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A two-helix motif positions the active site of lysophosphatidic acid acyltransferase for catalysis within the membrane bilayer

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Abstract

Phosphatidic acid is the central intermediate in membrane phospholipid synthesis and is generated by two acyltransferases in a pathway conserved in all life forms. The second step in this pathway is catalyzed by 1-acyl-*sn*-glycero-3-phosphate acyltransferase, called PlsC in bacteria. The crystal structure of PlsC from *Thermotoga maritima* reveals an unusual hydrophobic/aromatic N-terminal two-helix motif linked to an acyltransferase $\alpha\beta$ domain that contains the catalytic HX₄D motif. PlsC dictates the acyl chain composition of the 2-position of phospholipids, and the acyl chain selectivity 'ruler' is an appropriately placed and closed hydrophobic tunnel. This was confirmed by site-directed mutagenesis and membrane composition analysis of *Escherichia coli* cells expressing the mutated proteins. MD simulations reveal that the two-helix motif represents a novel substructure that firmly anchors the protein to one leaflet of the membrane. This binding mode allows the PlsC active site to acylate lysophospholipids within the membrane bilayer using soluble acyl donors.

The positional asymmetry of fatty acids in bacterial glycerophospholipids is governed by a universally conserved two step pathway for the sequential acylation of *sn*-glycerol-3-phosphate (G3P) to produce the central intermediate phosphatidic acid (PA) (Fig. 1a)^{1–4}. The first acylation step creates 1-acyl-glycero-3-phosphate (LPA) and is carried out by G3P acyltransferase (GPAT), which is called PlsB in bacteria. The second acylation step is catalyzed by 1-acyl-*sn*-glycero-3-phosphate acyltransferase (LPAAT), which is called PlsC in bacteria. The crystal structure of a plant GPAT has revealed an acyltransferase HX₄D active site that resides within a single $\alpha\beta$ domain^{5,6}. Recently, the same $\alpha\beta$ domain was

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AUTHOR CONTRIBUTIONS

R.M.R., J.Y., C.O.R. and S.W.W. designed the studies. R.M.R., S.G. and S.W.W. determined and interpreted the crystal structures. J.Y. and C.O.R. performed and analyzed the biochemical experiments. G.K. performed the modeling and docking studies. E.W.M. performed the molecular dynamics simulations. All authors contributed to and approved the manuscript.

COMPETING FINANCIAL INTERESTS

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identified in PatA, an interfacial acyltransferase that acylates a glycolipid mannose residue protruding from the mycobacterial membrane⁷. GPAT and PatA are soluble enzymes that use soluble acyl donors to acylate substrates in the aqueous phase. In contrast, LPAAT is an integral membrane enzyme that allows it to access its lysophospholipid LPA substrate that resides within the phospholipid bilayer. The LPAAT enzymes belong to the evolutionarily conserved lysophospholipid acyltransferase (AGPAT) family of intrinsic membrane proteins. This large family is not only responsible for phospholipid synthesis, but also for phospholipid re-modeling^{4,8,9}. In humans, mutations in AGPAT family members are associated with a number of diseases^{10,11}. Sequence analyses of AGPAT family members suggest that they also adopt the acyltransferase $\alpha\beta$ protein fold. Specifically, they contain four sequence motifs that are conserved in all GPAT structures, which includes the HX₄D motif at the active site^{10,12}.

The AGPAT family must all solve the topological problem of accessing and processing substrates in the membrane interior. Two structural solutions to this problem have been described. Soluble, interfacial enzymes, like phospholipase A₂ and diacylglycerol kinase (DgkB), bind to the polar surface of the membrane bilayer and create a hydrophobic diffusion pathway to 'extract' the lipid substrate from the bilayer into the active site^{13–15}. In contrast, intrinsic membrane proteins, like site 2 protease¹⁶, MraY¹⁷ and liponucleotide synthetase¹⁸ position their active sites along trans-membrane helices at the correct membrane depth to effect catalysis on the membrane-embedded substrate. It has always been assumed that the AGPAT enzymes use the latter mechanism, but trans-membrane α -helical predictions have not yielded a clear result and have produced topological models with either two¹⁹ or four membrane helices²⁰. Moreover, these models place the four conserved motifs on opposite sides of the membrane and are therefore inconsistent with a single acyltransferase $\alpha\beta$ protein fold. Finally, these models provide no obvious explanation for how the AGPAT enzymes can simultaneously access membrane-bound and soluble substrates and bring them to the active site.

To answer these questions, we have determined the crystal structure of PlsC from the thermophilic bacterium *Thermotoga maritima*. The structure reveals that PlsC does contain the $\alpha\beta$ acyltransferase domain and the canonical HX₄D active site, but it is linked to an unusual N-terminal two-helix motif that is highly populated with hydrophobic and aromatic residues. Modeling, MD simulations and mutagenesis reveal that this motif enables PlsC to be anchored to one leaflet of the membrane. This mode of binding to the membrane together with the location of the active site allows PlsC to simultaneously bind the membrane-associated LPA and the soluble acyl donor, and to directly release the PA product into the phospholipid bilayer. A biochemical feature of all AGPAT acyltransferases is their high degree of substrate selectivity that gives rise to the fatty acid positional distribution found in phospholipids. Our structure also reveals the structural basis for this substrate specificity that is confirmed by mutagenesis and biochemical analyses.

