In Vivo Interaction between the Polyprenol Phosphate Mannose Synthase Ppm1 and the Integral Membrane Protein Ppm2 from Mycobacterium smegmatis Revealed by a Bacterial Two-hybrid System*§

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Dolichol phosphate-mannose (Dol-P-Man) is a mannosyl donor in various eukaryotic glycosylation processes. So far, two groups of Dol-P-Man synthases have been characterized based on the way they are stabilized in the endoplasmic reticulum membrane. Enzymes belonging to the first group, such as the yeast Dpm1, are typical integral membrane proteins harboring a transmembrane segment (TMS) at their C terminus. In contrast, mammalian Dpm1, enzymes of the second group, lack the typical TMS and require the association with the small hydrophobic proteins Dpm3 to be properly stabilized in the endoplasmic reticulum membrane. In Mycobacterium tuberculosis, the Polyprenol-phosphate-synthase MtpPpm1 is involved in the biosynthesis of the cell wall-associated glycolipid lipoparabinomannan. MtpPpm1 is composed of two domains. The C-terminal catalytic domain is homologous to eukaryotic Dol-P-Man synthases. The N-terminal domain of MtpPpm1 contains six TMS that anchor the enzyme in the cytoplasmic membrane. In contrast, in Mycobacterium smegmatis, orthologs of the MtpPpm1 from M. tuberculosis are encoded by two distinct open reading frames, Msppm1 and Msppm2, organized as an operon. No TMS are predicted in Msppm1, and subcellular fractionation experiments indicate that this enzyme is cytosolic when produced in E. coli. Computer-assisted topology predictions and alkaline phosphatase insertions showed that Msppm2 is an integral membrane protein. Using a recently developed bacterial two-hybrid system, it was found that Msppm2 interacts with Msppm1 to stabilize the synthase Msppm1 in the bacterial membrane. This interaction is reminiscent of that of mammalian Dpm1 with Dpm3 and mimics the structure of MtpPpm1 as demonstrated by the capacity of the two domains of MtpPpm1 to spontaneously interact when co-expressed in E. coli.

Dolichol phosphate mannose (Dol-P-Man) synthesizes mannose (Man) from GDP-Man to polyprenoid dolichol phosphate (Dol-P). Dol-P-Man is a mannol-sulfur donor in pathways leading to N-glycosylation (reviewed in Refs. 1 and 2), the synthesis of glycosylphosphatidylinositol anchors (3, 4), O-mannosylation of fungal proteins (4), and the construction of bacterial cell walls and proteozoa glycoalyx (5, 6).

The well characterized Dol-P-Man synthases fall into two distinct groups. One group contains the Schizosaccharomyces pombe, Caenorhabditis briggsiae, and mammalian Dol-P-Man synthases. The other group includes the Saccharomyces cerevisiae, Ustilago maydis, and Trypanosoma brucei Dol-P-Man synthases. Enzymes of the mammalian class lack the C-terminal hydrophobic domain that is characteristic of the S. cerevisiae class of Dol-P-Man synthases (7). The C-terminal hydrophobic domain has been proposed to stabilize the yeast enzyme by anchoring it in the cytoplasmic membrane. More recently, the mammalian Dol-P-Man synthase Dpm1 has been shown to interact with the transmembrane protein Dpm3 that retains and stabilizes the synthase in the endoplasmic reticulum membrane (8, 9), thereby functionally replacing the missing C-terminal hydrophobic domain.

In mycobacteria, polyprenyl phosphate mannose (Polyprenol-P-Man), closely related to Dol-P-Man, is involved in lipoparabinomannan biosynthesis (10, 11), a key immunomodulator implicated in tuberculous pathogenesis (12). Based on its homology with the human Dol-P-Man synthase Dpm1, we recently identified and characterized a Polyprenol-P-Man synthase (MtpPpm1) from Mycobacterium tuberculosis (13). Unexpectedly, MtpPpm1 is composed of two domains. The Dol-P-Man homologous domain is located in the C-terminal part of the protein, whereas the N-terminal domain possesses a low degree of similarity with a variety of bacterial acyltransferases. Interestingly, when genetically disconnected from the N-terminal domain, the catalytic C-terminal domain of MtpPpm1 was still able to catalyze Polyprenol-P-Man synthase in mycobacteria. However, further experiments also demonstrated that the addition of the disconnected N-terminal domain somehow in-
creased the transferase activity of the C-terminal domain without displaying catalytic activity by itself (13).

