PHOSPHATIDYLMYOINOSITOL MONOMANNOSIDE IN *PROPIONIBACTERIUM shermanii*

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Certain morphological (1) and physiological (2) similarities between the genera *Mycobacterium* and *Propionibacterium* indicate a phylogenetic and taxonomic relationship. This is supported by chemical evidence, in particular similarities in cell wall constituents (3), in DNA structure (2) and in the presence of large amounts of free trehalose in both organisms (4, 5). We have reported on the characterization (6) and biosynthesis (7, 8) of the mannophosphoinositides of *Mycobacterium* species, lipids which have been detected mainly in the *Actinomycetales*. In this note, evidence is presented for the presence in and the biosynthesis by *Propionibacterium shermanii* of phosphatidylmyoinositol monomannoside (monomannophosphoinositide). This is further evidence for a close relationship between these organisms.

EXPERIMENTAL PROCEDURE

Propionibacterium shermanii (ATCC 9614) cells were obtained from Dr. H.A. Barker and their growth has been described (9). Phospholipid was extracted, washed with acetone, the sodium salts were formed and the material was lyophilized as for *Mycobacterium* (10). Of the dry cells, 2.5% of the weight was recovered as phospholipid and 1% as acetone-soluble neutral lipid. After hydrolysis of the phospholipid fraction with 1 N HCl at 100° for 8 hours, paper chromatography in two solvent systems (Whatman No. 1 paper, *n*-butanol-pyridine-water, 3:1:1; and ethylacetate-acetic acid-formic acid-water, 18:3:1:4, by volume) revealed the presence of about equal amounts of myoinositol, mannose and glycerol, and a trace of glucose. No ethanolamine, serine or any nitrogen-

containing product was detected in these hydrolysates, and thin-layer chromatography (Silica gel H, chloroform-methanol-acetic acid-water, 60:30:8:4, by volume) of the phospholipid fraction confirmed the absence of any ninhydrin-positive phospholipid.

Preparative thin-layer chromatography of the crude phospholipid yielded a major phospholipid with a phosphorus to mannose ratio near unity. Its rate of migration relative to phosphatidylmyoinositol was 1.3. Gas chromatography of the methyl esters of the fatty acids from the phospholipid showed two major peaks. One (64%) had a retention time relative to methyl palmitate of 0.77, consistent with 13-methyltetradecanoic acid which has been reported in *Propionibacterium freudenreichii* (11), or an unsaturated myristic acid. The second peak (31%), with a relative retention time of 1.2, was inhomogeneous and corresponded to unsaturated or methylated C_{16} acids.

To determine if *P. shermanii* could synthesize mannophosphoinositides, which the evidence above suggested were present in these cells, cell-free extracts were incubated with GDP-mannose-^{14}C in the presence and absence of yeast phosphatidylmyoinositol (Table I). This lipid stimulated incorporation of the radioactivity 5-fold into phospholipid, suggesting that it acted as an acceptor of mannose for the synthesis of phosphatidylmyoinositol mannoside.

The products of the reaction with endogenous acceptor and with added phosphatidylmyoinositol were analyzed by thin-layer chromatography (Fig. 1). The endogenous acceptor did not yield any product identifiable as a mannophosphoinositide. Yeast phosphatidylmyoinositol yielded a product which was shown to be identical with phosphatidylmyoinositol monomannoside by the experiments outlined below.

The radioactive lipid from the incubation containing phosphatidylmyoinositol was isolated from the chromatogram by extraction with a mixture of chloroform, methanol and water, and the extract was passed through Sephadex G25 (10). Paper chromatography of the deacylated product (Fig. 2) shows that it has the expected R_f of glycerylphosphorylmyoinositol monomannoside, since it ran be-

tween glyceryl phosphorylmyoinositol and the dimannoside (6). This product was

TABLE I

Incubation of P. shermanii extracts with GDP-mannose-^{14}C

Assay system	Incorporation of radioactivity	
Experiment I	dpm	µµmoles
Complete system	1450	249
minus phosphatidylmyoinositol	561	94
with boiled enzyme	106	18
Experiment II		
Complete system	8260	1390
minus phosphatidylmyoinositol	1640	276

In Experiment I, the complete assay system contained: Tris-HCl (pH 7.35), 80 µmoles; Cutscum, 1.5 mg; $MgCl_2$, 5 µmoles; GDP-mannose-^{14}C, 5.7 mµmoles; yeast phosphatidylmyoinositol, 1.8 gm; enzyme protein, 3.84 mg in a final volume of 1.0 ml. The mixture was incubated at 37° for 1 hour. In Experiment II, everything was increased by a factor of twenty, except GDP-mannose-^{14}C which was increased four-fold. Incubation was at 37° for 2 hours.

