that due to treatment of the purified product (data not shown). Radioactivity from the water-soluble fraction was identified to be

Table 1
Incorporation of [^{3}H]inositol into lipids using cell wall, membrane and cytosol fractions, obtained by an improved method of cell fractionation

Fraction	cpm	Specific activity (cpm/mg protein)	Percentage of total
Cell wall			
Subfraction 1	38	0	0
Subfraction 2	13 680	136 800	14.6
Subfraction 3	79 800	798 000	85.4
Membrane	79	520	0.07
Cytosol	26	0	0

Assay was performed as described in Section 2 with 20 μCi of [^{3}H]inositol (80 Ci/mmol), final concentration 1 μM, in a volume of 200 μl using subcellular fractions and cell wall subfractions obtained by sucrose gradient centrifugation (each assay contained 100 μg of protein). Incubation was for 60 min at 37°C. Lipids were extracted by the acid Bligh and Dyer procedure, analyzed by TLC using $CHCl_3$:CH_3OH:NH_4OH:H_2O (65:25:0.5:3.6) as a solvent system. Radioactive bands were extracted from the gel and counted by liquid scintillation counting.

non-modified *myo*-inositol, since it was not retained on the Dowex resin column.

The synthesis of PI observed in the membrane and cytosol fractions was probably due to cross-contamination with active cell wall/cell envelope materials, since in further experiments, using a similar separation procedure, no PI synthesis whatsoever was obtained in the cytosol fraction, and the yield of PI by the membrane fraction dropped to 0.15%. Cross-contamination of mycobacterial subcellular fractions is a common problem [17,18]. Possible cross-contamination between subcellular fractions of *M. smegmatis* was assayed by distribution of mycolic acids and NADH oxidase activity. Distribution of mycolic acids between fractions was calculated as a percentage of the total label in mycolic acids after labeling growing cells with [^{14}C]acetate for 24 h and subsequent extraction and TLC separation of lipids. The bulk of mycolic acids was found to be present in the cell wall preparation (86.0%); however, 13.8% found in membranes are known to closely correspond, for example, to the presence of diaminopimelic acid (DAP) in this fraction (12.6%), reported for *M. leprae* [18]. Thus, the detection of mycolic acids in the membrane is due to cross-contamination with the cell wall material. Also, the conclusion can be drawn that cross-contamination between cell wall and soluble fraction essentially does not exist, since very small amounts of mycolic acids (only up to 0.2%) were detected in cytosol. NADH oxidase activity was measured in the subcellular fractions in nmol/min/mg protein (cell wall, 29.4; membranes, 336.4; cyto-