Surprisingly, in related mycobacterial species, such as Mycobacterium leprae, Mycobacterium avium, and Mycobacterium smegmatis, orthologs of the two domains are encoded by two distinct open reading frames, organized as an operon. This observation suggests that MtPpm1 has resulted from the fusion of two ancestral neighboring open reading frames, still separated in some related mycobacterial species. According to the "equidistant genome" (14, 15), the presence of the M. smegmatis genome of adjacent genes encoding MtPpm1 and MtPpm2 that are both homologs of MtPpm1 encoded by a single gene in M. tuberculosis suggests that MsPpm1 and MsPpm2 interact with each other to exert a function similar to that of MtPpm1.

In this report, we show that MsPpm2 is an integral membrane protein and, using a bacterial two-hybrid system (16), we demonstrate that the synthase MsPpm1 binds to MsPpm2 in vivo. As observed with mammalian Dol-P-Man synthases, MsPpm1 also lacks the characteristic hydrophobic C terminus. Thus, the MsPpm1-MsPpm2 interaction is reminiscent of that of the mammalian Dpm1 with Dpm3 (8, 9). In contrast, MsPpm1 is functionally active when expressed in Escherichia coli, similarly to the S. cerevisiae group of Dol-P-Man synthases (7, 17). As a consequence, MsPpm1 may constitute a new intermediate group of Polypropenol-P-Man synthases.

EXPERIMENTAL PROCEDURES

Bioinformatics—The transmembrane segments (TMS) of MtPpm1 and MsPpm2 were predicted using the following seven methods available on the World Wide Web: TMHMM (www.cbs.dtu.dk/services/TMHMM-2.0), HMMTOP (www.enzimbi.utoronto.ca/HMMTOP), IARAS (www.sbc.su.se/~miklos/DAS), TMPRED (www.ch.embnet.org/software/TMPRED_form.html), MEMSAT2 (bioinf.cs.ucl.ac.uk/psiffm.html), TOPPRED (www.sbc.su.se/~erik/toppred2d), and PHDHTM (www.embl-heidelberg.de/predictprotein/submit_adv.html#top).

Bacterial Strains and Growth Conditions—All cloning steps were performed in E. coli XL1-Bl2 (Stratagene, La Jolla, CA). M. smegmatis mc2155 was a generous gift from W. R. Jacobs, Albert Einstein College of Medicine, Bronx, NY (18) and was transformed as described previously (19). Recombinant clones were selected on Middlebrook 7H10 agar supplemented with oleic acid–albumin–dextrose–catelase enrichment (Difco, Detroit, MI) containing 25 μg/ml kanamycin (Sigma). Liquid cultures of recombinant M. smegmatis were grown at 37 °C in Luria-Bertani (LB) broth (Difco) supplemented with 25 μg/ml kanamycin and 0.05% Tween 80. Liquid cultures of E. coli (pUC) and E. coli (pUC-MtPpm1/D2) were grown in LB broth at 37 °C with 100 μg/ml ampicillin to an optical density (OD) of 0.0 to 0.4 and induced for 4 h with 1 μM isopropyl-β-D-thiogalactoside. Large-scale cultures of bacteria were grown as described above, harvested by centrifugation, washed with phosphate-buffered saline, and stored at -20 °C until further use. E. coli DHP1 is an adenylate cyclase deficient (cya) derivative of DH1 (F’, glnV44AS, recA1, endA1, gyrA96 (Nalr), thi1, hsdR17, spoT1, rfdJ1) (16). Protein-protein interactions leading to the cytoplasmic production and assembly of a functional adenylate cyclase in E. coli DH1 were detected by the binary hybrid method, as described previously (16). Thus, clones were checked for their ability to form red colonies at 30 °C on freshly prepared MacConkey agar plates containing 1% maltose and supplemented with 25 μg/ml kanamycin, 25 μg/ml chloramphenicol, and 100 μg/ml ampicillin when appropriate.