Reactions were stopped by the addition of enough chloroform-methanol (2:1) to form a single phase. This was washed once with 0.2 volumes of 0.29% NaCl and three times with 0.1 volumes of chloroform-methanol-water (3:48:47) (13) containing 0.29% NaCl. The chloroform layer was evaporated to dryness, made up to volume and the radioactivity of an aliquot was counted.

Enzyme was obtained from a sonic extract of *P. shermanii* which were disrupted as described for *M. phlei* (7), then centrifuged at 3000 x g for 20 minutes and the clear supernatant was used. GDP-mannose-^{14}C (2.68 µc per µmole) was prepared by the method of Preiss and Greenberg (14). The preparation and characterization of yeast phosphatidylmyoinositol have been described (7).

eluted, dephosphorylated and analyzed in three chromatographic systems (Fig. 3). The radioactive material was identical with myoinositol monomannoside. Complete dephosphorylation took place under conditions which result in only 60-70% hydrolysis for dimannoside and higher homologs (12). This result is consistent with a glycerylphosphorylmyoinositol monomannoside, in which less steric hindrance of the phosphodiester bridge would be expected. Acid hydrolysis of the product of dephosphorylation and chromatography established that mannose was the only radioactive material present. Hydrolysis of the total crude phospholipid from Experiment I in Table I again showed that mannose was the major

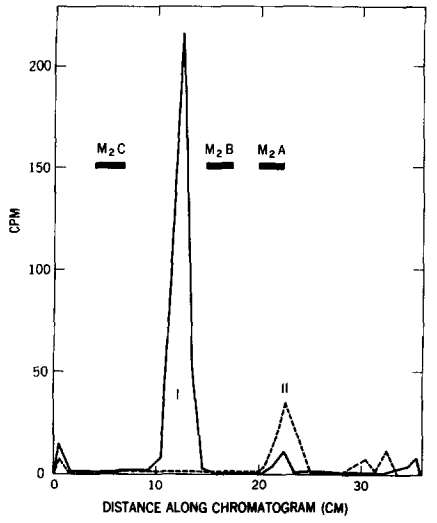

Fig. 1. Thin-layer chromatogram in chloroform-methanol-acetic acid-water (30:15:4:2, by vol.) of intact phospholipids synthesized by extracts of *P. shermanii* and GDP-mannose-^{14}C in the presence (solid line) and absence (dashed line) of yeast phosphatidylmyoinositol. Dimannophosphoinositide A (M_2A), B (M_2B) and C (M_2C) are the references (10) and they were detected with the phosphorus reagent (15).

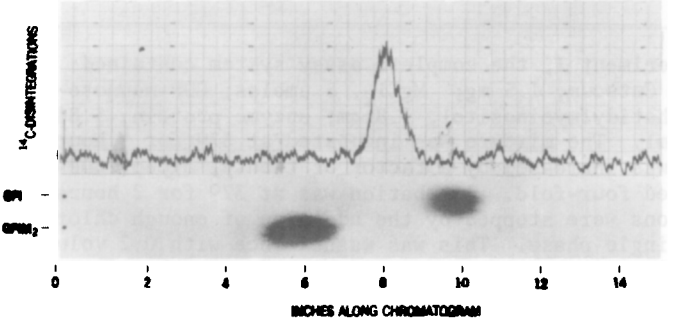

Fig. 2. Paper chromatogram of the deacylated phospholipid formed by extracts of *P. shermanii* from GDP-mannose-^{14}C and yeast phosphatidylmyoinositol. Radioactive lipid Peak I from the thin-layer chromatogram in Fig. 1 was deacylated with aqueous NaOH (16) and the product was chromatographed on Whatman 3MM paper with isopropanol-conc. NH_4OH (2:1, by vol.) descending for 4 days. A strip was examined for radioactivity on a Nuclear Chicago 2π radioscanner. Glycerylphosphorylmyoinositol (GPI) and glycerylphosphorylmyoinositol dimannoside ($GPIM_2$) from *M phlei* (10) were the reference standards and were located with the $AgNO_3$-NaOH reagent (17).

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radioactive product (Fig. 4). Some glucose may be present.