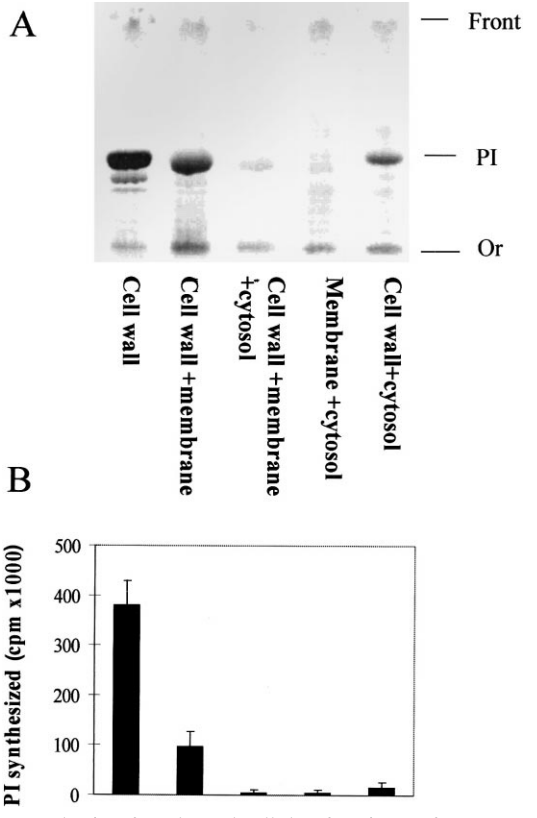

Fig. 2. Synthesis of PI by subcellular fractions of *M. smegmatis* added in different combinations. (A) Assay was performed with 20 μCi [^{3}H]inositol and 1 mg protein of each fraction (in different combinations) in a final volume of 400 μl as described in Section 2. All fractions were added to the assay prior to incubation for 1 h at 37°C. Lipids were extracted, analyzed by TLC using $CHCl_3$:CH_3OH:NH_4OH:H_2O (65:25:0.5:3.6) as a solvent system and plates were exposed to Kodak X-omat AR film. (B) Radioactive bands were extracted from the gel and counted by liquid scintillation counting.

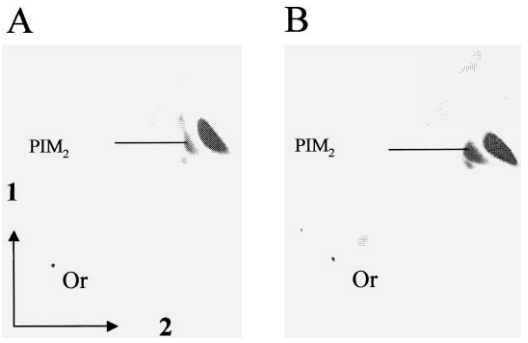

Fig. 3. Autoradiogram of two-dimensional TLC of [^{3}H]inositol labeled products. Assay was performed with 20 μCi [^{3}H]inositol in a final volume of 200 μl as described in Section 2. Lipids were extracted and run in $CHCl_3$:CH_3OH:H_2O (60:30:6) in the first and $CHCl_3$:CH_3COOH:CH_3OH:H_2O (40:25:3:6) in the second dimension. (A) Cell wall fraction added to the assay mixture for 60 min. (B) Cell wall fraction added for 30 min, then membrane fraction added for another 30 min. Total amount of radioactivity in A, 372 000 cpm; in B, 365 000 cpm. Or, origin.

sol, 34.7). Results of HADH oxidase assay revealed that cell wall fraction was contaminated by membrane fragments (8.7%).

We also resorted to another method of cell fractionation, which involved the purification of fractions by sucrose gradient centrifugation. Out of these efforts cell wall subfractions 1, 2 and 3, cytosol and membranes were obtained (Table 1). Membrane and cytosol were devoid of significant PI synthase activity. Again the majority of radioactivity was located in cell walls and specifically in the minor fraction lying between the 50% and 70% sucrose interface (Table 1). Thus we were able to achieve partial purification of the enzyme.

When subcellular fractions of *M. smegmatis* were added to the PI synthase assay in different combinations, activity was inhibited by different degrees (Fig. 2). Simultaneous addition of cytosol and cell wall fractions completely abrogated PI synthesis. Addition of membrane fraction considerably inhibited the PI synthase activity (by 74.7%). Membrane, cytosol and cell wall fractions added together had the same effect as cytosol alone. Thus, both cytosol and membranes are potent inhibitors of PI synthesis.

Two-dimensional TLC separation confirmed that PI was essentially the only major newly synthesized product in the cell wall preparation (Fig. 3A). The distribution of the incorporated label in the different products was as follows: PI, 92.3%; PIM_2, 3.6%; others, 4.1%. One of the [^{3}H]inositol labeled products synthesized exclusively in the cell wall fraction migrated with the solvent front in the polar TLC system used for separation of PI and PIMs. It was surprising, since no neutral inositol-containing lipids are known. Our attempts to purify and characterize

Table 2
Comparison of mycobacterial, rat liver and *S. cerevisiae* PI synthase properties with respect to activation by divalent cations, substrate specificity and inhibition pattern

	Mycobacterial PI synthase	Rat liver PI synthase	Yeast PI synthase
$ZnCl_2$ IC_{50} (μM)	0.4	0.38	1.5
scyllo-Inositol IC_{50} (μM)	0.04	800	850
epi-Inositol IC_{50} (μM)	8.0	200	370
Mn^{2+} and Mg^{2+} (fold stimulation)	Mg > Mn, ~2.32	Mn > Mg, ~5.78[a]	Mn > Mg, ~3.20[b]
Stimulation by exogenous CDP-DAG (dipalmitoyl)	none	×4.01	×1.52