Insertion of phoA Cassettes—To fuse phoA to codons 465 or 600 of MtPpm1, phoA was first amplified by PCR from E. coli XL1-Bl2 using primers N226 (5' -GCCATTAAGTCTCGATCCTACAC-3' ) and N225 (5' -CGGACACAGAGGGCGGCTTCT-3') and the TaqDNA polymerase (Qiagen, Courtaboeuf, France). The 1421-bp PCR fragment was inserted into the commercially linearized pCR2.1Topo (Invitrogen) to yield pT25-MtPpm1/D1. The resulting plasmid was termed pT18-MtPpm1/D2.

Random phoA Insertion into pT25-MsPpm2—TrnTap is a Tn5-based mini-transposon carrying the signal sequenceless phoA reporter gene, lacking a promoter and translation initiation signals (21). To construct a transposable element suitable for in vitro transposition, the phoA-neo cassette was amplified by PCR using tap-xba (5' -GCTCTAGATTTGC-AGGACTGCAGCGCAGCTACCCACCA-3') and tap-sac (5' -GCTCTAGATTGTAGCAGGACGGAACGGAGG-3') as primers and pmod-rp (5' -CGGATCCGTCGACGACGACGACGCAATCT-3') and pmod-db (5' -CGGATCCGTCGACGACGACGACGCAATCT-3') as primers. The random in vitro transposition of the ME-phoA neo-ME transposon element into pT25-MsPpm2 was performed by using the EZ::TN transposase according to the manufacturer's instructions (Epigenetix, Madison, WI). The transposition reaction product was introduced into E. coli 311 (araD139 (ara- leu7697 Δnix440 ΔphoA320 gale galk ih trpE1 rpsF2 argE recA1) by electroporation. Blue AmpR and KanR colonies were selected on agar plates containing corresponding antibiotics and 40 μg/ml of X-Phosphos (Sigma).

Alkaline Phosphatase Activity Assay—Alkaline phosphatase activity was measured in M. smegmatis. Enzyme activity was determined using the p-nitrophenyl phosphate (Sigma) hydrolysis in intact cells as described previously (22, 23). Enzymatic reactions were performed in triplicate in the dark at 37 °C. Reactions were stopped with 100 μl of 1 M KH2PO4, 0.1 M EDTA (pH 8.0), and the OD at the appropriate wavelength was measured. The activity is expressed in arbitrary units. Units of activity for M. smegmatis = (A600 nm - 1.75 × A550 nm) × 10-9 × min-1 × 106 nmol of culture-1 (24).

Preparation of Enzyme Fractions and Polypropenol Phosphate Mannose Synthesis Assay—E. coli (pT18-MtPpm1/D2), E. coli (pT25-MtPpm1/D2), E. coli (pT18-MsPpm2/D2) were grown at 37 °C in LB medium and subcultured as described previously (13). For further fractionation, the lysate of E. coli (pT18-MsPpm1/D2) was centrifuged at 27,000 × g for 20 min at 4 °C. The membrane fraction was obtained by further centrifugation of the 27,000 × g supernatant at 100,000 × g for 1 h at 4 °C. The supernatant was carefully removed, and the membranes were gently resuspended in buffer 50 mM MOPS (ad- justed to pH 8.0 with KOH) containing 0.12 M NaCl. The membranes were allowed to equilibrate at 4 °C for 1 h, and 10 μg of protein was added to a protein concentration of 20 mg/ml. Protein concentrations were determined using the BCA protein assay reagent kit (Pierce). Reaction mixtures for assessing [14C]Man transfer consisted of 2.4 μM GDP-[14C]Man (PerkinElmer Life Sciences, 321 μCi/mmol, 0.125 μCi), 62.5 μM ATP, 10 μM MgCl2, and crude or fractionated subcellular preparations corresponding to 80 mg of protein. Exogenous lipid monophosphate substrates (C15:0, dolichol monophosphate) were added to the reaction mixtures at a final concentration of 0.125 mM in 0.25% CHAPS. The reaction mixtures were then incubated at 37 °C for 30 min. The reaction was terminated, and the lipids were
Experimental Determination of the Cellular Localization of the Catalytic Domain of MtPpm1—In Gram-negative bacteria, the topology of integral membrane proteins has largely been studied using PhoA fusions since the sites at which alkaline phosphatase has high enzymatic activity normally correspond to periplasmic domains of the membrane protein. In a previous report, we have shown that PhoA fusions can be used in mycobacteria to determine the cytoplasmic or extracytoplasmic location of proteins or protein domains (22). To determine the cellular location of the catalytic domain of MtPpm1, we made use of PhoA fusions to various parts of the protein. The PhoA gene was thus inserted into pMV261-MtPpm1, a plasmid previously shown to allow for overexpression of MtPpm1 in mycobacteria (13). The PhoA gene was inserted in-frame with Mtppm1 either before (codon 465) or after (codon 600) the last predicted TMS (amino acids 509–527) of MtPpm1 (Fig. 1A). The resulting constructs, named pMV261-MtPpm1/PhoA465 and pMV261-MtPpm1/PhoA600, respectively, were introduced into M. smegmatis mc2155, and the recombinant clones were tested for their ability to hydrolyze X-phosphohexose. When compared with untransformed bacteria, M. smegmatis mc2155(pMV261-MtPpm1/PhoA465) exhibited an increased capacity to hydrolyze X-phosphohexose (25.7 ± 2.0 ± 0.011 OD unit(s)×min⁻¹×mg⁻¹). In contrast, M. smegmatis mc2155(pMV261-MtPpm1/PhoA600) did not hydrolyze X-phosphohexose (-0.178±0.014 OD unit(s)×min⁻¹×mg⁻¹). These results indicate that amino acid 465 of MtPpm1 is localized in a portion of the protein facing the outside of the cell membrane, whereas the region surrounding amino acid 600 is cytoplasmic. This topology is in agreement with four out of the seven computeraided predictions (HMMTOP, DAS, MEMSAT, and PHDHTM). Moreover, these results are consistent with the predicted cytoplasmic location of the MtPpm1/D2 orthologs, Ppm1 from M. smegmatis, M. avium, and M. leprae.