RESULTS

Confirming the biological role of *Thermotoga maritima* PlsC

The gene for *Thermotoga maritima* PlsC (TmPlsC) was optimized for expression in *E. coli* and first used for complementation, activity and membrane association studies. TmPlsC functions as an LPA acyltransferase based on its ability to complement the temperature-sensitive *E. coli* strain SM2-1 (*plsC*(Ts)) (Supplementary Fig. 1a,b). The *plsC* temperature sensitive *E. coli* strain SM2-1 grows at 30 °C but not at 42 °C unless complemented by an active PlsC. Strain SM2-1 containing the empty expression vector was viable at 30 °C but nonviable at 42 °C, whereas constructs expressing the previously characterized *E. coli* and *Chlamydia trachomatis* PlsC (EcPlsC and CtPlsC, respectively) also complemented growth at 42 °C. These studies confirmed that TmPlsC catalyzes the LPAAT reaction because its expression rescued the growth of strain SM2-1 at the non-permissive temperature.

PlsC substrate specificity is reflected by the phospholipid molecular species in the 2-position produced in the complemented strain^{21,22}. Consistent with the known specificity of EcPlsC^{21,22}, the major phosphatidylethanolamine molecular species in strain SM2-1 expressing EcPlsC was 16:0/16:1 (1-position/2-position) (Fig. 2a and Supplementary Fig. 2a). In contrast, complementation with CtPlsC yielded molecular species with 14:0 in the 2-position (Fig. 2b and Supplementary Fig. 2a) reflecting the selectivity of CtPlsC for *anteiso*15:0²². TmPlsC expression in strain SM2-1 resulted in predominately 16:0/16:0 and 18:1/16:0 phospholipids (Fig. 2c and Supplementary Fig. 2a), indicating a TmPlsC substrate selectivity for 16:0. This is consistent with the observation that 16:0 comprises 80–90% of *T. maritima* fatty acids²³.

To obtain purified TmPlsC for enzymatic studies, the gene was overexpressed in *E. coli* with a C-terminal 6xHis tag. As anticipated, it was necessary to purify TmPlsC from the detergent solubilized membrane fraction of the expressing cells, and the protein remained in the membrane fraction even after a high salt (0.5 M NaCl) wash (Fig. 2e). The isolated protein catalyzed the conversion of LPA into PA using either [¹⁴C]16:0-acyl carrier protein (ACP) or [¹⁴C]16:0-CoA as the acyl donor (Supplementary Fig. 3a,b).

Structural analysis of TmPlsC

For structural studies, TmPlsC was overexpressed with an N-terminal 6xHis tag and purified from the cell membrane by Ni²⁺ chelation chromatography and gel filtration. We exploited the gel filtration step to identify which detergent produced the most homogenous protein sample, and found n-dodecyl-β-D-maltoside (DDM, Anatrace) to be the optimal choice. Crystals suitable for X-ray analysis that diffracted to 2.8 Å in space group I23 were obtained in polyethylene glycol 3350 (PEG3350). Selenomethionine-substituted (SeMet) TmPlsC was purified using the same protocol and produced almost identical crystals, but they only diffracted to 4.0 Å despite extensive attempts to optimize their quality and improve their resolution. Using a combination of SAD phasing, phase extension and non-crystallographic symmetry averaging, a 2.9 Å structure containing two TmPlsC molecules in the asymmetric unit was obtained. The final model (Supplementary Fig. 4a,b) had Rwork/Rfree values of 0.22/0.28 and excellent geometry for this resolution (Table 1).

TmPlsC comprises two domains; residues 1-61 create a distinctive N-terminal two-helix motif, and the C-terminal residues 62-247 form an $\alpha\beta$ domain comprising a seven stranded β -sheet core surrounded by five α -helices and four 3_{10} helices (Fig. 3a and Supplementary Fig. 5a). The $\alpha\beta$ domain closely resembles the core structures of the soluble plant GPATs^{6,24} and a mycobacterial acyltransferase⁷ (Supplementary Fig. 6). This structural similarity includes the location of the conserved catalytic HX₄D sequence that spans His84 and Asp89 (Fig. 3a, Supplementary Fig. 5a and Supplementary Fig. 6). All four conserved motifs within the AGPAT family^{10,12,20} are within the $\alpha\beta$ domain (Supplementary Fig. 5a,b). Motifs 1, 3 and 4 are adjacent and create the active site locale. Motif 1 contains the HX₄D sequence, and Asn83 and Gln85 flank the catalytic His84 and fix its position via hydrogen bonds with neighboring backbone amide nitrogen atoms. Motif 3 contains Glu156 that forms two stabilizing hydrogen bonds to His84. Motif 4 is within strand β 6 and interacts with the adjacent strand β 2 to cap the active site and further stabilize motif 1. Motif 2 is in a separate part of the domain, and its putative role is discussed below.

The N-terminal two-helix motif comprises an anti-parallel two-helix bundle (α 1 and α 2) that is not present in the soluble GPAT structures^{6,24} or in the recently determined mycobacterial acyltransferase structure⁷. The motif has a very distinctive array of amino acids (Fig. 3a and Supplementary Fig. 5a). The central region of helix α 1 is very hydrophobic and contains many aromatic residues, while the N- and C-termini are populated with basic residues and are positively charged. Helix α 2 is sandwiched between helix α 1 and the $\alpha\beta$ domain, and its exposed surfaces are also populated with basic residues. There is a tight association between the two-helix motif and the $\alpha\beta$ domain and no indication that they are flexibly tethered. The association is mediated by distinct hydrophobic interfaces and a buried surface area of 1084 Å² that are identical in the two molecules in the crystal asymmetric unit (AU). The two molecules in the AU are very similar (RMSD on α -carbons of 0.85 Å) and the temperature factors also suggest no flexibility.

