Substrate Specificity and Membrane Topology of Escherichia coli PgpB, an Undecaprenyl Pyrophosphate Phosphatase*

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The synthesis of the lipid carrier undecaprenyl phosphate (C55-P) requires the dephosphorylation of its precursor, undecaprenyl pyrophosphate (C55-PP). The latter lipid is synthesized de novo in the cytosol and is also regenerated after its release from the C55-PP–linked glycans in the periplasm. In Escherichia coli the dephosphorylation of C55-PP was shown to involve four integral membrane proteins, BacA, and three members of the type 2 phosphatidic acid phosphatase family, PgpB, YbjG, and YejU. Here, the PgpB protein was purified to homogeneity, and its phosphatase activity was examined. This enzyme was shown to catalyze the dephosphorylation of C55-PP with a relatively low efficiency compared with diacylglycerol pyrophosphate and farnesyl pyrophosphate (C15-PP) lipid substrates. However, the in vitro C55-PP phosphatase activity of PgpB was specifically enhanced by different phospholipids. We hypothesize that the phospholipids are important determinants to ensure proper conformation of the atypical long axis C55 carrier lipid in membranes. Furthermore, a topological analysis demonstrated that PgpB contains six transmembrane segments, a large periplasmic loop, and the type 2 phosphatidic acid phosphatase signature residues at a periplasmic location.

Undecaprenyl phosphate (C55-P) is a 55-carbon-long poly- prenol (see Fig. 1). It is an essential bacterial lipid required for the synthesis of various cell wall polymers such as peptidoglycan, lipopolysaccharides, teichoic acids, osmo-regulated periplasmic glucans, capsular polysaccharides, and the enterobacterial common antigen (1–10). C55-P is utilized as a carrier lipid that allows the transport of the hydrophilic oligosaccharide precursors across the cytoplasmic membrane toward the periplasm where the elongation of the glycan chains takes place. Accordingly, the precursor is linked to the carrier lipid via a pyrophosphate linkage (C55-PP-substrate) through the action of a specific glycosyltransferase at the cytosolic side of the inner membrane; thereafter, the complex is translocated through the membrane by a yet unknown mechanism, and finally, the glycosyl moiety is transferred to the appropriate expanding polymer. De novo synthesis of C55-P implicates two enzymatic steps (11, 12); it originates from undecaprenyl pyrophosphate (C55-PP), itself being synthesized by successive condensations of eight isopentenyl pyrophosphates (C5-PP) with farnesyl pyrophosphate (C15-PP) (Fig. 1) catalyzed by the cytosolic UppS enzyme, a cis-prenyl-pyrophosphate synthase (13, 14). The C55-PP must then be dephosphorylated to yield the active monophosphate form of the carrier lipid (11). C55-PP is not solely generated by de novo synthesis, but it is also released and recycled after the transfer of the oligosaccharide unit to the growing polymer in the periplasm. It is yet unclear on which side of the membrane C55-PP dephosphorylation occurs and how the carrier lipid is translocated across the membrane before being reused.

The enzymatic dephosphorylation of C55-PP is the target of bacitracin, a cyclic polypeptide antibiotic that interacts tightly with C55-PP, thereby inhibiting the formation of C55-P through the sequestration of its precursor (15, 16). Cain et al. (17) earlier identified the Escherichia coli bacA gene whose overexpression conferred increased resistance to bacitracin. More recently, the BacA protein was demonstrated to catalyze C55-PP dephosphorylation in vitro (18). The resistance to bacitracin conferred by the overexpression of bacA was then explained by the fact that overproducing the C55-PP phosphatase activity should dramatically deplete the cellular pool of C55-P, the target of the antibiotic. The deletion of bacA was not lethal, which was consistent with the fact that the essential C55-PP phosphatase cellular activity present in ΔbacA cells was not totally abolished but was reduced by 75% compared with wild-type cells (18). El Ghachi et al. (19) have identified three other genes encoding putative integral membrane proteins whose overexpression also conferred resistance to bacitracin and a significant increase of C55-PP phosphatase activity in cell extracts: ybjG, yejU, and ppgB. The three genes could be independently disrupted without effect on growth in laboratory conditions. However, the simultaneous inactivation of ybjG and ppgB together with bacA
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could never be obtained, suggestive of a lethal effect. The lethality of the triple mutation was further demonstrated by the construction of a conditional thermosensitive mutant BWTS$_{bacA}$ ($\Delta$bacA, $\Delta$ybjG, $\Delta$pgpB) carrying the pMAKbacA plasmid with a thermosensitive replicon (19). These observations suggested that PgpB, YbjG, and BacA contributed to the total C$_{55}$-PP phosphatase activity in *E. coli*. Interestingly, the presence of only one of these chromosomal genes was sufficient to sustain normal cell growth, raising the question of the relevance of multiple C$_{55}$-PP phosphatases in *E. coli*. Surprisingly, the over-expression of yeiU that also led to a significant rise of C$_{55}$-PP phosphatase activity was not able to rescue cell viability of the triple mutant, suggesting that YeI has another function besides the dephosphorylation of C$_{55}$-PP. We recently demonstrated that YeI, renamed LpxT, not only dephosphorylates the carrier lipid C$_{55}$-PP but could also transfer the released phosphate group to lipid A (phosphotransferase activity) (20). PgpB together with LpxT and YbjG belongs to the type 2 phosphatidic acid phosphatase (PAP2) family previously identified by Stukey and Carman (21); they all possess a characteristic phosphatase signature in which three distinct conserved motifs are visible, designated C1, C2, and C3 (Fig. 2).