Assay on the smaller scale was performed as described in Section 2 with 5 μCi of [^{3}H]inositol in a final volume of 50 μl using P60 fraction from *M. smegmatis* (50 μM inositol final concentration, 250 μg protein), rat liver microsomes (1 mM inositol final concentration, 50 μg protein) and yeast microsomes (100 μM inositol final concentration, 50 μg protein). In case of mycobacterial PI synthase assay, endogenous CDP-DAG was utilized as a substrate in the inhibition (Zn^{2+}, inositol analogs) and cation (Mg^{2+}, Mn^{2+}) stimulation experiments. Range of concentrations (0–800 μM) of commercial CDP-dipalmitoyl-DAG was tested against mycobacterial PI synthase and 100 μM of CDP-dipalmitoyl-DAG was used to assess the stimulation of yeast and rat enzymes in the last experiment (stimulation by exogenous substrate). Final concentrations of the inositol were adjusted with 'cold' inositol to be close to reported K_m for *S. cerevisiae* (90 μM [15]) and rat liver PI synthases (1.3 mM [19]). Incubation was for 60 min at 37°C. Lipids were extracted by acid Bligh and Dyer procedure and counted by liquid scintillation counting. IC_{50}, inhibitor concentration which inhibited enzymatic activity by 50%.

[a]Also see Fischl and Carman [15].

[b]See Monaco et al. [19].