The catalytic mechanism and substrate binding sites

The HX₄D catalytic motif is positioned in a deep cleft that is the dominant surface feature of TmPlsC (Fig. 3b). As previously described^{6,7,24}, the role of Asp89 is to maintain the lone pair electrons on the N ϵ 2 nitrogen of His84 to abstract a proton, in this case, from the 2-hydroxyl of LPA for nucleophilic attack on the thioester substrate (Fig. 1b and Fig. 4a). This arrangement is reminiscent of the serine protease catalytic triad except that the 2-hydroxyl of LPA substitutes for the serine hydroxyl. The conserved Arg159 within motif 3 is ideally positioned to bind the 3'-phosphate of LPA. An energy minimized model of how the substrates engage the active site was generated based on the positions of His84 and Arg159 and the required geometry of the catalytic mechanism (Fig. 4b). The model also included the flexible regions of PlsC, including side chains, which were missing from the crystal structure.

In the model, the lower half of the cleft below the active site accommodates LPA while the upper half binds the phosphopantetheine arm of acyl-ACP/CoA. Residues 105-110 and 125-145 on one side of the binding cleft display high temperature factors, and residues 127-132 are not visible in both molecules in the crystal structure. This suggests that the cleft

opens to bind the substrates and release the product, and closes to perform catalysis. Lys105 is conserved and ideally located to interact with the 3'-phosphate of bound LPA when the pocket is closed (Fig. 4b). An oxyanion hole is required to stabilize the negatively charged oxygen atom in the transition state and the backbone amide nitrogen of Lys105 would be positioned to fulfill this role upon closure of the pocket (Fig. 4b). The basic and largely disordered residues 128-133 (RKNPRR) are positioned to mediate binding of the negatively charged acyl-ACP as observed in other proteins that are required to dock with ACP²⁵⁻²⁸. Motif 2 is directly N-terminal to this sequence (Supplementary Fig. 5a), which suggests that it contributes to binding the phosphopantetheine arm of the acyl donor.

Acyl chain specificity

Any proposed catalytic mechanism for PlsC must be consistent with the observation that the enzyme dictates the acyl chain species that is incorporated into the 2-position of bacterial phospholipids. In our substrate bound model, a hydrophobic tunnel directly adjacent to the active site is ideally positioned to bind the acyl chain of acyl-ACP/CoA (Fig. 3b and Fig. 4c). Electron density within this tunnel is consistent with a low occupancy bound acyl chain in the crystal structure (Supplementary Fig. 4c). Most significantly, the 16:0 acyl chain that we have shown to be selected by TmPlsC fits exactly into this tunnel while preserving the necessary interactions at the active site. The tunnel includes residues Tyr20, Ile21, Gly25, Ile49 and Phe52 from the N-terminal two-helix motif, which supports our proposal that its tight association with the $\alpha\beta$ catalytic domain is crucial to the function of the enzyme.

In our model, Gly25 sits at the end of the tunnel and creates space for the terminal methyl group of 16:0. Therefore, introducing bulky side chains at Gly25 should alter the acyl donor selectivity without impairing catalysis. This was directly verified by site-directed mutagenesis of Gly25 and testing the mutants in the complementation assay. Each of the mutated enzymes was able to complement growth of strain SM2-1 at the non-permissive temperature (Supplementary Fig. 1c,d). However, the phospholipid molecular species composition showed that the ratio of 16:0 to 14:0 in the 2-position of phospholipids decreased with increasing size of the side chain (Supplementary Fig. 2b,c,d). TmPlsC[G25M] was predicted to completely fill the end of the tunnel, and cells expressing this mutant indeed placed 14:0 in the 2-position rather than 16:0 (Fig. 2d). These experiments confirmed that the dead-end hydrophobic tunnel in TmPlsC is the acyl chain ruler that governs the substrate selectivity of PlsC.

The N-terminal two-helix motif associates with the membrane

The hydrophobic/aromatic nature of the N-terminal two-helix motif and its absence from the soluble acyltransferase structures suggest that it is the motif that interacts with the membrane. This is supported by the way in which TmPlsC packs in the crystal lattice. The two-helix motifs of both molecules in the asymmetric unit are packed around the 3-fold axis and surround electron density that is consistent with aligned phospholipid molecules that apparently co-purified with the protein (Supplementary Fig. 7). In contrast, the $\alpha\beta$ domains are exposed to the solvent channels. To test this role, truncated versions of TmPlsC missing either $\alpha 1$ (TmPlsC-H1) or both $\alpha 1$ and $\alpha 2$ (TmPlsC-H12) were expressed and analyzed. Neither of the two truncated proteins complemented the growth of strain SM2-1 (*plsC*(Ts)),

and the purified proteins were catalytically inactive (Supplementary Fig. 3c). We previously established that full length TmPlsC is membrane bound and not removed by a 0.5 M NaCl wash (Fig 2e). TmPlsC-H1 is also membrane-bound, but a proportion was extracted by 0.5 M NaCl (Fig. 2e). TmPlsC-H12 was removed from the membrane by 0.5 M NaCl (Fig. 2e) indicating primarily electrostatic membrane association. We acknowledge that the truncated proteins may be partially unfolded, but the removal of helices $\alpha 1$ or $\alpha 1/\alpha 2$ clearly impacts the ability of TmPlsC to associate with the membrane. These data firmly support our proposal that the N-terminal two-helix motif is responsible for the classification of PlsC as an intrinsic membrane protein.