Originally, an *E. coli* pgpB mutant was isolated in a screen designed to isolate cells defective in phosphatidylglycerol phosphate (PGP) phosphatase activity (22). Further analyses suggested that the pgpB product had a broad substrate spectrum, as *in vitro*-measured phosphatase activities toward PGP, phosphatidic acid (PA), but also lysoPA and diacylglycerol pyrophosphate (DGPP) were significantly increased in pgpB-overexpressing cells (23, 24). In the present study the *E. coli* PgpB membrane protein was purified to homogeneity, and a biochemical characterization of the enzyme was performed to elucidate its specificity. We show here that PgpB efficiently dephosphorylates various lipid pyrophosphate molecules, thereby confirming its broad substrate spectrum. In particular, PgpB dephosphorylates C$_{55}$-PP, and this reaction is dependent upon the addition of phospholipids. Several lines of evidence suggest that the phospholipids do not regulate PgpB activity *per se* but proceed through their interaction with the long chain C$_{55}$-PP lipid. A topological map of PgpB was also established by using the $\beta$-lactamase fusion procedure; it shows that PgpB is constituted of six transmembrane segments, with its

![Figure 1. Structures of isoprenoids pyrophosphate and diacylglycerol pyrophosphate lipids.](image)

![Figure 2. Amino acid sequence alignment of PAP2 enzymes.](image)
TABLE 1
Oligonucleotides used in this study
Restriction sites introduced in oligonucleotides are underlined.

| Name         | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| pgpBNcoI     | 5’-GCCCCTATGCAATGCTCATGCAACATGATGAC-3’                                  |
| pgpBNcoI     | 5’-GCCCCTATGCAATGCTCATGCAACATGATGAC-3’                                  |
| pgpBBglIII   | 5’-GCCCCTATGCAATGCTCATGCAACATGATGAC-3’                                  |
| pgpBBglIII   | 5’-GCCCCTATGCAATGCTCATGCAACATGATGAC-3’                                  |
| pgpB5’       | 5’-GCCCCTATGCAATGCTCATGCAACATGATGAC-3’                                  |
| pgpBBglIII   | 5’-GCCCCTATGCAATGCTCATGCAACATGATGAC-3’                                  |

N- and C-terminal ends localized in the cytoplasm and the active site located on the outer side of the cytoplasmic membrane.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The E. coli strains DH5α (Invitrogen) and C43(DE3) (Avidis) were used as hosts for plasmids and PgpB overproduction, respectively. The temperature-sensitive strain BWTSbacA was previously constructed (19). The plasmid vector pTrc99A was obtained from Amersham Biosciences, and the plasmid pTrcHis60 has been previously constructed (25). The pNF150 plasmid vector used for membrane topology analysis of PgpB was kindly provided by J. P. Bohin (26). Bacteria were grown at 37 °C in 2YT medium (27), and growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. When appropriate, cultures were supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (25 μg/ml).

Chemicals—DNA restriction enzymes were purchased from New England Biolabs, the Pfu Turbo DNA polymerase was from Stratagene, and oligonucleotides were from MWG-Biotec. Plasmid purification and PCR clean up kits were delivered from Stratagene, and oligonucleotides were from MWG-Biotec. Plasmid purification and PCR clean up kits were delivered from Stratagene, and oligonucleotides were from MWG-Biotec. phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, and proteinase K were purchased from Sigma. N-ethyl-N,N′-diisopropyl-N-fluorobenzene sulfonamide (Naf) was from Calbiochem. Restriction enzymes were purchased from DNA restriction enzymes were purchased from New England Biolabs. The 5-ethylthio-1H-tetrazolium-1,2,3-β-driodide (MTT) assay was from Sigma. The pNF150 plasmid vector used for membrane topology analysis of PgpB was kindly provided by J. P. Bohin (26). Bacteria were grown at 37 °C in 2YT medium (27), and growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer.

Construction of Plasmids—For the construction of plasmids, the gene product to the mature form of PgpB was amplified using the pgsB5′ oligonucleotide (5′ end of the gene) and various others (3′ end) listed in this table. The oligonucleotide sequences are listed in Table 1.

| Plasmid name     | Fusion point | Amp’ MIC | β-lactamase location |
|------------------|--------------|----------|----------------------|
| pNFpgpB11       | pGS11        | S <10 In  |                      |
| pNFpgpB39       | pGS19        | R >100 Out|                      |
| pNFpgpB73       | pGS73        | S <10 In  |                      |
| pNFpgpB97       | pGS97        | R >100 Out|                      |
| pNFpgpB120      | pGS120       | R >100 Out|                      |
| pNFpgpB140      | pGS140       | R >100 Out|                      |
| pNFpgpB160      | pGS160       | R >100 Out|                      |
| pNFpgpB84       | pGS84        | S <10 In  |                      |
| pNFpgpB210      | pGS210       | R >60 Out |                      |
| pNFpgpB236      | pGS236       | S <10 In  |                      |
| pNFpgpB254      | pGS254       | S <10 In  |                      |

Restriction sites introduced in oligonucleotides are underlined. After cleavage by the appropriate endonucleases, the PCR product was inserted into the corresponding sites of the expression vector pTrc99A. Similarly, for the expression of the C-terminal His6−tagged form of PgpB, the plasmid pPgPHis was created. In that case, the pgsB5′ gene was amplified using oligonucleotides pgsBNcoI and pgsBBglIII, and after restriction of the PCR product by NcoI and BglII, the fragment was inserted between the corresponding sites of the plasmid vector pTrcHis60. In both recombinant plasmids, pgsB5′ expression was under the control of the IPTG-inducible trc promoter.

