Crystal structure of lipid phosphatase *Escherichia coli* phosphatidylglycerophosphate phosphatase B

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Membrane-integrated type II phosphatidic acid phosphatases (PAP2s) are important for numerous bacterial to human biological processes, including glucose transport, lipid metabolism, and signaling. *Escherichia coli* phosphatidylglycerol-phosphate phosphatase B (ecPgpB) catalyzes removing the terminal phosphate group from a lipid carrier, undecaprenyl pyrophosphate, and is essential for transport of many hydrophilic small molecules across the membrane. We determined the crystal structure of ecPgpB at a resolution of 3.2 Å. This structure shares a similar folding topology and a nearly identical active site with soluble PAP2 enzymes. However, the substrate binding mechanism appears to be fundamentally different from that in soluble PAP2 enzymes. In ecPgpB, the potential substrate entrance to the active site is located in a cleft formed by a V-shaped transmembrane helix pair, allowing lateral movement of the lipid substrate entering the active site from the membrane lipid bilayer. Activity assays of point mutations confirmed the importance of the catalytic residues and potential residues involved in phosphate binding. The structure also suggests an induced-fit mechanism for the substrate binding. The 3D structure of ecPgpB serves as a prototype to study eukaryotic PAP2 enzymes, including human glucose-6-phosphatase, a key enzyme in the homeostatic regulation of blood glucose concentrations.

Type II phosphatidic acid phosphatases (PAP2s) are a large family of phosphatases important for lipid metabolism and signaling (1, 2). PAP2 proteins have been found in all life kingdoms from bacteria to mammals. They catalyze dephosphorylation of broad substrates by specifically hydrolyzing phosphoric monoester bonds. Their substrates include variety of phosphorylated carbohydrates, peptides, and lipids. PAP2s are involved in vesicular trafficking, secretion, and endocytosis (e.g., the enzyme phosphatidate phosphatase APP1 in yeast) (3); protein glycosylation [e.g., dolichyl pyrophosphate phosphatase 1 (DOLPP1) in the mouse] (4); energy storage (e.g., triacylglycerol biosynthesis) (5); and stress response (6). In contrast to type I PAP enzymes, which are Mg^{2+} -dependent and usually soluble, PAP2 proteins are Mg^{2+} -independent, and many of PAP2 enzymes are integral transmembrane (TM) proteins (7). Whereas the soluble branch of PAP2s is called class A nonspecific acid phosphatases (NSAPs) (8), the TM branch of the PAP2 family is also called the lipid phosphatase/phosphotransferase family (2) or lipid phosphate phosphatase family (9). Human glucose-6-phosphatase (G6Pase), the key enzyme in the homeostatic regulation of blood glucose concentrations, belongs to the TM PAP2 subfamily (10). Thus, the TM property is unique to the PAP2 family.

In *Escherichia coli*, undecaprenyl phosphate (C_{55} -P), a 55-carbon single-lipid chain phospholipid, serves as a carrier lipid to transfer a variety of phosphate-linked polymers across the periplasmic membrane from the cytosol to the periplasmic space. Recently, the crystal structure of phospho-*N*-acetylmuramic acid-pentapeptide translocase (MraY) from *Aquifex aeolicus*, which catalyzes the transfer of substrates to C_{55} -P, was reported (11). For this process to be sustainable, the transport intermediate, undecaprenyl pyrophosphate, needs to be dephosphorylated on

the periplasmic side by a phosphatase, for example, phosphatidyl-glycero-phosphatase B (PgpB, EC 3.1.3.27) (12), which may also be involved in the biogenesis of phosphatidylglycerol from phosphatidylglycerol phosphate (13). Similar dephosphorylation mechanisms of carrier lipids (e.g., dolichyl pyrophosphates) for glycan transport have been observed in yeast (14) and mammalian cells (4), and the corresponding PAP2 phosphatases (Cwh8 and DOLPP1, respectively) have been found to be located in the endoplasmic reticulum and are essential for luminal *N*-glycosylation of newly synthesized proteins in eukaryotic cells. Therefore, PAP2-facilitated recycling of carrier lipids is of fundamental interest to cell biology.