To test this membrane-association model and to computationally establish the preferred binding orientation within the bilayer, we performed an MD simulation of TmPlsC embedded in one leaflet of the bilayer using the coordinates of the complete PlsC molecule bound to its substrates described above. The starting position was based on the observed interaction with three bound phospholipids in the best resolved molecule in the crystal structure asymmetric unit (Supplementary Fig. 7e,f). During the initial stages of the simulation, the PlsC-substrate complex quickly adopted a preferred position in the leaflet, and this remained stable with respect to protein depth (Supplementary Fig. 8) and orientation. However, PlsC was able to diffuse freely in the plane of the membrane (Supplementary Fig. 8). As regards the LPA substrate, the phosphate group initially relocated to the phosphate level of the leaflet via a rotation of the Arg159 side chain, but it moved back to the active site during the final stages of the simulation. The final model revealed two additional features of the protein-membrane complex. First, three exposed aromatic residues on helix $\alpha 2$ (Tyr44, Trp59 and Phe61) and two exposed aromatic residues in the $\alpha\beta$ domain close to the two-helix motif (Trp116 and Trp200) are appropriately positioned to further stabilize the interaction of TmPlsC with the membrane. Also, basic residues on the exposed surfaces of helix $\alpha 2$, notably at its N-terminus, also interact with the surface phosphates of the membrane. The final position and orientation of PlsC within the membrane is shown in Fig. 5.

DISCUSSION

Our structural analysis of TmPlsC has revealed a new motif by which proteins can tightly associate with membranes. The motif comprises an N-terminal pair of antiparallel α -helices in which the first long α -helix $\alpha 1$ is characterized by an unbroken string of 25 hydrophobic/aromatic residues, a central kink mediated by a pair of glycine residues (Gly25-Gly26), and basic termini. The structurally conserved $\alpha\beta$ acyltransferase catalytic domain found in related soluble enzymes is firmly anchored to the membrane bilayer by this two-helix motif. Consistent with this, we have demonstrated that truncation of the motif reduces the association with the membrane, and complete removal prevents the association. The motif is fully sequestered in the crystal lattice and only interacts with bound phospholipid and detergent molecules. We propose that the motif functions like the bow of ship that brings the catalytic domain of TmPlsC into close proximity to one leaflet of the membrane bilayer. The kink in helix $\alpha 1$ allows it to enter and exit the same side of the membrane, while the central hydrophobic residues and the terminal basic residues interact with the apolar interior and negatively charged surface of the membrane, respectively. Helix $\alpha 1$ is reminiscent of so-

called ‘reentrant helices’ that also re-emerge on the same side of the membrane as they enter^{29,30}. However, unlike the continuous helix $\alpha 1$ in TmPlsC, a typical reentrant helix comprises two short hydrophobic helices connected by a hairpin loop that sharply reverses direction after reaching the bilayer center.

To gain more insight into how the two-helix motif engages one leaflet of the membrane, we performed an MD simulation of the complex. The simulation fully supported the model and identified the preferred orientation of TmPlsC within the leaflet that is further stabilized by additional interactions with the surface of the $\alpha\beta$ domain adjacent to the two-helix motif. The model also showed that TmPlsC is able to diffuse freely in the plane of the membrane as required by the need to search for its LPA substrate. Biophysical experiments³¹ have shown that each leaflet of the membrane is divided into five zones beginning with the outer zone populated by water, followed by the phospholipid head group, the phosphate, the carbonyl glycerol and the inner waterless hydrocarbon zones. The model reveals that the two-helix motif and the surrounding surface of TmPlsC are ideally structured to interact with these zones (Fig. 5). Most notably, aromatic and basic residues are known to favorably interact with the carbonyl and phosphate zones, respectively, as observed in the MD-derived model^{32,33}.

The structure of TmPlsC and the MD-derived model of the membrane-bound complex explain how the enzyme solves the difficult topological problem of acylating a membrane-embedded substrate with a soluble acyl donor. The proposed ACP/CoA binding surface adjacent to motif 2 is fully solvent accessible and not sterically blocked by the membrane surface. The upper half of the adjacent surface crevice accommodates the phosphopantetheine arm of ACP or CoA, and the attached acyl chain is tucked away in the acyl donor binding tunnel that also serves as the length and shape selectivity device to ensure that the correct acyl chain becomes attached to LPA. The acyl donor is the leading substrate in GPAT catalysis³⁴, and we therefore envisage that TmPlsC is preloaded with acyl-ACP/CoA and that this complex with the thioester positioned at the active site moves through the membrane leaflet seeking LPA. LPA can directly enter the lower half of the surface crevice via the flexible gateway loop, and the binding of the phosphate group adjacent to the active site signals closure of the loop via the interaction with Lys105 and prompts catalysis by the generation of the oxyanion hole provided by the backbone amide nitrogen of the same lysine residue. An intriguing aspect of the mechanism suggested by our MD model is that the two-helix motif acts like a fishing bobber to suspend the PlsC active site near the glycerol-carbonyl level of the leaflet to directly access the 2-hydroxyl of LPA *in situ*. This would facilitate the entry to, and the exit from, the active site by the LPA substrate and the PA product, respectively. The MD simulation model suggests that the active site actually hovers 5–8 Å above the glycerol-carbonyl level (Fig. 5), and the movement of the LPA into the active site would result from interactions between its phosphate group and the conserved Arg159 and Lys105. In this scenario, the lack of both a head group and a 2' acyl chain on LPA compared to the surrounding mature phospholipids may facilitate its selection by the PlsC active site.

All AGPATs are structurally and functionally related to PlsC^{8–10} and are likely to associate with the membrane in a similar manner. They each contain an N-terminal hydrophobic

extension, a catalytic HX₄D motif, and the four motifs that we have shown to be structurally and functionally important to the conserved $\alpha\beta$ acyltransferase catalytic domain. PlsC is a unique member of the family because its substrate is LPA whereas most AGPAT family members are specific for lysophospholipids with different head groups (choline, inositol, etc). However, it would not be difficult to structurally modify TmPlsC to create the spectrum of mammalian AGPATs with defined biochemical roles in membrane lipid homeostasis. This would simply involve altering the phosphate binding pocket to allow the binding of chemically different head groups, and changing the length and shape of the acyl selectivity tunnel to accommodate their particular acyl-CoA(ACP) substrates.