For topology analysis, various truncated forms at the 3′ end of the pgsB5′ gene were PCR-amplified from the E. coli chromosome using oligonucleotides listed in Table 1 and cloned into pNF150, a plasmid vector carrying the lac promoter, a fragment of the β-galactosidase gene, and the mature form of the β-lactamase gene (26). For all constructs, the oligonucleotide pgsB5′ was used as the primer for the 5′ end of the gene, introducing a BamHI site downstream of the initiation codon of pgsB5′. Different oligonucleotides were then used as 3′ end primers introducing a KpnI site (Tables 1 and 2). The generated PCR products were cleaved by BamHI and KpnI enzymes and inserted into the corresponding sites of the pNF150 vector, creating in-frame fusions of the truncated forms of the pgsB5′ gene product to the mature form of β-lactamase via a short

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peptide linker, AVPHAISSSPLR, originating from the sequence of the pNF150 plasmid vector. The nucleotide sequence of all constructs was verified by automated sequencing (MWG-Biotech).

**Expression and Purification of PgpB—** *E. coli* C43(DE3) cells carrying the plasmid pPgpBH1s were grown at 37°C in 2YT medium (1 liter) containing ampicillin. When the optical density (A600) of the culture reached 0.8, IPTG was added at the final concentration of 1 mM, and growth was continued for 3.5 h. Cells were then harvested (4000 × g, 10 min) and resuspended in 40 ml of 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 20 mM 2-mercaptoethanol, 0.5 mM NaCl, and 10% glycerol (buffer A). They were disrupted by 3 successive passages through a French press, and the membrane and soluble proteins were separated by ultracentrifugation at 100,000 × g for 1 h. The resulting pellet was washed 3 times in 20 ml of buffer A, and membranes were then solubilized by incubation in 20 ml of buffer A supplemented with 2% (w/v) DDM detergent for 2 h at 4°C. The latter solution was centrifuged (100,000 × g, 1 h), and the supernatant was incubated with 2 ml Ni2+-NTA and 10 mM imidazole at 4°C overnight. The resin was washed successively with 20 volumes of 10 mM and 30 mM imidazole solutions prepared in buffer A supplemented with 0.2% DDM. Final elution was performed with buffer A supplemented with 400 mM imidazole and 0.2% DDM, yielding a pure fraction of PgpB protein. This fraction was concentrated up to 1.5 mg/ml by ultrafiltration on Amicon Ultra centrifugal filter devices (Millipore) and thoroughly dialyzed against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.02% DDM before being stored at −20°C. Typically, up to 2.5 mg of pure PgpB protein were obtained from 1 liter of bacterial cell culture. Protein concentration was determined using the Sigma bicinchoninic acid (BCA) assay system (28).

**Mass Spectrometry—** Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of PgpB samples was performed on a PerSeptive Voyager-DE STR instrument (Applied Biosystems). 0.5 μl of the PgpB solution was deposited on the plate and allowed to dry. Subsequently, 0.5 μl of matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid) was applied to the dried sample and again allowed to dry. Spectra were recorded in a positive ion mode at an acceleration voltage of +25 kV and an extraction delay time of 300 ns. Enolase was used as an external calibrant.

**Gel Filtration—** Gel filtration chromatography was performed using a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with two column volumes of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% DDM, and 5 mM 2-mercaptoethanol. Elution of proteins was followed at 280 nm. Protein standards, whose elution profiles were known to be unchanged in the presence of DDM (29–31), were used to calibrate the column: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa). The void volume was determined using dextran blue. A 100-μl aliquot of the protein sample was loaded onto the column at a flow rate of 0.5 ml/min. The PgpB-DM1 complex was eluted as a single peak at an elution volume of 12.6 ml corresponding to an apparent molecular mass of 110 kDa.

**Phosphatase Assays—** Standard C55-PP phosphatase assays were performed in a 20-μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 150 mM NaCl, 0.6% DDM, and [32P]C55-PP (2305 Bq, varying concentrations). Pure PgpB protein was added to initiate the reaction, and after 30 min of incubation at 37°C the reaction was stopped by heat denaturation of the enzyme. Appropriate dilution of the enzyme was used so that the amount of hydrolyzed substrate did not exceed 30% at the end of the reaction. The samples were analyzed by TLC as already described (18). When the phosphatase activity was investigated at various pH values, buffering was achieved in sodium acetate (pH 5–5.5), Bis-Tris (pH 6–6.5), or Tris-HCl buffer (pH 7–9). To test the phosphatase activity of PgpB toward various mono- and pyrophosphate molecules, the release of inorganic phosphate was measured during catalysis. Phosphatase reaction mixtures (100 μl) containing 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 150 mM NaCl, 0.6% DDM, the appropriate substrate, and PgpB were incubated for 30 min at 37°C. The reactions were quenched by adding 0.9 ml of Malachite green (Biomol Green), and the released phosphate was measured at 620 nm and quantified relative to a phosphate standard curve. With this approach, we could not distinguish between the removal of the β-phosphate from that of the α-phosphate when pyrophosphate molecules were used. Therefore, when measuring the initial rate of dephosphorylation of pyrophosphates, we ensured that no more than 10% of the phosphate was removed, thereby limiting the amount of the released α-phosphate taking into account that PgpB has higher activity toward pyro- than monophosphates (see below). All enzyme assays were performed at least in triplicate. For the determination of kinetic constants, initial velocity data were fitted to the equation $v = VS/(K_m + S)$ by the Levenberg-Marquardt method (32), and values ± S.D. at 95% of confidence were calculated; the MDFltt software developed by M. Desmadril (UMR 8619 CNRS, Orsay, France) was used for this purpose.