Experimental Determination of the Location of MsPpm1—Computer predictions suggested a cytoplasmic location for MsPpm1. These predictions are consistent with the cytoplasmic location of MtPpm1/D2 orthologs, Ppm1 from M. smegmatis, M. avium, and M. leprae.

In Vivo Interaction between MsPpm1 and MsPpm2—The existence of a strictly conserved operon architecture for ppm2-ppm1 in M. leprae, M. avium, and M. smegmatis suggests that the two corresponding proteins participate in a common structural complex or metabolic pathway, consistent with the presence in M. tuberculosis of a single gene (Mtppm1) resulting from the fusion of the two corresponding open reading frames. It is thus likely that MsPpm1 and MsPpm2 are able to directly interact with each other. Among the general methodologies to identify interactions between proteins, the yeast two-hybrid system represents the most powerful in vivo approach. However, the detection of the interaction between MsPpm1 and

MtPpm1, nor were signal sequences predicted in the N-terminal part of these proteins. These results suggest a cytoplasmic localization for these proteins and, by analogy, a cytoplasmic localization for the catalytic domain of MtPpm1.

Computer-predicted Topology of Mycobacterial Polyprenol-P-Man Synthases—Consistent with the previously shown membrane association of the Polyprenol-P-Man synthase activity in M. tuberculosis (13), MtPpm1 contains TMS located in its N-terminal domain (D1). Since MtPpm1 uses GDP-Man as a substrate (11), its catalytic domain (D2) would be expected to be cytoplasmic where GDP-Man is readily available. To predict the topology of MtPpm1, we used seven different programs available on the internet (TMHMM, HHMPTOP, DAS, TMPRED, MEMSAT2, TOPPRED, and PHDHTM) since the reliability of topology predictions increases substantially when different prediction methods are compared (25). When applied to MtPpm1, all seven programs converged to the presence of several TMS, most of which was located in the N-terminal half of the protein (D1), strongly suggesting that MtPpm1 is an integral membrane protein (Fig. 1A). However, the algorithms did not converge on the precise number of TMS and on the orientation of the catalytic domain (D2) of MtPpm1.