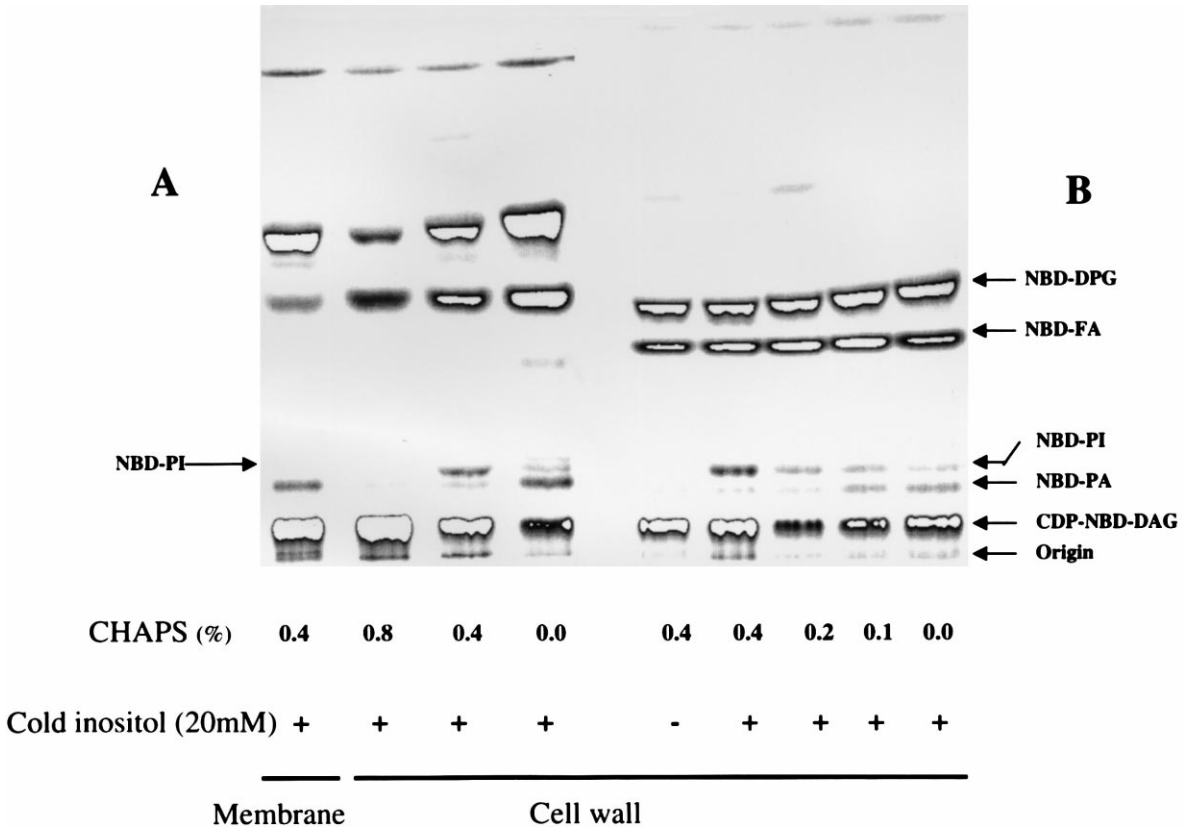

Fig. 4. Metabolism of CDP-NBD-DAG by different subcellular fractions of *M. smegmatis*. *M. smegmatis* P60, membranes or cytosol (1 mg protein) were incubated with 25 μM CDP-NBD-DAG in the presence of different concentrations of CHAPS (0–0.8%) in a final volume of 300 μl for 1 h at 37°C. Lipids were extracted and analyzed by TLC using $CHCl_3$:CH_3OH:NH_4OH (65:25:5) as a solvent system. The positions of authentic NBD-lipid standards are indicated by the arrows.

this unknown compound were unsuccessful, due to the small amounts synthesized.

3.2. Substrate requirements for mycobacterial PI synthase

CDP-DAG is clearly a required substrate for the synthesis of PI in eukaryotic cells [6]. This reaction involves the simple exchange of the CMP moiety of CDP-DAG for inositol. In some experiments, sonicated CDP-dipalmitoyl-DAG (150 μM) was added to the assay mixture and no stimulation of product formation was observed (Fig. 1). Addition of increasing concentrations of the theoretical substrate (30–800 μM) did not result in stimulation of incorporation, though addition of dipalmitoyl-CDP-DAG to the rat liver and *S. cerevisiae* PI synthase assays resulted in dose-dependent increases in yield (Table 2). However, even successful stimulation of [^{3}H]inositol incorporation by 'cold' CDP-DAG or other compound does not provide absolute proof that this compound is the substrate. We devised other means to address the question. Since radioactive CDP-DAG is unavailable commercially, we synthesized the fluorescent analog of CDP-DAG, CDP-C_6-NBD-DAG, with a short chain C_6-NBD fluorescent fatty acid attached to the *sn*-2 position of diacylglycerol. Fluorescent C_6-NBD-PI was also synthesized from CDP-NBD-DAG to be used as a standard, since fluorescent and endogenous lipids have different R_f values [12]. When CDP-NBD-DAG was added to the reaction mixture, trace amounts of NBD-PI were produced with mycobacterial P60 as an enzyme source and a very high yield of fluorescent PI (20–30% from the added substrate) was obtained with rat liver microsomes and yeast microsomes. The result means that fluorescent CDP-DAG is a good substrate for both rat liver and *S. cerevisiae* PI synthases. Upon