**RESULTS**

**Expression and Purification of PgpB—** The *pgpB* gene was cloned under the control of the IPTG-inducible trc promoter in the expression vectors pTrcHis6A and pTrcHis60, yielding plasmids pPgpB and pPgpBH1s, which allow the expression of wild-type and C-terminal His6-tagged proteins, respectively. The plasmids were transformed into the *E. coli* BW3tsbA thermo-sensitive strain, which carries deletions of the three *pgpB*, *ybjG*, and *bacA* chromosomal genes and bears an intact copy of *bacA* on a plasmid (pMAKbacA) whose replication is impaired at 42°C (19). The two constructed PgpB-expressing plasmids allowed growth of this mutant strain at the restrictive temperature with and without addition of IPTG in the culture medium, showing that they both expressed an active form of PgpB. Interestingly, the fact that the inducer IPTG was not required to allow complementation indicated that a basal expression level of PgpB was sufficient to ensure the supply of C55-P in vivo. Overproduction of the His6-tagged form of PgpB was then performed in the C43(DE3) strain in the conditions detailed under
Characterization of the Purified PgpB Preparation—MALDI-TOF mass spectrometry analysis of the pure PgpB protein in the presence of DDM detergent was performed using α-cyano-4-hydroxycinnamic acid as the matrix, which was previously shown to be suitable for mass spectrometry analysis of integral membrane proteins (18, 33). We obtained a sharp signal (Fig. 3B) allowing the observation of peaks with m/z ratios of 30,151, 15,084, 10,044, and 7,555, which were assigned to be the [M + H]⁺, [M + 2H]²⁺, [M + 3H]³⁺, and [M + 4H]⁴⁺ PgpB ions, respectively. The observed mass was, therefore, in perfect agreement with the form of the protein without the N-terminal methionine (calculated, 30,158 Da). The absence of any other significant peak in the spectrum further revealed that the PgpB sample was pure, and the protein was not degraded.

The PgpB preparation was further analyzed by gel filtration chromatography using a Superdex 200 HR 10/30 column previously calibrated with protein standards whose elution profiles were known to be unaffected by the presence of the DDM detergent. The PgpB-DDM complex eluted as a single, sharp, and symmetrical peak corresponding to an apparent molecular mass of 110 kDa (Fig. 3C). These data indicated that the PgpB preparation was homogeneous and monodisperse, further demonstrating that PgpB did not aggregate and existed in a single oligomeric state in the DDM detergent. For several membrane proteins with various numbers of membrane-spanning helices and different oligomeric states, the amount of bound DDM was reported to be in the range of 160–200 detergent molecules per monomer, contributing to 80–100 kDa of the total protein–detergent mass. Considering the mass of the PgpB polypeptide, ~30 kDa, these data suggest that PgpB exists as a monomer in DDM solution.

Kinetic Analysis of the C₅₅-PP Phosphatase Activity of PgpB—To test the C₅₅-PP phosphatase activity of PgpB, assays were carried out in presence of 100 μM [¹⁴C]C₅₅-PP and 0.6% DDM as described under “Experimental Procedures.” PgpB catalyzed a time-dependent hydrolysis of the C₅₅-PP β-phosphate (the distal phosphate), with a specific activity of 3.0 μmol/min/mg of protein (Fig. 4, A and B). When the reaction went to completion, 100% of the C₅₅-PP was hydrolyzed into C₅₅-P as shown by TLC analysis of the reaction mixtures. It was shown earlier that PgpB could remove consecutively both the β- and the α-phosphates from DGPP, yielding PA and diacylglycerol, respectively (24). Here we show that the product C₅₅-P was not used itself as a substrate since no formation of undecaprenol could be detected, even after prolonged incubation or in the presence of higher amounts of enzyme (Fig. 4B). These results also indicated that PgpB was not able to remove in block the pyrophosphate moiety. When the optimal reaction conditions were investigated by activity measurements at various pH values, a broad pH optimum was found between 6.5 and 7.5, and the reaction velocity was only partially decreased, by less than 50%, at the other pH values tested (pH 5–9) (Fig. 4C). The C₅₅-PP phosphatase activity was also measured in the presence of var-

“Experimental Procedures.” Accumulation in the membrane fraction of a protein of about 30 kDa was observed by SDS-PAGE analysis (Fig. 3A), a finding in agreement with the calculated molecular mass of the recombinant PgpB protein, 30,290 Da, taking into account the Arg-Ser-His₆ extension. This protein was successfully solubilized with the DDM detergent and purified as detailed under “Experimental Procedures,” yielding an apparently pure PgpB sample, as judged by SDS-PAGE (Fig. 3A).