In other mycobacterial species, such as M. leprae, M. avium, and M. smegmatis, the orthologs of the two MtPpm1 domains, referred to as D1 and D2, are encoded by separate genes. The orthologs of the catalytic domain (D2) of MtPpm1 are named Ppm2, and the orthologs of the N-terminal non-catalytic domain of MtPpm1 (D1) are named Ppm1. Application of the topology prediction algorithms on Ppm2 from M. smegmatis (MsPpm2) (Fig. 1B), M. leprae (MtPpm2), and M. avium (MaPpm2) (data not shown) strongly suggests that these proteins, similar to the MtPpm1/D1 domain, are anchored in the bacterial membrane. In contrast, none of the seven methods predicted the presence of TMS in MsPpm1, MtPpm1, or MaPpm1, nor were signal sequences predicted in the N-terminal part of these proteins.
**Fig. 2. Bacterial two-hybrid system assays.**

A, *in vivo* interaction between MsPpm1 and MsPpm2. pT18-MsPpm1 carries a fusion between Msppm1 and a fragment corresponding to the T18 fragment of *B. pertussis* CyaA. pT25-MsPpm2 carries a fusion between Msppm2 and a fragment corresponding to the T25 fragment of CyaA. No red color on McConkey/Maltose agar plates was detected when pT18-MsPpm1 + pT25 or pT25-MsPpm2 + pT18 were introduced into *E. coli* DHP1. B, *in vivo* interaction assay between the two domains of MtPpm1. When co-introduced into *E. coli* DHP1, pT25-MtPpm1/D1 and pT18-MtPpm1/D2 yielded red transformants on McConkey/maltose. C, trans-species *in vivo* interaction assay. The two domains of MtPpm1 (MtPpm1/D1 and MtPpm1/D2) were tested for their capacity to interact with MsPpm1 and MsPpm2, respectively.

**MsPpm2** using a yeast two-hybrid system may be impaired by the association of MsPpm2 with the membrane. Therefore, to test whether MsPpm2 can interact with MsPpm1, a recently described bacterial two-hybrid system (16) was used, which is suitable to study protein interactions even if one of the partners is membrane-associated. In this system, the interaction of two proteins results in functional complementation between two domains of the adenylate cyclase (CyaA) from *Bordetella pertussis*, leading to cAMP synthesis. As a soluble regulatory molecule, cAMP is then able to activate cAMP-dependent transcriptional events, which can be easily monitored (16).

The entire MsPpm1 was thus genetically fused to the 18-kDa domain of CyaA to produce pT18-MsPpm1, and MsPpm2 was fused to the 25-kDa domain of CyaA to produce pT25-MsPpm2. The cyclase-deficient *E. coli* strain DHP1 was co-transformed with the two constructs, and the resulting recombinant colonies...
were tested for their ability to metabolize maltose on McConkey agar plates supplemented with ampicillin and chloramphenicol. As shown in Fig. 2A, E. coli DHP1 containing both fusions were able to metabolize maltose and appeared red, whereas colonies containing pT18-MsPpm1 with pT25 or pT25-MsPpm2 with pT18 were not able to metabolize maltose and remained white on McConkey-maltose plates. These results demonstrate specific interactions between MsPpm1 and MsPpm2.

Interaction between the Two Domains of MtPpm1 Is Conserved—Since MsPpm1 and MsPpm2 correspond to domains D2 and D1 of MtPpm1, respectively, encoded by a single gene, we wanted to test whether D1 and D2 can also interact with each other, even in the absence of a covalent link between them. We therefore genetically disconnected the two domains and co-expressed them within the bacterial two-hybrid system. MtPpm1/D2 was fused to T18, and MtPpm1/D1 was fused to T25. After introduction of both constructs into E. coli DHP1, the colonies were found to be able to metabolize maltose on McConkey agar plates, in contrast to the cells containing pT18-MtPpm1/D2 and pT25 or pT25-MtPpm1/D1 and pT18 (Fig. 2B). This result demonstrates that the two domains D1 and D2 of MtPpm1 can specifically interact with each other, even if they are not covalently linked.

Trans-species in Vivo Interaction—We have previously shown that MtPpm1/D1 has no Dol-P-Man synthase activity but increases Dol-P-Man production when its gene is overexpressed in M. smegmatis, suggesting that the M. tuberculosis MtPpm1/D1 may interact with the M. smegmatis MsPpm1 enzyme and may thereby increase its enzymatic activity. To test this hypothesis, pT18-MsPpm1 and pT25-MtPpm1/D1 were both introduced into E. coli DHP1. The recombinant bacteria showed a red color on McConkey-maltose plates (Fig. 2C), demonstrating that MsPpm1 can interact with MtPpm1/D1. Vice versa, when pT18-MtPpm1/D2 and pT25-MsPpm2 were introduced into E. coli DHP1, the recombinant bacteria also displayed a red color on McConkey-maltose plates (Fig. 2C).