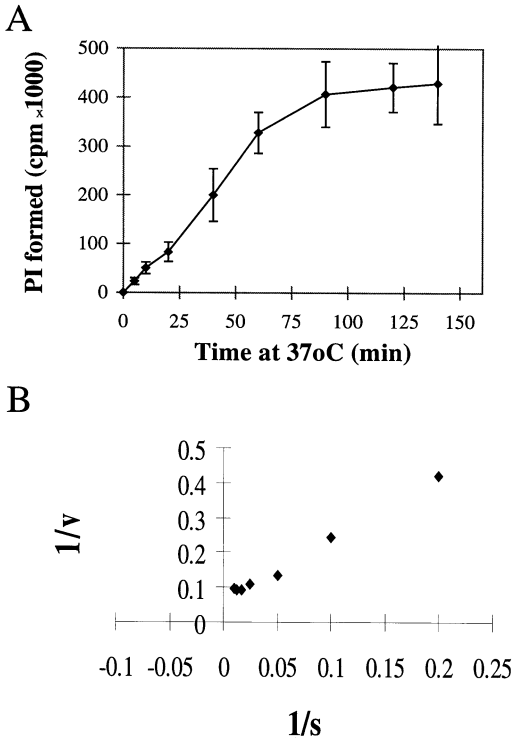

Fig. 5. Kinetics of PI synthesis by *M. smegmatis* cell wall preparation. (A) Time dependence of PI synthesis. Enzymatic assay was performed as described in Section 2 (5 μCi [^{3}H]inositol per assay, final concentration 50 μM, 250 μg of P60 fraction). After the indicated time of incubation at 37°C, lipids were extracted, and the amount of radioactivity was counted by liquid scintillation counting. (B) Lineweaver-Burk plot of PI synthase activity. Enzymatic assay was performed as described in Section 2. Amounts of the substrate consumed did not exceed 10% in each case. The data are from a single typical experiment; each point represents the mean of duplicate determinations.

addition of CHAPS to the reaction mixture containing mycobacterial P60, synthesis of NBD-PI increased manyfold. Fluorescent PI was formed in a CHAPS concentration-dependent manner (Fig. 4B). Maximum NBD-PI yield was obtained at a CHAPS concentration of approx. 0.4%; increase above 0.4% resulted in attenuation of the synthesis with complete deactivation of the enzyme at 0.8% CHAPS (Fig. 4A). NBD-PI, like radioactive PI (when [^{3}H]inositol was used as a substrate) was produced exclusively by the cell wall (P60) fraction. As expected, when excess cold inositol was not added to the reaction, no NBD-PI was formed (Fig. 4B). At CHAPS concentration of 0.4% both CDP-dipalmitoyl-DAG and CDP-NBD-DAG stimulated the formation of [^{3}H]PI and NBD-PI, respectively, in a concentration-dependent manner (data not shown). Other NBD fluorescent lipids (NBD-PA, NBD-PG, NB-PS, NBD-PE) when added to the reaction instead of CDP-NBD-DAG did not cause the formation of NBD-PI. Hence we proved for the first time that CDP-DAG is the general precursor of PI not only in eukaryotic cells, but also in bacterial cells. The results also demonstrate the strict selectivity of the enzyme toward endogenous species of CDP-DAG. Solubilization of the enzyme by CHAPS resulted in the apparent loss of the selectivity toward CDP-DAG. In addition to NBD-PI, other fluorescent lipids, such as NBD-cardiolipin and a couple of unidentified lipids were synthesized (Fig. 4A,B). Cardiolipin constitutes about 50% of all phospholipids in mycobacteria [4]. NBD-free fatty acid was also one of the major products in all fractions (cell wall, membrane, cytosol), thus demonstrating that phospholipase A_2 activity is prominent in mycobacteria. CDP-NBD-DAG appears to be a good substrate for general lipid synthesis in mycobacteria.

3.3. Kinetic data, divalent cation activation and inhibitors

Fig. 5A shows the kinetics of PI synthesis by the

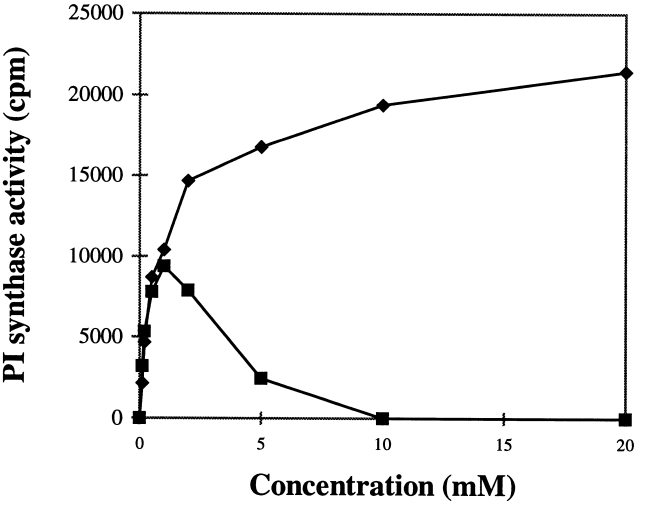

Fig. 6. Effect of Mg^{2+} and Mn^{2+} on PI synthase activity. Enzymatic assays were performed with 5 μCi of [^{3}H]inositol (final concentration 50 μM) in a final volume of 50 μl in the presence of increasing concentrations of Mg^{2+} (♦) and Mn^{2+} (■). Lipids were extracted and incorporated radioactivity counted in a liquid scintillation counter. Each point is the mean of duplicate determinations.