Online Methods Section

Molecular Biology and Mutagenesis

THEMA_05775 is annotated as the putative PlsC gene from *Thermotoga maritima* (NCBI Bacterial Genome Database). A version of this gene optimized for expression in *E. coli* was obtained using GeneArt Gene Synthesis Technology (ThermoFisher Scientific). For the complementation and membrane association studies, the gene was cloned into the pPJ131 vector²² via the *Nde*I (5') and *Eco*RI (3') restriction sites. For the enzyme assay studies, the gene was cloned into the pET21a vector (Novagen) via the *Nde*I (5') and *Eco*RI (3') restriction sites so as to introduce a C-terminal 6xHis tag. For structural studies, the gene was cloned into the pET28b(+) vector (Novagen) via the *Nde*I (5') and *Xho*I (3') restriction sites so as to introduce an N-terminal 6xHis tag. Subsequent to the structure determination, several variants of the pPJ131-TmPlsC construct were generated for functional analyses. These included TmPlsC-H1 and TmPlsC-H12 missing one and two N-terminal α -helices, respectively, and point mutants G25A, G25V, G25L, and G25M. The mutations were introduced using the QuikChange Lightning mutagenesis kit (Agilent Technologies), and the mutagenesis primers were designed using the QuikChange Primer Design software (Agilent Technologies). All constructs were verified via Sanger DNA sequencing.

Complementation Assays

The function of the predicted TmPlsC was verified using the previously described complementation method²². pPJ131 expression plasmids were generated to express the following; no protein, *E. coli* PlsC, *Chlamydia trachomatis* PlsC, TmPlsC, TmPlsC-H1, TmPlsC-H12, TmPlsC(G25A), TmPlsC(G25V), TmPlsC(G25L) and TmPlsC(G25M). Each construct was transformed into the *plsC* temperature sensitive *E. coli* strain SM2-1 and plated at 30 °C or 42 °C on Luria Bertani plates (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar per liter) with 100 μ g/ml carbenicillin (LB-CBC). Strain SM2-1 containing the empty pPJ131 vector was viable at 30 °C but nonviable at 42 °C, and viability at 42 °C provided a read out of complementation by the various genes. The constructs containing *E. coli* and *C. trachomatis* PlsC genes fully complemented growth at 42 °C and served as positive controls.

Membrane Association

SM2-1 cells expressing pPJ131-TmPlsC, pPJ131-TmPlsC-H1, and pPJ131-TmPlsC-H12 were grown overnight at 30 °C, diluted back to OD₆₀₀ = 0.05 in a 200 ml LB-CBC culture,

and grown at 30 °C to OD₆₀₀ = 0.8–1.2. Cells were washed twice with phosphate buffered saline (PBS) and re-suspended in 15 ml PBS. The cells were lysed using a French press and divided into 900 µl aliquots. For the isotonic set, an additional 100 µl of PBS was added. For the high salt set, an additional 100 µl of 3.63 M NaCl was added to make the solution a 500 mM final NaCl concentration. The lysed cells were centrifuged at 20,000 g for 30 min to remove the cell debris, and 800 µl of the supernatant was further centrifuged at 80,000 x g for 30 min to separate the cell membrane and soluble fractions, both of which were saved. The cell membrane fraction was further washed to remove contaminating soluble proteins by re-suspension in 800 µl of PBS for the isotonic set or PBS with 500 mM NaCl for the high salt set, and centrifuged at 80,000 g for 30 min and stored in 800 µl of PBS. Equal volumes of the membrane or soluble fraction for each construct were electrophoresed on a NuPAGE Novex 10% bis-Tris protein gel (Life Technologies), and the TmPlsC construct was visualized via Western blot analysis using a primary rabbit polyclonal IgG His-probe antibody (Santa Cruz Biotech) and a secondary goat alkaline phosphatase linked anti-rabbit IgG antibody (Sigma Aldrich). This antibody to detect His-tagged proteins has been used in >129 papers. The blot was visualized using the ECF substrate (GE Healthcare) on a Typhoon FLA 9500 imager in the fluorescence detection mode.

Protein Production and Purification

To obtain purified protein for structural analyses, the pET28b(+)-TmPlsC expression vector was transformed into *E. coli* One Shot BL21(DE3) competent cells (Thermo Fisher Scientific). Cells were grown in 1 l of LB media containing 50 µg/ml kanamycin at 37 °C to OD₆₀₀ = 0.6, and protein expression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C for 12–14 hours. Cells were harvested by centrifugation at 3,200 x g for 30 min, and the cell pellets were washed in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, re-suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2% glycerol, 2.5 mM EDTA, 4 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride (PMSF)) containing complete protease inhibitor cocktail (Roche), and lysed by sonication. Cellular debris was removed by centrifugation at 30,000 x g for 20 min, and the membrane fraction was isolated by ultracentrifugation at 120,000 x g for 2 hours and then solubilized overnight at 4 °C in buffer A (20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 4 mM β-mercaptoethanol) containing 20% glycerol and 20 mM (~1%) n-dodecyl-β-D-maltoside (DDM, Anatrace)). The membrane suspension was centrifuged at 120,000g for 80 min, diluted 10-fold in buffer B (20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 10% glycerol, 1 mM DDM, 2.5 mM β-mercaptoethanol) and loaded onto a pre-equilibrated HisTrapHP column (GE Healthcare) at 4 °C. The column was washed with buffer B and the protein was eluted using a 0–500 mM imidazole gradient in buffer B. Final cleanup was achieved by size exclusion chromatography on a Superdex S200 16/60 column (GE Healthcare) in buffer C (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% glycerol, 2.5 mM β-mercaptoethanol). Selenomethionine-substituted (SeMet) TmPlsC was purified using the same method except that the plasmid was transformed into *E. coli* 834(DE3) cells (New England Biolabs) and grown in SelenoMet medium (Molecular Dimensions) supplemented with 50 µg/l L-selenomethionine (Sigma-Aldrich).