Partial Determination of the Topology of T25-MsPpm2 in E. coli—MsPpm2 and MtPpm1/D1 were both predicted to contain many TMS, suggesting that they are integral membrane proteins. Moreover, PhoA fusions demonstrated that MtPpm1 is associated with the membrane. Here, PhoA fusions were used to determine the global topology of MsPpm2. TnTap (21) was randomly transposed in vitro into pT25-MsPpm2 using the EZ::TN transposase (Epicentre, Madison, WI), and the recombinant plasmids were introduced into E. coli CC118. Blue colonies obtained as a result of PhoA activity were selected on agar plates containing X-phosphate. The insertion positions of the mini-transposon were determined by DNA sequencing for 25 blue colonies that contained the phoA cassette in the Msppm2 gene. Activities were measured according to Manoil (24). The results confirm that MsPpm2 is an integral membrane protein and provide some insight into the topology of the protein (Fig. 3). The blue color obtained by insertion of phoA after codon 495 is similar to the results obtained after insertion of phoA in the corresponding region of MtPpm1, indicating the extracytoplasmic location of this region for both proteins. Four of the seven algorithms predicted the existence of a TMS at approximately residue 450 (Fig. 1B). However, the PhoA fusion data indicate that this TMS is unlikely since PhoA fusions upstream and downstream of residue 450 resulted in strong phosphatase activity. No TnPhoA insertions leading to a PhoA-positive phenotype were obtained in the region between Ala160 and Val222. However, based on the phosphatase activity of the PhoA insertions in the Gly88 to Gly116 region and the unanimous prediction of TMS Tyr117 to Ser134 and Ala160 to Ala139, this region of MsPpm1 is likely to be periplasmic. It is possible that insertions of TnTap in this region lead to unstable hybrid proteins. One colony with a very low phoA activity (phoA activity = 1.15 units) obtained after transposition revealed an insertion of TnTap in the region corresponding to Trp139 to Ala252, confirming that this portion of the protein is cytoplasmic (Fig. 3). Interestingly, the topology determined by the PhoA fusions together with the TMS predicted by all the algorithms used indicate that only very few amino acids of MsPpm2 are located at the cytoplasmic side of the membrane and may thus be available for the interaction with MsPpm1 (Figs. 3 and 5).
Fig. 4. Incorporation of $[^{14}C]$Man from GDP-$[^{14}C]$Man into exogenous C$_{95}$ Dol-P using extracts from E. coli(pT18-MtPpm1/D1), E. coli(pT25-MtPpm1/D1), and E. coli(pT18-MtPpm1/D2 + pT25-MtPpm1/D1). Arrows indicate the origin of the migration, the solvent front, and the C$_{95}$ Dol-P-Man. Radioactivity was quantified using a Phosphorlmager detector (Storm, Amersham Biosciences). The average incorporation of $[^{14}C]$Man was as follows: E. coli(pT18-MtPpm1/D2), 18,080 cpm; E. coli(pT18-MtPpm1/D2 + pT25-MtPpm1/D1), 87,330 cpm; and E. coli(pT25-MtPpm1/D1), 205 cpm.

Influence of T25-MtPpm1/D1 on the Catalytic Activity of T18-MtPpm1/D2—To test whether the physical interaction between MtPpm1/D1 and MtPpm1/D2 has an impact on the Polyprenol-P-Man synthase activity, we measured Dol-P-Man synthesis in E. coli transformed with pT18-MtPpm1/D2 and compared it with that measured in E. coli co-transformed with both pT18-MtPpm1/D2 and pT25-MtPpm1/D1. As shown in Fig. 4, E. coli co-producing T18-MtPpm1/D2 and T25-MtPpm1/D1 synthesized 4.8-fold more Dol-P-Man than E. coli cells producing T18-MtPpm1/D2 alone, demonstrating that the interaction between MtPpm1/D1 and MtPpm1/D2 leads to an increase in the catalytic activity of MtPpm1/D2.