DISCUSSION

These experiments show that *P. shermanii* extracts synthesize a monomannophosphoinositide from GDP-mannose-^{14}C with yeast phosphatidylmyoinositol as a

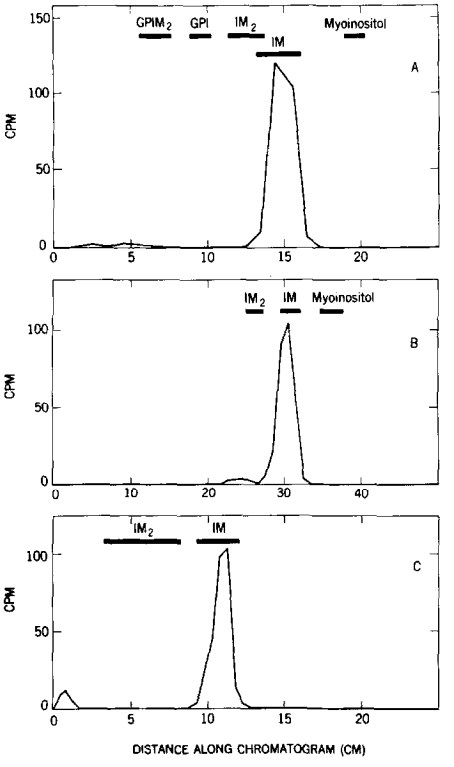

Fig. 3. Chromatograms of the dephosphorylated phospholipid synthesized by *P. shermanii* extracts, GDP-mannose-^{14}C and yeast phosphatidylmyoinositol. (A) Paper chromatography in ethyl acetate-pyridine-water (5:3:2, by vol.) descending for 2 days. (B) Paper chromatography in isopropanol-conc. NH_4OH (2:1, by vol.) descending for 4 days. (C) Thin-layer chromatography in n-butanol-acetic acid-diethylether-water (9:6:3:1, by vol.) on Silica gel H (0.25 mm thick) developed 6 times. Authentic myoinositol 2-mannoside was obtained by partial acid hydrolysis of myoinositol pentamannoside from *M. phlei* (6). Paper chromatograms were cut into 1 cm bands and analyzed for radioactivity by scintillation counting. The thin-layer chromatogram was divided into 0.5 cm bands and markers were located by charring after spraying the plate with 50% H_2SO_4. IM and IM_2 are myoinositol mono- and dimannoside.

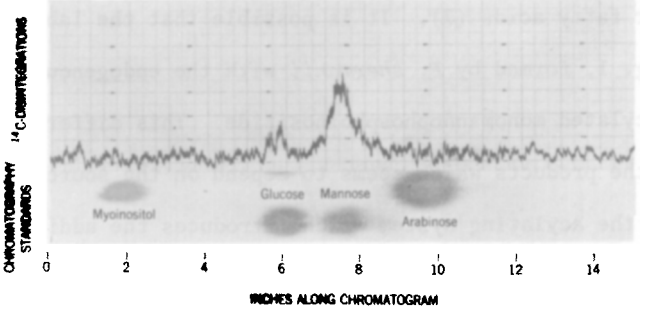

Fig. 4. Paper chromatography of acid hydrolyzed radioactive phospholipid synthesized by *P. shermanii*, GDP-mannose-^{14}C and yeast phosphatidylmyoinositol. The solvent was ethylacetate-acetic acid-formic acid-water (18:3:1:4, by vol.).

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mannose acceptor. In contrast to our studies with *M. phlei*, in the present system it is possible to demonstrate clearly the synthesis of a monomannophosphoinositide without the complication of the formation of higher mannosides. Our previous work on the biosynthesis of a monomannophosphoinositide by *M. phlei* (8) has been difficult to reproduce (7), and in view of the properties of the mono-

mannophosphoinositide reported here it now seems likely that the radioactive component obtained previously (8) was a more highly acylated mannophosphoinositide, perhaps containing more than one mannose unit. We find that the dimannophosphoinositides are the major phospholipids synthesized by extracts of *Mycobacterium*, and this is consistent with the high level of these compounds in the cell (10). While monomannophosphoinositide is certainly a biosynthetic intermediate in *Mycobacterium*, only a very small amount must accumulate in the cell. Apparently little or no dimannophosphoinositide occurs in *P. shermanii*, since deacylation of the extractable phospholipid did not yield a product corresponding to glycerylphosphorylmyoinositol dimannoside. The major product was glycerylphosphorylmyoinositol monomannoside. Further work is required to establish completely the nature of the mannophosphoinositides in *Propionibacterium*, particularly the degree of acylation.

When extracts of *M. phlei* are incubated with GDP-mannose-^{14}C and yeast phosphatidylmyoinositol, the major product is a dimannophosphoinositide with two fatty acids (7). The products from the endogenous lipid acceptor have three and four fatty acids (7). It is possible that the labeled product in Peak II of Fig. 1, formed by *P. shermanii* with the endogenous acceptor, is a more highly acylated monomannophosphoinositide. This difference in degree of acylation of the products which seems to depend on the source of the acceptor suggests that the acylating system which introduces the additional acyl groups may be sensitive to the nature of the fatty acids that are already present on the glycerol moiety of the phospholipid.

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