Based on amino acid sequence analysis, members of the PAP2 family share a signature sequence “ $KX_6RPX_{12-54}PSGHX_{31-54}SRX_5HX_3D$,” which is often divided into three motifs: C1, “ KX_6RPF ”; C2, “ $PSGH$ ”; and C3, “ SRX_5HX_3D ” (1, 2) (Fig. S1). The PAP2 family also includes one group of haloperoxidases [e.g., vanadium-dependent chloroperoxidase (CPO)] (1). The CPO of the fungus *Curvularia inaequalis* (ciCPO) has also been shown to possess phosphatase activity (15). Crystal structures of soluble ciCPO [Protein Data Bank (PDB) ID code 1VNC] (16) and NSAP from *Escherichia blattae* (ebNSAP; PDB ID code 1D2T) (17) from the PAP2 family have been reported. These 3D structures share a similar folding topology for a core helix bundle, with residues from the signature sequence residing on the same end of the helix bundle. Structural variations may occur as insertions in the connecting loops and even within an essential

Significance

Phosphatases regulate many aspects of cellular function-homeostasis and signal transduction. Using X-ray crystallography methods, we determined the structure of phosphatidylglycerol-phosphate phosphatase B (PgpB) from *Escherichia coli*, a member of the type II phosphatidic acid phosphatase (PAP2) family and a homologue of human glucose-6-phosphatase, which plays a variety of physiopathological roles. Our structure of PgpB showed that the membrane-integrated and soluble members of the PAP2 family share the same catalytic mechanism. The mechanism of recognition of lipid substrates was postulated based on analyses of enzymatic activities and thermal stabilities of PgpB variants. This work presents an important structural model for studying eukaryotic PAP2s.

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The authors declare no conflict of interest.

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Data deposition: Coordinates of the crystal structure of *Escherichia coli* phosphatidylglycerol-phosphate phosphatase B have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4PX7).

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identity). For instance, the α 1-helices and TMs 4, 5, and 6 of ecPgpB showed a 1.4-Å rmsd with ebNSAP (PDB ID code 1EOI) (17) for ~60 C α atom pairs, including most of the signature motifs except the N-terminal region of the C1 motif (see below). On the other hand, TMs 1, 2, and 3 and most of the periplasmic domain significantly deviate from the corresponding structural elements of the soluble counterparts in ebNSAP/1EOI (Fig. 2). These differences are partially due to the structure requirement of membrane proteins [e.g., the length of TM helices and the positive-inside rule (19)], and may also be required by the lipid substrates. Similarly, the crystal structure of soluble ciCPO (PDB ID code 1VNC) shares a homologous core structure around the active site with ecPgpB as well as ebNSAP (Fig. 2). Nevertheless, ciCPO has an extra N-terminal region of over 230 amino acid residues compared with ecPgpB, and this extra structural region essentially blocks the surface corresponding to the lipid substrate entrance in ecPgpB (i.e., the front side in Fig. 1A). In addition, ciCPO contains a 48-residue insertion inside the helix corresponding to TM6 of ecPgpB and C-terminal to the conserved motif C3 (i.e., the back side in Fig. 1A). Between TMs 4 and 5, ciCPO contains another insertion of *ca.* 50 amino acid residues (i.e., the bottom). Thus, the conserved core of TMs 4–6 is essential for the active site formation of PAP2 enzymes.

The overall sequence homology for TM PAP2 proteins is usually low (*ca.* 10–15% identity). However, based on sequence analyses and prediction of locations of TM helices, all known TM PAP2 proteins are likely to share the same folding topology with ecPgpB, assuming that the three conserved motifs, C1–C3, have similar locations in their 3D structures (Fig. S1A). Human glucose-6 phosphatase possesses an additional four TM helices C-terminal to the “canonical” six-TM helix topology. Therefore, our crystal structure of ecPgpB strongly supports the notion that PAP2 proteins, both soluble and TM forms, are evolutionarily related.

Conserved Active Site in ecPgpB. To verify that our recombinant protein of ecPgpB was functional, we performed an *in vitro* kinetic analysis of the dephosphorylation catalysis of WT ecPgpB toward 1,2-dioleoyl-*sn*-glycero-3-phosphate [PA (18:1)] using detergent-solubilized protein (Fig. 3A). The results showed that the k_{cat} was 24 ($\pm$ 2) s^{−1} and the K_m was 0.30 ($\pm$ 0.09) mM, which are roughly comparable with previously reported data (k_{cat} = 61 s^{−1} and K_m = 1.7 mM) (12). This catalytic activity is believed to be associated with the signature motifs of the PAP2 family (1). In the crystal structure of ecPgpB, the C1 and C2 motifs are located at the

two ends of the periplasmic insertion connecting TMs 3 and 4 (Fig. 2B). The C3 motif forms the region from the C-terminal end of TM5 to the N-terminal end of TM6. In the following, we refer to a residue from a given motif by its motif number (1, 2, or 3), combined with the position number in that motif, for the convenience of structural comparison between different PAP2 enzymes. For example, the catalytic His163 of ecPgpB is located at the fourth position in the C2 motif and is referred to as His163^(2.04). Catalytic residues His163^(2.04) and His207^(3.08), and most other residues essential for the activity, are located near the putative solvent–membrane interface, and both their main-chain and side-chain atoms are able to be superimposed with those in the crystal structure of ebNSAP/1EOI (Fig. 2B). An exception is the conserved Lys97^(1.01), which may be involved in an induced-fit mechanism. Therefore, ecPgpB is likely to share a similar phosphate hydrolysis mechanism with both ebNSAP and ciCPO.