cell wall fraction of *M. smegmatis*. At 37°C, the incorporation of inositol was essentially linear for about 80 min, after which time the reaction reached a plateau. To calculate the apparent K_m of the process, a time span of 40 min was used. Kinetic studies yielded an apparent K_m for [^{3}H]inositol of 25 μM (Fig. 5B).

As for cation requirements, both Mg^{2+} and Mn^{2+} stimulated the conversion, as was expected, but in a different manner (Fig. 6). Maximal activity was obtained at high concentrations of Mg^{2+} (>20 mM) and medium concentrations of Mn^{2+} (approx. 1 mM). Higher concentrations of the latter inhibited the reaction completely. Maximal activity obtained with Mg^{2+} was approx. 2.3-fold higher than the activity obtained with Mn^{2+}. Zn^{2+} was extremely effective in inhibiting the enzyme, with an IC_{50} of 0.4 μM (Table 2). When rat liver and *S. cerevisiae* PI synthases were assayed for activation by divalent cations, results in good agreement with those in the literature were obtained (Table 2). In both rat liver and *S. cerevisiae*, Mn^{2+} was more potent than Mg^{2+} in activating the enzyme [19,15]. However, in some cases in mammalian cells and yeast, Mg^{2+} is a better activator. For example, human placental [27] and *Candida albicans* PI synthases [28] demonstrated patterns of activation by divalent cations closer to mycobacterial than to yeast enzymes, i.e. Mg^{2+} was better at activating the enzyme.

Different *myo*-inositol structural analogs were checked for their ability to inhibit PI synthase in mycobacteria, yeast and rat liver. *scyllo*-Inositol and *epi*-inositol were found to be extremely potent competitive inhibitors in *M. smegmatis*, and much less effective in yeast and mammalian cells (Table 2). The inhibitory effects of *scyllo*- and *epi*-inositol in the presence of increasing concentrations of *myo*-inositol were analyzed using a reciprocal plot ($1/v$ versus S); the lines intercepted in a manner resembling of a competitive type inhibition. For *scyllo*-inositol, the difference in IC_{50} values for mycobacterial PI synthase and rat liver and yeast enzyme was approx. 1:20 000. In the case of *epi*-inositol, this ratio dropped to 1:25, but the compound still was very potent toward the mycobacterial enzyme and yielded 50% inhibition at 8.0 μM. Zn^{2+} was nearly equally active against all three enzymes with very low IC_{50} values. It was previously reported to be an inhibitor of PI synthase in *S. cerevisiae* (1 mM gave 95–97% inhibition [15]) and less potent against human PI synthase ($K_i = 2$ mM [27]). It should be remembered that in all experiments with mycobacterial PI synthase, unless otherwise indicated, endogenous CDP-DAG was utilized, ensuring the supply of 'natural' substrate.