For the activity analyses of the full-length and N-terminally truncated proteins, the pET21a-TmPlsC, pET21a-TmPlsC-H1 and pET21a-TmPlsC-H12 expression vectors were transformed into BL21AI cells. The cells were grown at 37 °C to OD₆₀₀ = 0.6–0.8, induced with arabinose (0.2% w/v) and IPTG (1 mM), and incubated overnight at 16 °C. The cells were washed with and re-suspended in 20 mM Tris-HCl, pH 8.0 and 500 mM NaCl buffer and lysed using a French press. The C-terminal 6xHis-tagged protein was purified via nickel chelation chromatography as described above.

TmPlsC Assay

The activities of the purified TmPlsC, TmPlsC-H1 and TmPlsC-H12 proteins were assayed in a detergent-based system using [¹⁴C]16:0-acyl carrier protein (ACP) from *E. coli* or [¹⁴C]16:0-coenzyme A (American Radiolabelled Chemicals), and 16:0-lysophosphatidic acid. [¹⁴C]16:0-ACP was synthesized from purified *E. coli* ACP using ACP synthetase from *Streptococcus pneumoniae* and acyl-ACP synthetase from *Vibrio harveyi*¹. The assays were performed for 15 min at 42 °C in 20 µl solutions containing 20 µM [¹⁴C]16:0-CoA or [¹⁴C]16:0-ACP, 150 µM 16:0-lysophosphatidic acid, 2 mM MgCl₂, 0.1% Brij-58, and 100 mM Tris-HCl, pH 8.0. The reaction was extracted via the Bligh and Dyer method³⁵ to isolate the phosphatidic acid product, which was quantified by liquid scintillation counting (Tri-Carb 2910 TR, PerkinElmer Life Sciences). The identity of the extracted phosphatidic acid was confirmed by thin-layer chromatography on Analtech Silica Gel G plates developed with chloroform/methanol/acetic acid (90/5/5, v/v/v).

Molecular Species Analysis

SM2-1 cells expressing the relevant pPJ131 constructs were grown overnight in LB-CBC medium at 30 °C, diluted back to OD₆₀₀ = 0.01 in a 10 ml LB-CBC culture, and grown at 42 °C to OD₆₀₀ = 0.8–1.2. The lipids were extracted from the cells via the Bligh-Dyer method³⁵ and the phospholipid molecular profiling was performed as previously described^{22,36,37}. Phospholipid molecular species fingerprints were determined using direct infusion electrospray ionization-mass spectrometry technology. Mass spectrometry analysis was performed using a QTrap 4500 (Sciex, Framingham, MA) equipped with a Turbo V ion source. Lipid extracts were resuspended in 50:50 (v/v) chloroform/methanol + 1% formic acid. The instrument was operated in the positive ion mode for phosphatidylethanolamine scan analysis. The ion source parameters were ion spray voltage, 5500 V; curtain gas, 15 psi; temperature, 270 °C; collision gas, medium; ion source gas 1, 15 psi; and ion source gas 2, 25 psi. Parameters for phosphatidylethanolamine analysis are the following: scan range, 600–900 *m/z*; declustering potential, 30 V; collision energy, 45 V; peak width, Q1 and Q3 0.7 FWHM. Data were processed using Analyst[®] software (AB Sciex).

Positional distribution analysis

Phospholipids were extracted from SM2-1 cells expressing the various PlsC constructs. Lipids were chromatographed on silica gel H thin-layer plates (heated activated at 90 °C for 1 h) in chloroform:methanol:acetic acid (55:20:5, v/v). Phosphatidylethanolamine was isolated from the plate and added to 50 mM Tris pH 8.5 and 1 mM CaCl₂. Phospholipase A₂ from *Naja mossambica mossambica* (250 µg/ml final concentration for 4 hours) was used to selectively deacylated the 2-position fatty acid. The reaction was chromatographed on silica

gel H thin-layer plates (heated activated at 90 °C for 1 h) in chloroform:methanol:acetic acid (55:20:5, v/v) to separate the fatty acid from the lysophospholipids. The fatty acid fraction was converted to fatty acid methyl esters through incubation in anhydrous methanol/acetyl chloride overnight and analyzed by gas chromatography. The free fatty acid contribution from phospholipase A₂ was determined by subjecting phospholipase A₂ alone to the same treatment as the phospholipid samples. The free fatty acid contribution from phospholipase A₂ was subtracted from the sample chromatograms to determine the 2-position acyl chain composition of the different samples.

Crystallization, Data Collection and Structure Determination

Initial crystallization trials were performed at 18 °C using the sparse matrix screens JCSG suites (I–IV, JCSG +), PEGs Suite, and PEGs II Suite (Qiagen), and several conditions produced crystals that were subsequently optimized. Crystals suitable for X-ray analysis were obtained in 18–22% (w/v) polyethylene glycol 3350 (PEG3350), 0.1 M HEPES, and 0.2 M MgCl₂. Crystals were cryo-protected in mother liquor supplemented with 25% (v/v) glycerol and flash-frozen in liquid nitrogen. SeMet protein produced crystals with very similar morphology in 18–20 % (w/v) PEG3350 and 0.2 M CaCl₂. Native and SAD diffraction data, at 1.0 Å and 0.97895 Å, respectively, were collected at the SER-CAT ID22 beam line at the Advanced Photon Source (APS) and processed using XDS³⁸. The native and SeMet crystals were both in space group I23 with very similar cell dimensions (Table 1).