**Characterization of E. coli C₅₅-PP Phosphatase PgpB**

![Image](image_url)
TABLE 3

| Substrate | Activity | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------|----------|-------|-----------|--------------|
| $C_{15}-PP$ | 3.0 ± 0.3 | 530 ± 90 | 9.0 ± 0.7 | 0.017 ± 0.003 |
| $C_{55}-PP$ | 300 ± 5 | 96 ± 15 | 290 ± 17 | 3.0 ± 0.5 |
| DGPP | 310 ± 8 | 80 ± 14 | 280 ± 20 | 3.5 ± 0.7 |
| PPI | 2.3 ± 0.1 | 3900 ± 500 | 48 ± 5 | 0.012 ± 0.002 |
| $C_{15}-PP$ | 1.0 ± 0.2 | 3600 ± 350 | 19 ± 1 | 0.0053 ± 0.0007 |
| PA | 6.7 ± 1.0 | 1700 ± 340 | 61 ± 8 | 0.036 ± 0.009 |
| G6P | ND | ND | ND | ND |
| PNPP | ND | ND | ND | ND |
| $C_{55}-PP$ | 20 ± 5 | 360 ± 80 | 50 ± 6 | 0.14 ± 0.03 |

* The activity was measured with 100 μM substrate. Values represent the mean ± S.D. of triplicate determinations.
* Values ± S.D. at 95% of confidence were calculated as described under "Experimental Procedures.
* The kinetic of $C_{55}$-PP dephosphorylation was measured in presence of 200 μM DGPP.

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FIGURE 4. $C_{55}^-PP$ phosphatase activity of PgpB. A, time dependence of PgpB $C_{55}^-PP$ phosphatase activity. After incubation for the indicated time intervals of 2 nmol of $[^{14}C]C_{55}^-PP$ with 1.7 pmol of purified PgpB, substrate and product were separated by TLC and quantified with the radioactive scanner. B, TLC separation of the PgpB substrate ($C_{55}^-PP$) and product ($C_{55}^-P$). C, effect of pH on the chromatogram. D, effect of $C_{55}^-PP$ on PgpB C55-PP phosphatase activity. After incubation for the indicated time (Fig. 5A and Table 3). In comparison, the apparent $K_m$ and $k_{cat}$ values (Table 3), the activities were determined as a function of the bulk concentration of the different substrates, with the molar concentration of DDM held constant (0.6%).

This analysis yielded an apparent $K_m$ value for $C_{55}^-PP$ of 530 μM and a $k_{cat}$ of 9 s$^{-1}$ (Fig. 5A and Table 3). In comparison, the apparent $K_m$ for DGPP was determined to be 80 μM, and the $k_{cat}$ was 280 s$^{-1}$ (Fig. 5B and Table 3). Both $K_m$ and $k_{cat}$ values revealed that DGPP was apparently the preferred substrate for PgpB in vivo; the enzyme efficiency ($k_{cat}/K_m$) was 206-fold greater for DGPP than for $C_{55}^-PP$. When the PA phosphatase activity of PgpB was examined, a high apparent $K_m$ value of 1.7 mM and a $k_{cat}$ value of 61 s$^{-1}$ were found, yielding an enzyme efficiency about 100-fold lower for PA than for the corresponding pyrophosphate form, viz. DGPP. Other soluble phosphomonoester molecules, glucose 6-phosphate and $p$-nitrophenyl phosphate, were tested, but none was dephosphorylated by PgpB. We next tested pyrophosphate isoprenoids, which are precursors of $C_{55}^-PP$: $C_{15}^-PP$ and $C_{15}^-PP$ (Fig. 1). The 15-carbon chain isoprenoid was apparently a good substrate for PgpB, yielding similar kinetic constants than for DGPP (Table 3). The 5-carbon isoprenoid was also dephosphorylated by PgpB; however, the enzyme efficiency was about 600-fold lower than for $C_{15}^-PP$ due in particular to a high $K_m$ value of 3.6 mM. The main difference between $C_{15}^-PP$ and $C_{55}^-PP$ is the length of the lipids, probably mimicking the physiological surface of the membrane.

PgpB Phosphatase-specific Activity—It was previously reported that upon PgpB overexpression, phosphatase activities toward DGPP, PA, lysoPA, and PgpB were all increased in cell extracts, suggesting that PgpB had a broad substrate spectrum (23, 24). It also appeared that PgpB activity toward DGPP was about 10-fold higher than toward PA (pyrophosphate versus monophosphate) (24). Here, we demonstrated that the purified PgpB could dephosphorylate the carrier lipid precursor $C_{55}^-PP$, yielding the active monophosphate form but that the latter, $C_{55}^-P$, was not used as a substrate. To elucidate the specificity of purified PgpB, different mono- and pyrophosphate molecules, either lipidic- or water-soluble, were tested as substrates. In this case the activity was measured by following the release of inorganic phosphate during catalysis ("Experimental Procedures"). To enable the calculation of apparent $K_m$ and $k_{cat}$ values (Table 3), the activities were determined as a function of the bulk concentration of the different substrates, with the molar concentration of DDM held constant (0.6%).
Figure 5. Kinetics of PgpB for C_{55}PP and DGPP substrates. The phosphatase activity of PgpB on C_{55}PP and DGPP was measured as a function of substrate concentration, as detailed under “Experimental Procedures.” The inset is the replot of 1/v versus the reciprocal of the substrate concentration. A, C_{55}PP dephosphorylation assays. B, DGPP dephosphorylation assays.

The apparently low efficiency of PgpB to dephosphorylate C_{55}PP in vitro indicated either that this activity level is insufficient to ensure C_{55}PP supply or that the cellular context influences the C_{55}PP phosphatase activity of PgpB.