DISCUSSION

Due to the lack of a transporter, GDP-Man, a widely used mannosyl donor, is generally unable to cross biological membranes. Consequently, the enzymes that use GDP-Man are predicted to be located in the cytosol. In eukaryotes, Dol-P-Man synthase (EC 2.4.1.83) catalyzes the transfer of Man from GDP-Man to dolichol monophosphate, forming Dol-P-Man, which is subsequently translocated through the endoplasmic reticulum membrane to be used as a mannosyl donor in the lumen of the endoplasmic reticulum. The Dol-P-Man synthases are usually associated with the cytoplasmic side of the membrane, and two different mechanisms have been proposed for this membrane association (7, 17). In S. cerevisiae, U. maydis, and T. brucei Dpm1 contains a C-terminal hydrophobic domain proposed to anchor the enzyme into the lipid bilayer of the membrane. In contrast, mammalian Dol-P-Man synthases are associated with the endoplasmic reticulum membrane through specific interactions with the hydrophobic protein Dpm3, in turn, stabilized by Dpm2 (8, 9). In mycobacteria, the biosynthesis of cell wall glycoconjugates, such as lipoarabinomannan, also requires the transfer of Man residues through the bacterial membrane. The Man carriers in mycobacteria are Polyprenol-P-Man, isoprenoid derivatives that are shorter than Dol-P-Man and contain an unsaturated α-isoprene residue.

In this study, we found that the synthesis of Polyprenol-P-Man in M. smegmatis is also mediated by two proteins, one being the catalytic MsPpm1 and the other being the “helper” protein MsPpm2. MsPpm1 and MsPpm2 interact in vivo when co-produced in E. coli, and MsPpm2 enhances the catalytic activity of MsPpm1. Thus, as for mammalian Dpm1, MsPpm1 lacks the C-terminal hydrophobic domain but interacts instead with the integral membrane protein MsPpm2. This finding suggests a role for the two-domain structure of Ppm1 from M. tuberculosis, in which the first domain would anchor the protein into the bacterial membrane. As shown by the bacterial two-hybrid assay, the two domains of MtPpm1 are able to interact with each other even in the absence of covalent linkage.
between them. Moreover, the interaction between the two domains of MtPpm1 independently produced in E. coli results in enhanced Polyprenol-P-Man production. In addition, the capacity of interaction has been conserved between two mycobacteria that differ with respect to the genetic structure of their ppm1 genes, as illustrated by the capacity of MsPpm1 and MsPpm2 to interact with MtPpm1/D1 and MtPpm1/D2, respectively.

By analogy with the S. cerevisiae Dpm1 (ScDpm1), MtPpm1 contains a hydrophobic region responsible for its attachment to the bacterial membrane. However, in M. tuberculosis, the hydrophobic region is located in the N-terminal portion of the protein and is larger than the C-terminal hydrophobic domain of ScDpm1. On the other hand, MsPpm1 may be, to some extent, compared with the human Dpm1 as they both lack the C-terminal transmembrane domain and interact with a polypeptide (MsPpm2 versus human Dpm3) that is localized in the membrane. In contrast, MtPpm1/D2 and MsPpm1 are active when produced in E. coli, whereas human Dpm1 is not. Thus, as illustrated in Fig. 5, we propose to extend the family of Polyprenol-P-Man synthases by including two new members that use two original strategies of membrane association.

MtPpm1 is a cytoplasmic soluble protein, whereas MsPpm2 is a hydrophobic protein containing 6–8 predicted TMS. Due to the hydrophobic nature of MsPpm2, we have not been able to purify MsPpm2 to test physical interactions with MtPpm1 in vitro. However, the interactions could be studied in vivo by using a bacterial two-hybrid system based on the functional complementation between two domains of the cyaA gene from B. pertussis, which catalyzes the production of cAMP from ATP. Both the substrate and the product of the reaction are soluble in the cytosol, even if one of the partners of the two-hybrid system is anchored in the inner face of the membrane. This approach allowed us to demonstrate a specific interaction between the transmembrane protein MsPpm2 and its soluble catalytic partner MtPpm1. The bacterial two-hybrid system did not abolish the enzymatic activity of MtPpm1/D2 or MsPpm1, nor did it affect the enhancing effects of MtPpm1/D1 and MsPpm2 on the enzymatic activities of MtPpm1/D2 and MtPpm1, respectively.