Two SeMet data sets were collected, and these were individually processed and then merged into a single dataset using Aimless³⁹. An initial 11 atom Se substructure (CC_{all}/CC_{weak} 31.20/14.16 and PATFOM 29.94) was identified with the SHELXC/D/E 2014.1 pipeline⁴⁰ that generated a clear molecular envelope, and this was eventually reduced to a 4 atom substructure by rounds of phasing and map inspection using Phaser 2.5.5⁴¹ and Coot 0.8.8⁴². Final phasing using phenix.autosol 1.10.1⁴³ resulted in an interpretable map with overall Figure of Merit of 0.237 and two molecules in the asymmetric unit. Phase extension to 2.8 Å using the initial SAD phases, the non-crystallographic symmetry and the higher resolution native data was performed using resolve⁴⁴. Secondary structures were initially built into the map manually, and the majority of the structure was subsequently built using phenix.autobuild 1.10.1⁴⁵ and Coot 0.8.8⁴² and refined using phenix.refine 1.10.1⁴⁶ with TLS definitions and NCS restraints.

Substrate Docking

Two substrates were docked into the TmPlsC active site locale, 16:0-phosphopantetheine and 1-acyl-*sn*-glycerol-3-phosphate (lysophosphatidic acid or LPA). These substrates were built and energy-minimized using the Builder module of the molecular modeling program MOE (*Molecular Operating Environment (MOE)*, 2013.08; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2016). Regions missing from the TmPlsC crystal structure were built using the Biopolymer module of MOE and included the loop from residues 127–132, and the side chains of residues 1–5, 135 and 193. Following the addition of hydrogen atoms, the full structure was energy-minimized to optimize the geometry. Based on our understanding of the reaction mechanism and geometry, the two substrates were manually docked into the active site. The model of

the complex was then optimized by energy-minimization using the Merck molecular force field (MMFF94), keeping the protein rigid but the two substrates fully flexible. The final model contained no van der Waals clashes or unfavorable geometries.

Molecular Dynamics Simulations of Membrane Association

The substrate-docked model of TmPlsC incorporating the missing regions of the crystal structure (see previous section) was used to generate the initial coordinates for the MD simulations. The system was created using the Visual Molecular Dynamics (VMD) program 1.9.2⁴⁷ and comprised the TmPlsC model imbedded into a lipid bilayer using the well resolved bound phospholipid from the crystal structure as a guide. The model was placed into a POPC membrane bilayer using the VMD package Membrane. Water molecules were added above and below the lipid bilayer using the VMD package Solvate to create a total simulation box of 110x110x130 Å³ allowing for approximately 30 Å between the box edge and protein or lipid. 101 chloride and 85 sodium ions were added to neutralize the system and create pseudo-physiological, 150 mM ionic strength. Lipids molecules were removed if they were located within 0.8 Å of protein or substrates. Overlapping water molecules were selected and deleted using a cutoff of 3.4 Å between the water oxygen and all non-water atoms. The final simulation box contained 130,387 atoms. Molecular dynamics simulations were performed in program NAMD 2.9⁴⁸ using the Charmm36 force field for proteins, lipids and nucleic acids⁴⁹. Parameters for the substrates LPA and acyl-ACP/CoA were assigned using the Charmm General Force Field (CGenFF) 3.0.1 and the parachem server 1.0.0^{50–52}. Parameters for acyl-CoA were supplemented by those previously derived by Aleksandrov and Field⁵³. In the case of acyl-ACP/CoA, charges with high penalties were optimized using Gaussian 09 (Revision A.02; Gaussian, Inc: Wallingford CT, 2009) using B3LYP hybrid functional and 6-31G* basis set. The simulation box was equilibrated under three conditions, each using the previous as an input. (1) NVT simulation for 1 ns with the heavy atoms of lipid headgroups, and nearby water constrained with a harmonic potential of 4 kcal/mol/Å², water molecules greater than 5 Å from lipid atoms, protein and substrate coordinates fixed and lipid tail atoms free to move. (2) NPT simulation for 1 ns with protein and substrate heavy atoms constrained by a harmonic potential of 2 kcal/mol/Å² and all remaining atoms free to move. (3) NVT simulation with the temperature increased from 5 to 310 K in 1 K steps every ps using 0.5 fs stepsize and held at 310 K for 2 ns. The final equilibrated system was simulated as a NPT ensemble using periodic boundary conditions at 310 K. Constant temperature control was achieved using Langevin dynamics with 5 ps⁻¹ damping. Pressure was held at 1 atm by a Nose-Hoover Langevin piston with a decay period of 100 fs and a damping time of 50 fs. After equilibration, a total trajectory of 100 ns was calculated.

Data availability

The structure factors and coordinates of the TmPlsC structure have been deposited in the Protein Data Bank under accession code PDB 5KYM. Source data for Figure 2e are provided in the Supplementary Data Set 1. All other data that support the findings of this study are available from the corresponding authors upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chemical reactions involving LPA acyltransferase (PlsC)

(a) Pathway for the synthesis of phosphatidic acid (PA). The 1-position of *sn*-glycero-3-phosphate (G3P) is acylated by G3P acyltransferase (PlsB). The intermediate, 1-acyl-*sn*-glycero-3-phosphate (LPA) is then esterified by PlsC to form PA, the precursor to all membrane glycerophospholipids. (b) A schematic of the PlsC active site mechanism. The solid arrows show the first step of the reaction leading to a negatively charged oxygen atom in the transition state stabilized by the backbone amide of Lys105. The dotted arrows show the resolution of the transition state leading to acyl transfer.