Effect of Phospholipids on PgpB C_{55}PP Phosphatase Activity—Both PgpB substrates C_{55}PP and DGPP are present as minor lipids in E. coli inner membranes. In light of their respective kinetic constants, DGPP was expected to inhibit competitively C_{55}PP dephosphorylation. Therefore, we tested in vitro the effect of various concentrations of DGPP, from 20 to 800 µM, on the rate of C_{55}PP dephosphorylation (Fig. 6); in these assays [^{14}C]C_{55}PP was held constant at 100 µM and the DDM detergent at 0.6%. To our surprise, the addition of DGPP, up to 400 µM, did not result in enzyme inhibition but resulted in a dose-dependent stimulation of C_{55}PP phosphatase activity of PgpB; above 400 µM, the stimulation effect reached a plateau value representing ~400% of the control C_{55}PP phosphatase activity. To further examine the mechanism of DGPP activation, we determined the apparent kinetic constants of PgpB toward C_{55}PP in the presence of 200 µM DGPP. This analysis yielded apparent K_m and k_cat values of 360 µM and 50 s^{-1}, which are ~1.5- and 5.6-fold greater than those obtained in the absence of DGPP, respectively (Table 3).

DGPP stimulation of PgpB C_{55}PP phosphatase activity raised several question; (i) does DGPP have a specific regulator effect or does it act as any other phospholipid?, (ii) does it activate PgpB directly?, or (iii) does it influence the recognition of C_{55}PP by PgpB? We then examined the effect of the three major E. coli phospholipids; that is, PG, PE, and cardiolipin on PgpB C_{55}PP phosphatase activity. Assays were performed in a mixed phospholipids/DDM/PgpB micelle model; the lipids were added at different concentrations (from 125 µM to 1 mM), and DDM and [^{14}C]C_{55}PP concentrations were fixed at 0.6% and 100 µM, respectively. All three phospholipids exerted a dose-dependent stimulatory effect on PgpB C_{55}PP phosphatase activity, comparable to DGPP, except that we did not observe a saturation phase at high lipid concentrations (Fig. 6). The most potent stimulation was obtained with the two anionic phospholipids cardiolipin and PG, with the presence of 1 mM concentrations of these lipids increasing the enzyme activity by 5.4- and 5.2-fold, respectively. At the same concentration, the zwitterionic phospholipid PE increased the activity by 3.4-fold.

To test whether the phospholipids activated PgpB activity per se, we next tested the effect of the three lipids on the PgpB activity using this time C_{15}PP as substrate. No stimulatory effect was observed in this case. In contrary, the presence of high concentrations of PG and PE significantly decreased the PgpB activity as compared with the control, an effect that could be due to the dilution of C_{15}PP at the micellar surface (Fig. 6). We also examined the PgpB activity toward the C_{35}PP pyrophosphate isoprenoid in the presence or not of phospholipids. In the absence of phospholipids, PgpB dephosphorylated C_{35}PP as efficiently as C_{55}PP (data not shown). The phospholipids cardiolipin and PG exerted a stimulatory effect that was about twice less pronounced with C_{35}PP than with C_{55}PP, and PE had no significant effect on C_{35}PP dephosphorylation (Fig. 6). In conclusion, our data show that the phospholipids are important determinants for the PgpB C_{55}PP phosphatase
activity that do not seem to activate the enzyme activity per se as judged from C15-PP dephosphorylation assays. We can, therefore, hypothesize that they should be involved in long chain isoprenoids arrangement within the mixed micelles, rendering C55-PP a better substrate for PgpB. The fact that DGPP did not inhibit C55-PP dephosphorylation also suggests that C55-PP is a preferred substrate for PgpB in the mixed phospholipids/detergent model. A model membrane is now required for proper determination of kinetic parameters for C55-PP toward PgpB; for that purpose PgpB reconstitution in model membranes is under way.

PgpB Topology Determination—C55-PP is de novo synthesized by the UppS synthase in the cytosol, where it must partition into the inner layer of the membrane, but it is also released on the outer side of the membrane after transfer of the oligosaccharide moieties to the growing polymers. In both cases C55-PP must be dephosphorylated to supply the active form of the carrier lipid. It is still unclear on what side of the inner membrane dephosphorylation of C55-PP occurs. Different programs predicted that PgpB should contain six transmembrane segments. A topology analysis was carried out by fusion experiments between various truncated forms of PgpB (at the 3' end of the gene) and the β-lactamase. The β-lactamase breaks down ampicillin, whose targets are the penicillin-binding proteins located in the periplasm. Therefore, if β-lactamase is fused to a point in PgpB that is located in the periplasm, ampicillin is hydrolyzed, and the cells become ampicillin-resistant. Conversely, if the β-lactamase is fused to a point in PgpB that is cytoplasmic, cells remain ampicillin-sensitive (26, 34). Eleven fusion sites were chosen to be located all along PgpB at approximately every 20 residues (approximately the average size of a transmembrane) (Fig. 7). Fusions were constructed by PCR amplification of truncated forms of the pgpB gene, which were cloned in-frame with the β-lactamase blaM gene in plasmid vector pNF150, yielding different fused pgpB-blaM recombinant genes whose expression was under the control of the IPTG-inducible lac promoter (“Experimental Procedures”).