Based on both the TMS prediction methods and the PhoA insertions, we deduced a topological model of MsPpm2 (Fig. 3). Surprisingly, the proposed model suggests that only few amino acids of MsPpm2 are located at the cytoplasmic side of the membrane and may thus be available for the interaction with MtPpm1. Mutagenesis of residues of the N-terminal tail and of the first and second intracellular predicted loops of MsPpm2 may help to identify amino acids implicated in the interaction with MtPpm1.

The physical interaction demonstrated between MsPpm1 and MsPpm2 and the existence of MtPpm1 in M. tuberculosis containing the two domains fused into a single protein are an illustration of the Rosetta stone theory (15). This theory proposes that interactions between a protein of unknown function and a well-characterized protein suggest that the function of the former is somewhat related to that of the latter. In accordance with this theory, the implication of MsPpm2 in the Polyprenol-P-Man synthesis pathway may have been inferred from the architecture of MtPpm1 (14).

In conclusion, the interaction of MtPpm1 with MsPpm2 allows us now to understand how the Polyprenol-P-Man synthase activity of M. smegmatis is associated with the membrane in the absence of a TMS in MsPpm1. Therefore, M. smegmatis uses a membrane-targeting strategy similar to that of the mammalian Dol-P-Man synthases.

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REFERENCES

1. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
2. Herscovics, A., and Orlean, P. (1993) FASEB J. 7, 540–550
3. Menon, A. K., Mayor, S., and Schwarcz, R. T. (1990) EMBO J. 9, 4249–4258
4. Orlean, P. (1990) Mol. Cell. Biol. 10, 5796–5805
5. Beverley, S. M., and Turco, S. J. (1998) Trends Microbiol. 6, 35–49
6. Mengeling, B. J., and Turco, S. J. (1998) Curr. Opin. Struct. Biol. 8, 572–577
7. Colussi, P. A., Taron, C. H., Mack, D. C., and Orlean, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7873–7878
8. Maeda, Y., Tomita, S., Watanabe, R., Oishi, K., and Kinoshita, T. (1998) EMBO J. 17, 4920–4929
9. Maeda, Y., Tanaka, S., Hino, J., Kangawa, K., and Kinoshita, T. (2000) EMBO J. 19, 2475–2482
10. Schultz, J., and Elbein, A. D. (1974) Arch. Biochem. Biophys. 160, 311–322
11. Besra, G. S., Morehouse, C. B., Rittner, C. M., Waechter, C. J., and Brennan, P. J. (1997) J. Biol. Chem. 272, 18460–18466
12. Chatterjee, D. (1997) Curr. Opin. Chem. Biol. 1, 579–588
13. Gurcha, S. S., Baulard, A. R., Kremer, L., Locht, C., and Brennan, P. J. (1997) J. Biol. Chem. 272, 13181–13187
14. Pellegrini, M., Marcotte, E. M., Thompson, M. J., Eisenberg, D., and Yeates, T. O. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4285–4288
15. Marcotte, E. M., Pellegrini, M., Ng, H. L., Rice, D. W., Yeates, T. O., and Eisenberg, D. (1999) Science 285, 751–753
16. Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5752–5756
17. Kruusewetzka, J. S., Salosheko, M., Migalska, A., Orlean, P., Penttila, M., and Palamarczyk, G. (2000) Glycobiology 10, 983–991
18. Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T., and Jacobs, W. R., Jr. (1990) Mol. Microbiol. 4, 1911–1919
19. Lugosi, L., Jacobs, W. J., and Bloom, B. R. (1989) Tubercle 70, 159–170
20. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennet, L. T., Bansal, G. P., Young, J. F., Lee, M. H., Hatfull, G. F., Snapper, S. B., Barletta, R. G., Jacobs Jr., W. R., and Bloom, B. R. (1995) J. Bacteriol. 177, 460–469
21. Kremer, L., Baulard, A., Estaquier, J., Content, J., Capron, A., and Locht, C. (1995) J. Bacteriol. 177, 642–653
22. Mansil, C., and Beckwith, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8129–8133
23. Mansil, C. (1991) Methods Cell Biol. 34, 61–75
24. Nilsson, J., Persson, B., and von Heijne, G. (2000) FEBS Lett. 486, 267–269
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