3.4. Further steps in PI metabolism

LM and LAM contain the mannan core linked to PI, with the structure D-*myo*-inositol 2,6-bis-α-mannose [29] and are thus derived from PIM_2 [30]. In order to follow the synthesis of PIMs and LM/LAM, the following system was designed [5]: first the membrane fraction was utilized for the synthesis of [^{14}C]Man-labeled lipids with GDP-[U-^{14}C]Man as a mannose donor and endogenous membrane PI as a metabolic precursor. The major products synthesized were PIM_2s, C_{35}-P-Man and C_{50}-P-Man. No incorporation of radioactivity into higher PIMs or LM/LAM occurred. On the second stage of the experiment, membranes were washed to remove residual GDP-Man and cell wall fraction (P60) was added. Immediate incorporation of radioactivity into the products with longer sugar backbones like PIM_{3-5} and LM was observed, at the expense of C_{35}/C_{50}-P-[^{14}C]Man. Since this paper demonstrates that PI was synthesized in the cell wall fraction exclusively, it must undergo transfer to the cytoplasmic membrane prior to the first and second mannosylation to yield PIM_2. To corroborate this idea, we designed an experiment involving in the first stage the synthesis of [^{3}H]inositol-labeled PI by the cell wall fraction for 30 min, and then membrane fraction was added for an additional 30 min. The results are shown in Fig. 3A,B. The amount of PIM_2 yielded as a product by the cell wall fraction was insignificant compared to that synthesized after the addition of membranes to the reaction (3.6% vs. 26.2% of the total lipid-incorporated label). In both cases the total incorporation of [^{3}H]inositol into lipids was approximately equal (A, 372 000 cpm; B, 365 000 cpm). It should be emphasized that in no case was there incorporation of the label into more polar products, not extractable with $CHCl_3$:CH_3OH (2:1) and extractable with $CHCl_3$:CH_3OH:H_2O (10:10:3), such as higher PIMs and LM. Thus, the small amount of PIM_2 in

Fig. 3A cannot be explained by its utilization for the synthesis of more complex compounds.

4. Discussion

For the first time we have characterized PI synthase in prokaryotic cells and early steps in inositol metabolism with regard to subcellular localization, substrate specificity, kinetic data, and cation requirements. We are confident that PI synthesis in the cell wall fraction is not a result of contamination by membrane fragments and that PI synthase may be localized in the cell wall, unlike other enzymes of phospholipid biosynthesis.

In a recent paper on *M. leprae* fractionation [18], experiments were performed to assess the distribution of different markers and enzymes in subcellular fractions of *M. leprae*. The amount of peptidoglycan, a constitutive marker of the cell wall, was assessed by assaying DAP. Purified membrane fractions contained 12.6% of total DAP, probably due to the presence of appreciable amounts of lipid-linked peptidoglycan precursors and contamination of membrane fraction by cell wall fragments. The soluble fraction was devoid of detectable DAP. Similar data were obtained after measuring the distribution of arabinogalactan (marker of the cell wall) and mannose (which is found primarily in membranes and cytosol). Galactose was also detected in the membrane, which can be attributed to the sizable quantities of lipid-linked arabinogalactan, a precursor of the insoluble form [31]. Altogether, the results for *M. smegmatis* fractionation were found to be in accordance with previous results obtained on *M. leprae* [18] and residual PI synthase activity in membrane fraction can be satisfactorily explained by the presence of the enzyme within cell wall fragments co-purified with cell membranes.

We should emphasize that our knowledge of mycobacterial lipid and lipoglycan metabolism is quite limited. PI and PIM_2 can be utilized for the synthesis of other inositol-containing compounds and polymers, or free inositol can be incorporated independently into unidentified neutral lipids. Kozloff et al. [9] mentioned that while using [^{3}H]inositol as a substrate for PI synthesis in *E. coli*, they discovered 30% of the radioactivity in PI and a lesser amount of ^{3}H label in every phospholipid component. This phenomenon was explained by exchange and release of ^{3}H from the labeled inositol ring and subsequent incorporation into other compounds. We found no non-specific exchange of ^{3}H, only metabolic incorporation. When [^{3}H]inositol was used as a substrate for PI synthase in rat liver microsomes and yeast microsomes, absolutely no labeling of other phospholipids in the lipid extract was detected, and PI was the only labeled product. The conclusion can be drawn that labeling of the neutral compound was not an artifact, or due to non-specific atomic exchange, but the result of metabolic labeling. We also showed for the first time the existence of extremely neutral inositol-containing lipid compounds in mycobacteria. The metabolic role and physiological importance of this compound(s) remains to be elucidated.