According to the established two-step catalytic mechanism of soluble PAP2 enzymes, of the residues critical to catalysis, His207^(3.08) and Asp211^(3.12) of ecPgpB form a charge–relay pair and are responsible for nucleophilic attacking and formation of a phosphate/enzyme intermediate in the first step of the reaction (20). His163^(2.04) stabilizes the transition state intermediate and catalyzes the cleavage of the terminal PO₄ group from the substrate (18). Mutations at the equivalent position of His119^(2.04) in human G6Pase to any other amino acid residue have been shown to abolish its enzyme activity (21). In ecPgpB, conserved Arg104^(1.08) interacts with the catalytic His207^(3.08) and potentially with the substrate. Mutations at the equivalent position of Arg83^(1.08) in human G6Pase (alignment is shown in Fig. S1A) to any other amino acid residue, including Lys, also abolished enzyme activity (21). In particular, mutation of R83C has been directly related to glycogen storage disease type 1a (10, 22). Similarly, in ecPgpB, Arg201^(3.02) interacts with His163^(2.04). The N-terminal end of TM4 also potentially contributes to binding of the PO₄ group in a charge–dipole interaction. In particular, lack of the side chain of Gly162^(2.03) at the N-terminal end of TM4 favors binding of the substrate PO₄ group. In agreement, active site mutations H163^(2.04)A, H207^(3.08)A, D211^(3.12)E, R104^(1.08)A, and R201^(3.02)A of ecPgpB showed only background level activity toward the substrates (Fig. S3B) dioleoylglycerol pyrophosphate [DGPP (18:1)], dioctanoylglycerol pyrophosphate [DGPP (8:0)], 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate [LPA (18:1)], and PA (18:1), similar to negative controls (Fig. 3B). These results confirmed the catalytic roles of the corresponding residues in the WT ecPgpB.

Fig. 2. Structural comparison of ecPgpB with soluble PAP2 enzymes. (A) Superposition of the core helices of ecPgpB (blue) with ebNSAP/1EOI (wheat) and ciCPO/1VNC (cyan, with large insertions omitted). Backbones of the core helices and signature motifs are shown in ribbons, and the remaining parts are shown in thin lines. A molybdate group in the active site from the ebNSAP crystal structure is shown in a sphere model. (B) Superposition of the active site of ecPgpB with ebNSAP/1EOI. Conserved residues in signature motifs are labeled. Residue numbers of ebNSAP are shown in parentheses. The figures were generated with the program PyMol.

Fig. 3. Activity measurements. (A) Enzyme activity of recombination WT ecPgpB. The protein concentration was 0.1 μ M, and the reaction time was set to 1 min. (B) Phosphatase activity of variants of ecPgpB with different substrates. The substrates used were DGPP (18:1) (blue), DGPP (8:0) (red), LPA (18:1) (green), and PA (18:1) (purple). Because most mutations showed only background activity, the reaction time was set to 10 min. Absorbance at 795 nm ($A_{795 \text{ nm}}$) was reported as a measurement of the relative activity. Multiple experiments were performed (*Methods*), and the average results and SDs are shown.

In thermofluor assays, most purified variants showed comparable melting temperatures (Tms) with that of the WT Tm in the absence of substrate, ranging between 39 °C and 48 °C (Table S2). Interestingly, only the WT protein, but not its active site mutant forms H163^(2.04)A, H207^(3.08)A, and D211^(3.12)E variants, was further stabilized by a transition state analog, molybdate (Na_2MoO_4) (Table S2), whereas Na_2SO_4 showed no effect and NaH_2PO_4 destabilized all variants slightly. In particular, in the presence of 10 mM Na_2MoO_4 , the Tm of WT ecPgpB increased from 43 °C to 55 °C. This observation is in agreement with the previously reported crystal structure of the ebNSAP–Mo complex (PDB ID code 1EOI), showing covalent bonding of the MoO_4^{2-} group with His189^(3.08) (17). Moreover, the H207^(3.08)A variant of ecPgpB, which is presumably not able to form the phosphate/enzyme intermediate, could be stabilized by additional substrate [1 mM PA (18:1)] with a melting temperature change (ΔTm) of approximately +12 °C (Fig. S34), whereas other variants, as well as the WT, displayed a smaller change of thermal stability (ΔTm of ~ 5 °C) in response to substrate binding. These observations suggest that although most of the residues within the conserved active site contribute to the substrate loading, His207^(3.08) is more important for catalysis in ecPgpB.