Figure 2. Acyl chain specificity and membrane binding of TmPlsC

The temperature-sensitive *E. coli* strain SM2-1 (*plsC*(Ts)) was transformed with *E. coli* PlsC, *Chlamydia trachomatis* PlsC, *T. maritima* PlsC, and the *T. maritima* PlsC(G25M) mutant. These constructs rescued the growth of SM2-1 at non-permissive temperature (Supplementary Fig. 1). The PE molecular species of the complemented SM2-1 strains were determined to assess PlsC substrate selectivity. (a) Molecular species profile of strain SM2-1 complemented with the *E. coli* PlsC. (b) Molecular species profile of strain SM2-1 complemented with the *C. trachomatis* PlsC. (c) Molecular species profile of strain SM2-1 complemented with the *T. maritima* wild-type PlsC. (d) Molecular species profile of strain SM2-1 complemented with the *T. maritima* PlsC (G25M) mutant. The spectrum shown is representative from two biological replicates. (e) Localization of the TmPlsC, and the TmPlsC-H1 and TmPlsC-H12 deletion mutants. Strain SM2-1 was transformed with plasmids expressing these three constructs. The cells were grown to $OD_{600} = 1$, and re-suspended in either isotonic solution (PBS) or high salt solution (PBS with 500 mM NaCl). Cells were lysed via the French press, and the membrane fraction (Mem) was separated from the soluble fraction (Sol). Western blot analyses were used to determine whether a particular TmPlsC construct was associated to the membrane or the soluble fraction. The western blot is representative from two biological replicates. See Supplemental Data Set 1 for additional information.

Figure 3. The tertiary structure of TmPlsC

(a) Overall structure of TmPlsC in cartoon representation. The N-terminal two-helix motif is colored orange, and the $\alpha\beta$ core catalytic domain is colored light blue (α -helices) and light green (β -strands and loops). Functionally important residues are colored as follows: yellow, active site residues; magenta, exposed aromatic residues proposed to interact with the lipid bilayer; blue, basic residues proposed to interact with the membrane phospholipids. (b) Charged surface representation of TmPlsC showing the substrate (LPA) binding groove, the entrance to the acyl chain selectivity tunnel, the two-helix motif and the active site. The electrostatic surface was calculated using the APBS program. The extreme ranges of red (negative) and blue (positive) represent electrostatic potentials of -7 to $+7$ kbT where kb is the Boltzmann constant and T is the temperature.

Figure 4. Active site locale of TmPlsC

(a) Close up of active site locale. (b) Model of the TmPlsC-substrate complex. The membrane-bound LPA substrate is shown with salmon carbon sticks and the soluble acyl-ACP/CoA substrate is shown with teal carbon sticks. Note that the putative oxyanion hole (orange asterisk) is proposed to be created by the movement (orange arrow) of the amide nitrogen of Lys105 that is located on a flexible loop. (c) The acyl chain specificity tunnel created by aromatic and hydrophobic residues shown in light purple carbon stick representation. The base of the tunnel comprises five residues (labeled) from helices $\alpha 1$ and $\alpha 2$ of the N-terminal two-helix motif. The transparent spheres show the G25M mutation that changes the tunnel specificity from a 16- to a 14-carbon chain. His84 identifies the position of the active site.

Figure 5. MD-derived model of TmPlsC bound in the lipid bilayer

This snapshot is taken at 97 nanoseconds after equilibration of the molecular dynamics simulation. The coloring is as follows; the N-terminal two-helix motif (orange), the $\alpha\beta$ core catalytic domain (blue/green), active site histidine (yellow); LPA substrate (salmon carbons), acyl donor (teal carbons), membrane phospholipids (yellow acyl chains, red/orange phosphate groups, blue head groups). The blue to green scale reflects the water to lipid occupancy of one half of the lipid bilayer relative to the proposed location of the TmPlsC-substrate complex. The scale is based on biophysical studies³¹ in which H₂O, phospholipid head group (PG), carbonyl glycerol (CG), and hydrocarbon CH₂ and CH₃ zones identify the highest probability of finding these moieties within one leaflet of the bilayer.

Table 1

Data collection and refinement statistics

	Native	Se-Met
Data collection[#]		
Space group	I23	I23
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	173.8, 173.8, 173.8	171.0, 171.0, 171.0
α , β , γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å) *	50-2.8 (2.9-2.8)	50-4.0 (4.47-4.0)
<i>R</i> _{merge}	0.1879 (2.25)	0.259 (2.403)
<i>R</i> _{meas}	0.1994	0.266 (2.469)
<i>R</i> _{pim}		0.062 (0.565)
CC(1/2)	0.996 (0.167)	0.998 (0.739)
<i>I</i> / σI	10.18 (0.60)	9.2 (2.1)
Completeness (%)	99.94 (99.58)	100 (100.0)
Multiplicity	9.0 (4.6)	18.7 (19.0)
Refinement		
Resolution (Å)	46.46 - 2.8	
No. reflections	194239 (9832)	
<i>R</i> _{work} / <i>R</i> _{free}	0.219/0.277	
No. atoms	4138	
Protein	3859	
DDM	35	
Acyl chains	18	
Phospholipids	225	
Water	1	
<i>B</i> -factors	81.12	
Protein	78.73	
DDM	114.38	
Acyl chains	66.31	
Phospholipids	118.27	
Water	36.62	
R.m.s. deviations		
Bond lengths (Å)	0.015	
Bond angles (°)	1.16	

[#]The Se-Met dataset was collected from two crystals and the native dataset from a single crystal.

* Values in parentheses are for highest-resolution shell.