The expression of the hybrids proteins in E. coli DH5α cells was highlighted by their ability to grow when patched (patch screening) onto 2YT agar plates containing 10 μg/ml ampicillin and 1 mM IPTG. Under these conditions, ampicillin resistance should be independent of the localization of the β-lactamase moiety, since after limited cells lysis, β-lactamase is released in the milieu leading to a hydrolysis of ampicillin that allows neighboring cells to grow (34). All the fusions conferred resistance in patch screening, clearly indicating that they all express PgpB-BlaM hybrids. To assess the location of the β-lactamase moiety of the recombinant proteins, ampicillin resistance conferred by each fusion was then tested for single cells (single cells screening) by plating appropriate dilutions of exponential cell cultures on 2YT agar plates containing 10 μg/ml ampicillin and 1 mM IPTG. Under these conditions, the fusions at amino acids Lys39, Lys97, Glu120, Glu140, Pro160, and Arg210 conferred ampicillin resistance, and their minimal inhibitory concentration (MIC) for ampicillin was further determined to be over 100 μg/ml, except for the R210 recombinant protein, whose MIC was estimated at 60 μg/ml (Table 2). The latter fusions conferring ampicillin resistance were considered to have the β-lactamase moiety, and consequently the PgpB junction sites to which β-lactamase had been fused, exposed in the periplasm (Fig. 7). The fusions at amino acids Gly11, Lys73, Thr184, Pro236, and Ser254 did not confer ampicillin resistance and, thus, were considered to have the β-lactamase moiety in the cytoplasm (Table 2). From the ampicillin resistance data, a six-transmembrane topological model of E. coli PgpB was obtained, which was in agreement with the predicted one (Fig. 7). These data further indicated that the PgpB PAP2 conserved motifs, C1, C2, and C3 had a periplasmic localization.

**DISCUSSION**

C55-P is a key lipid in bacterial metabolism that is shared by different pathways leading to the formation of various cell wall components, in particular peptidoglycan, whose inhibition of synthesis causes rapid cell lysis. A cis-prenyl pyrophosphate synthase (UppS) catalyzes the formation of its precursor, C55-PP, by successive additions of C5-PP units onto C15-PP; this enzyme has been biochemically and structurally characterized (13, 14, 35–37). The subsequent step consists of the dephosphorylation of C55-PP. Recently, two different classes of integral membrane proteins that can catalyze this reaction have been identified in E. coli; that is, the BacA enzyme on the one hand and several members of the PAP2 phosphatase family on
the other hand, PgpB, YbjG, and YeiU/LpxT (18–20). The simultaneous inactivation of at least PgpB, YbjG, and BacA provoked cell lysis through disruption of C₅₅-P metabolism. Therefore, at least these three proteins were involved in C₅₅-P synthesis in *E. coli*. Previously, PgpB was also shown to exhibit phosphatase activity toward DGPP, PA, PGP, and lysoPA, raising the question of the specific role of this protein (23, 24). In this study the PgpB protein was overproduced, extracted from membranes, and purified to homogeneity, and its ability to dephosphorylate different monophosphate and pyrophosphate molecules was examined. We showed that PgpB was able to dephosphorylate with similarly high efficiencies two very different pyrophosphate lipids, phospholipid DGPP and isoprenoid C₁₅-PP, which only have in common their pyrophosphate head group, providing strong evidence that PgpB has a relatively low specificity toward lipid pyrophosphate substrates. In contrast, we showed that PgpB had a relatively low efficiency toward lipid monophosphate (PA) and water-soluble pyrophosphate compounds (inorganic pyrophosphate, C₅-P), whereas it has no activity on water-soluble monophosphate molecules (glucose 6-phosphate and p-nitrophenyl phosphate). To our surprise, C₅₅-PP primarily appeared as a relatively poor substrate as judged from kinetic constants measured in DDM/PgpB mixed micelles. We then demonstrated that the presence of phospholipids was required to elicit the maximal turnover for C₅₅-PP dephosphorylation. In contrast, PgpB dephosphorylation of the shorter acyl chain isoprenoid C₁₅-PP was not stimulated by phospholipids. These data suggested that PgpB activity and/or structure was not regulated by lipid effectors per se, whereas binding and/or dephosphorylation of C₅₅-PP by PgpB was specifically enhanced by phospholipids. Previously, NMR studies demonstrated that isoprenoids can modulate membrane bilayer structure through direct interaction with phospholipids via hydrophobic contacts, and molecular modeling calculations suggested that these long axis lipids may adopt very atypical conformation in membranes, the phosphorus atoms being anchored near the aqueous interface of the bilayer (38–40). In detergent/PgpB-mixed micelles, C₅₅-PP may adopt an unusual or “non-native” conformation or orientation, which could prevent the head group, which is the reactive site of the C₅₅-PP, from being correctly positioned with respect to the enzyme active site. The addition of phospholipids in the mixed micelles may mimic membrane bilayers and favor C₅₅-PP binding and/or dephosphorylation by PgpB. The effect of phospholipids on the dephosphorylation of the C₅₅-PP isoprenoid by PgpB was less pronounced than that measured with C₅-PP, suggesting that a definite size of the isoprenoid is the determinant. It is likely that the length, geometry, and membrane orientation of isoprenoids in membrane bilayers are relevant to their function.