Cleft of Substrate Entrance Is Located in the Membrane–Solvent Interface of PgpB. One of the fundamental questions about TM PAP2 enzymes is about the entrance for their lipid substrates. As mentioned above, the main structural differences between soluble

PAP2 enzymes and ecPgpB include TMs 1–3, which surround the core formed by TMs 4–6 and the periplasmic insertion between TMs 3 and 4. In particular, we observed a V-shaped TM helix pair (TMs 2 and 3) with its periplasmic opening close to the active site (Fig. 44). This TM helix pair forms a surface cleft, presumably allowing membrane-associated lipid substrates to enter the active site. With such an accessing mode, a lipid substrate would insert its phosphate head into the bottom of the active pocket and toward the nucleophilic residue His207^(3.08) to form the phosphate–enzyme complex and would allow His163^(2.04) to complete the second step of dephosphorylation by recruiting a water molecule from the solvent-accessible side of the active site. To test this hypothesis, we constructed a few more point mutations at the putative entrance cleft of this substrate binding pocket (Fig. 44). Most of the variants in this group (e.g., V54F, F61W, V88F, Q90A, K93E) showed reduced activity to some extent (Fig. 4B). As a control, Leu58 is located outside of the V-shaped opening, and the H57F and L58F variants behave essentially like WT. In contrast, the A83F variant was predicted to block the opening from the TM3 side, and it did indeed exhibit significantly reduced activity. However, this variant remained capable of binding molybdate (Table S2), suggesting that the reduction in activity is the result of weaker binding of the lipid chain(s) of the substrate. Similarly, the G89L variant lost nearly all activity toward all four substrates tested. Therefore, we conclude that the opening of the V-shaped cleft that faces the outer leaflet of the lipid bilayer is the substrate entrance of ecPgpB. The wide cleft formed by the

Fig. 4. Putative substrate entrance. (A) V-shaped substrate entrance. The main body of the enzyme is shown as a molecular surface model. The V-shaped TM helix pair, TMs 2 and 3, are shown as cylinders. Putative movements of the substrate and TM3 are indicated as open arrows. Selected residues that were mutated to define the substrate entrance are shown as sphere models. The positions of those residues that showed an effect on enzyme activity are highlighted in yellow; otherwise, they are wheat-colored. (B) Relative activities of the variants in the putative substrate entrance. The reaction time was set to 1 min to compare initial reaction speeds of the variants. All substrate concentrations were 1 mM, and all protein concentrations were 0.1 μ M.

V-shaped TM helix pair may explain the promiscuous substrate preference of ecPgpB. Similar loading mechanisms of lipid substrates have been proposed for several membrane-integrated enzymes (23, 24).

Compared with known structures of soluble PAP2 proteins, our crystal structure appears to represent an apo-form of ecPgpB. Specifically, TM3 packed loosely with the rest of the protein (Fig. 1A). As a consequence, the conserved Lys97^(1.01) residue is located *ca.* 9 Å away from its putative active position near the C2 motif at the N-terminal end of TM4. Although we cannot rule out the possibility that the observed Lys97^(1.01) conformation is a crystallization artifact, the region surrounding this residue is not involved in crystal packing. Thus, we attempt to postulate that Lys97^(1.01) of ecPgpB must move from its current position in the crystal structure to complete the formation of the active site and to interact directly with the substrate PO₄ group. Such a movement of Lys97^(1.01) is likely to be driven by both substrate binding to the V-shaped cleft and a subsequent movement of TM3 toward TM2. This hypothesis of conformational change upon induction by a substrate is supported by the following observations: (i) Mutation K97^(1.01)A lost its ability to bind the transition-state analog MoO₄²⁻ (Table S2), which suggests that the side-chain tip of Lys97^(1.01) in ecPgpB directly interacts with MoO₄²⁻; (ii) K97^(1.01)A lost the enzyme activity (Fig. 3B), suggesting that the conformation of the canonical active site observed in soluble enzymes also functions in ecPgpB; and (iii) the variant G89L, which was designed to block the movement of TM3 toward TM2

without disturbing the conformation observed in the crystal structure, behaved in the same way as the Lys97^(1.01)A variant in terms of changes in thermal stability and activity (Fig. 4 and Table S2). A potential advantage of such an induced-fit mechanism is to restrict the enzyme activity to properly bound lipid substrates only.

Methods

The gene of full-length PgpB was cloned from the *E. coli* BL21 genome. Recombinant PgpB protein was expressed in *E. coli* and purified in a detergent combination of nonyl-β-D-glucopyranoside (Jiejing Tech, Inc.) and n-dodecyl-N,N-dimethylamine-N-oxide (Anatrace). The protein sample was crystallized using the vapor diffusion method. Initial phases of the structure factors were determined using the selenium-based SAD method, and the structure model was refined at 3.2 Å (Fig. S4). Thermal stability of PgpB variants was analyzed using a count per second-based thermofluor method, and activities of the PgpB variants were analyzed using a phosphor-molybdic acid colorimetric method. More information on materials and methods can be found in *SI Methods*.

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