Reviews suggest the presence in prokaryotic cells of the same metabolic pathway for the synthesis of PI as described for mammalian cells. For instance, there is the statement that PI is formed from inositol and CDP-DAG under strict stereospecific conditions [4,32], but no definite proof was supplied. Mycobacterial PI has a unique fatty acid composition; tuberculostearic acid (D-10-methylstearic acid) is in the *sn*-1 position and palmitic acid is in the *sn*-2 position of the glycerol backbone [2,5]. If CDP-DAG is the metabolic precursor of PI in mycobacteria, some or all of the CDP-DAG pool must have a similar fatty acid composition. Walsh et al. [33,34] reported the purification of a diacylglycerol kinase strictly selective for *sn*-1-acyl-2-arachidonoyl diacylglycerols. Keeping in mind the marked enrichment of animal cells phosphatidylinositols in arachidonate at the glycerol *sn*-2 position, substrate selectivity of arachidonoyl-DAG kinase suggests a special role for this isoform in PI synthesis in mammalian cells. The same may be true in the case of mycobacteria. Selective (fatty acid-dependent) substrate specificity can explain the prevalence of tuberculostearic acid-rich isoforms of PI in all mycobacterial species. Only certain species of CDP-DAG (with tuberculostearic acid in the *sn*-1 position) should be utilized for PI biosynthesis. As a matter of fact, addition of both exogenous substrates (CDP-DAG and fluorescent CDP-NBD-DAG) did not stimulate the [^{3}H]inositol incorporation into PI; only enzyme solubilization with CHAPS altered the PI synthase substrate specificity.

Fluorescent lipid analogs have been very useful for studying lipid metabolism and trafficking in animal [35,36] and bacterial cells [37] and were shown to be suitable substrates. Also selective activity of PI synthase from maize coleoptiles before and after solubilization toward different CDP-DAG substrates has been reported [38]. Before solubilization only endogenous CDP-DAG species with a polyunsaturated fatty acid in the second position were utilized in this study even under conditions of excess exogenous substrate in the system [38]. After solubilization, with increasing concentrations of exogenous CDP-dipalmitoyl-DAG, increasing amounts of labeled dipalmitoyl-PI appeared. It seems that in case of enzymes involved in lipid metabolism, fatty acid specificity is a common phenomenon and this specificity plays an important regulatory role. These findings emphasize the importance of a critical approach to the use of exogenous substrates to measure the activity of enzymes of lipid metabolism. All data generated using CDP-dipalmitoyl-DAG as a PI synthase substrate should be treated very cautiously.

BLAST search [39] revealed 98% sequence similarity between human and rat PI synthases (95% of amino acids are identical) and 61% similarity between rat and *S. cerevisiae* PI synthases (39% identical). In contrast, only weak homology was found between yeast or mammalian phosphatidylinositol-synthesizing enzymes and any mycobacterial sequences in the *M. tuberculosis* and *M. leprae* genomic databases. Recent completion of *M. tuberculosis* genome yielded a great deal of information on mycobacterial proteins and genome organization [1]. The authors tentatively identified three open reading frames (ORFs) as mycobacterial phosphatidylglycerophosphate (PGP) synthase genes (Rv2612c, pgsA; Rv1822, pgsA2; Rv2746c, pgsA3). The reason for this redundancy is unknown. PGP synthases are the closest to PI synthase by homology. Two proteins from *M. tuberculosis* (pgsA2 and pgsA) were tentatively identified among all *M. tuberculosis* proteins as potential candidates for the detected phosphatidylinositol-synthesizing activity based on the results of homology searches, sequence alignment with known eukaryotic PI synthases and phylogenetic analysis. However, PI synthase activity may also be attributed to other uncharacterized ORFs. Analysis of the biochemical data from Table 2 and the homology data allows one to conclude that PI synthases form two distinct groups with respect to inhibition pattern, activation by divalent cations, substrate specificity and homology.

4.1. Summary

Lack of sequence homology, different enzyme characteristics and the existence of highly selective inhibitors (*scyllo*-inositol) suggest that mycobacterial PI synthase, probably being an essential enzyme, is an excellent potential target for anti-tuberculosis (TB) therapy. Its inhibition should abrogate the synthesis of PIMs, LM, LAM – important components of the cell wall. Assays and protocols described in this paper pave the way for the development of high throughput screens for prospective anti-TB drugs able to fight multidrug resistant tuberculosis. The value of this approach is supported by the important structural, physiological and immunological role PI and PI-based compounds play in mycobacterial physiology and mycobacteria-host interaction.

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