PgpB, LpxT, and YbjG belong to the PAP2 phosphatase superfamily from the Pfam data base (PF01569), whose members are largely widespread among all kingdoms and are characterized by a conserved signature in which three distinct motifs are visible, designated C1, C2, and C3 (Fig. 2). The crystal structure of one soluble PAP2 protein, the acid phosphatase

![Proposed topological model of *E. coli* PgpB protein. Red stars indicate the positions of the different PgpB-BlaM fusion points that have been constructed in this study. The residues indicated by blue, violet, and green circles are the signature residues found in the PAP2 family: C1, C2, and C3 motifs, respectively.](image)

**FIGURE 7.** Proposed topological model of *E. coli* PgpB protein. Red stars indicate the positions of the different PgpB-BlaM fusion points that have been constructed in this study. The residues indicated by blue, violet, and green circles are the signature residues found in the PAP2 family: C1, C2, and C3 motifs, respectively.
Characterization of E. coli C$_{55}$-PP Phosphatase PgpB

from *Escherichia blattae* (EB-NSAP), has been solved in complex with molybdate, showing that the active site is effectively constituted by the three motifs (Fig. 8A) (41). The membrane topology of PgpB determined in the present work is consistent with a similar organization since the three motifs are localized on the same side with respect to the inner membrane. From the EB-NSAP structure, it was proposed that a catalytic triad mediating the nucleophilic attack on the phosphate ester bond constituted of residues C3 histidine, C2 histidine, and C3 aspartate (41). This "catalytic triad" is conserved in all three *E. coli* phosphatases (Fig. 2), suggesting that the catalytic mechanism is also conserved in the membrane homologs. Interestingly, the other signature residues that were assumed from the EB-NSAP structure to be important for attraction of the negatively charged phosphate group and/or for stabilization of the transition state are in red, gray, and pink, respectively. The catalytic triad residues and the molybdate ion are shown in lines models; molybdenum, carbon, nitrogen, and oxygen atoms are in blue, green, dark blue, and red, respectively. B, a simplified representation of the topological model of *E. coli* PgpB protein is shown for comparison.

Strates. C$_{55}$-PP may interact with the enzyme after lateral diffusion in the membrane, so that the pyrophosphate group should dock directly into the active site. After hydrolysis, the C$_{55}$-P product should diffuse away in the membrane plan, freeing the active site for another cycle of dephosphorylation.

C$_{55}$-PP is released at the inner side of the membrane during *de novo* synthesis but also at the outer side after the transfer of the glycosidic moieties to the acceptor polymers, raising the question of the localization of the dephosphorylation reaction. Here, we show that the PgpB active site faces the periplasm. Recently, Tatar et al. (42) demonstrated that the signature residues of LpxT and YbjG were oriented toward the periplasmic space. However, the localization of the BacA active site remains still unknown. We could speculate, considering the periplasmic localization of the active site of the PAP2 phosphatases, that these enzymes participate exclusively in C$_{55}$-PP recycling. However, the fact that only one chromosomal copy of either *bacA*, *ybjG*, or *pgpB* gene was sufficient for C$_{55}$-P supply and cell viability strongly suggests that either of the three proteins can ensure dephosphorylation of *de novo* synthesized C$_{55}$-PP. Therefore, in the presence of only one PAP2 C$_{55}$-PP phosphatase, C$_{55}$-PP and C$_{55}$-P must be efficiently translocated across the inner membrane to reach the sites of dephosphorylation and glycosylation, respectively. This is supported by the fact that a high rate of transbilayer movement of C$_{55}$-P was observed in *Micrococcus lysodeikticus* (43, 44), which has to match the high rate of peptidoglycan synthesis (45). It was hypothesized that the long chain of the carrier lipid enables spontaneous diffusion of the latter across membranes (46). However, a simple diffusion could not account for the observed rate of trans-bilayer passage, suggesting the existence of an essential membrane machinery responsible for the transport of the lipid carrier across the membrane. Whether C$_{55}$-PP-linked substrates are translocated through the membrane by the same mechanism remains to be determined. It is well established that translocation of C$_{55}$-PP-linked substrates requires specific flippases/translocators (O-antigen biosynthesis, some types of bacterial protein glycosylation) (47–49), but in the case of peptidoglycan synthesis this translocator is not known (50).

The assembly of glycans intermediates onto a polyphosphoryl phosphate carrier lipid is a universal process (46, 51). In eukaryotic cells, the dolichyl phosphate (C$_{75}$ to C$_{100}$) is used for the biogenesis of glycoproteins (52). In this case, the glycan chains are linked to the carrier lipid on the cytoplasmic side of the endoplasmic reticulum membrane; thereafter, the complexes are translocated toward the luminal side where the glycan chains are transferred to selected asparagine residues of target.

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T. Touzé, D. Blanot, and D. Mengin-Lecreux, unpublished data.
proteins. Comparable to what is observed in bacterial cells, dolichyl pyrophosphate is released at the luminal side after the transfer of the glycans to the acceptor molecules and then is recycled. In Saccharomyces cerevisiae, the CwH8 membrane protein with a luminal-oriented active site catalyzes the dephosphorylation of the so-formed dolichyl pyrophosphate to yield the active form of this carrier lipid (53). The fact that the ΔcwH8 yeast mutant is deficient in protein N-glycosylation suggests that the recycling contributes significantly to the pool of dolichyl phosphate available for synthesis of the lipid intermediates. Homologues of the CwH8 protein have since been found in mammalian cells, where they likely ensure the same function (54). It was recently shown that the luminal-formed dolichyl phosphate was translocated back to the cytoplasmic leaflet where it can be reused (55). However, the mechanism by which this translocation occurs is yet to be established as this is the case in bacteria. These observations provide strong evidence that procaryotic and eucaryotic cells utilize a highly conserved mechanism to recycle dolichyl phosphate available for synthesis of the lipid intermediates.

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