Substrate Selectivity of Lysophospholipid Transporter LplT Involved in Membrane Phospholipid Remodeling in *Escherichia coli* 

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Lysophospholipid transporter (LplT) was previously found to be primarily involved in 2-acyl lysophosphatidylethanolamine (lyso-PE) recycling in Gram-negative bacteria. This work identifies the potent role of LplT in maintaining membrane stability and integrity in the *Escherichia coli* envelope. Here we demonstrate the involvement of LplT in the recycling of three major bacterial phospholipids using a combination of an *in vitro* lysophospholipid binding assay using purified protein and transport assays with *E. coli* spheroplasts. Our results show that lyso-PE and lysophosphatidylglycerol, but not lysophosphatidylcholine, are taken up by LplT for reacylation by acyltransferase/acyl-acyl carrier protein synthetase, demonstrating the first evidence of cardiolipin remodeling in bacteria. These findings support that a fatty acid chain is not required for LplT transport. We found that LplT cannot transport lysophosphatidic acid, and its substrate binding was not inhibited by either orthophosphate or glycerol 3-phosphate, indicating that either a glycerol or ethanolamine headgroup is the chemical determinant for substrate recognition. Diacyl forms of PE, phosphatidylglycerol, or the tetra-acylated form of cardiolipin could not serve as a competitive inhibitor in *vitro*. Based on an evolutionary structural model, we propose a “sideways sliding” mechanism to explain how a conserved membrane-embedded α-helical interface excludes diacylphospholipids from the LplT binding site to facilitate efficient flipping of lysophospholipid across the cell membrane.

Diacylphospholipids are the major component of bacterial membranes. Microorganisms have evolutionarily adapted to maintain a relatively constant lipid composition in the membrane. For instance in *Escherichia coli*, membrane lipids are composed of 70% phosphatidylethanolamine (PE), 20% phosphatidylglycerol (PG), and 5–10% cardiolipin (CL) (1). These diacylphospholipids form a stable bilayer structure to maintain cell integrity and to perform normal membrane activities (2, 3).

However, as a protective barrier, bacterial membranes must withstand harsh conditions and challenges from external perturbations, particularly threats from diverse phospholipases. Disrupting bacterial membranes by phospholipases is a potent mechanism in host cell defense (4). In polymorphonuclear neutrophils, phospholipase A2 (PLA$_2$) is delivered into the ingested bacterial cells by bacterial permeability protein, causing degradation of >50% of target cell membranes and consequent membrane disorganization and cell disassembly (4, 5). Phospholipases are also involved in interbacterial antagonistic interactions. A recent study showed that microorganisms can deliver a new class of PLA$_2$ and PLA$_3$ proteins via the type VI secretion system to different bacterial species to decompose PE, serving as a specific antibacterial effector (6). All of these PLA-mediated reactions utilize a common chemical mechanism in which a diacylphospholipid molecule is deacylated by hydrolyzing the ester bond at the sn-1 or sn-2 position to form 2-acyl- or 1-acyl-lyso derivatives. Furthermore, lysophospholipids can be generated by lipoprotein acyltransferase activity in γ proteobacteria (7). Lipoprotein acyltransferase transfers the sn-1 acyl chain from a diacylphospholipid to the N terminus of the major outer membrane lipoprotein, releasing 2-acyl lysophospholipid into the membrane (7).

Lysolipids are considered to be nonbilayer-forming lipids, found only in a trace amount in normal bacterial membranes (8–10). Accumulation of lysophospholipids markedly disrupts membrane structure by increasing membrane permeability and inducing membrane curvature due to their detergent-like physicochemical properties.
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tives. We utilized transport assays to demonstrate the essential role of the LplT-Aas system in membrane phospholipid repair in bacteria. We recently, Rock, Saier, and co-workers identified a group of lysophospholipid transporter LplT proteins in the inner membrane of Gram-negative bacteria. They found that LplT promotes an energy-independent flipping of 2-acyl lyso-PE, but not 2-acyl glycero phosphocholine (lyso-PC), into the cells. The imported lyso-PE is subsequently reacylated by a bifunctional peripheral enzyme, 2-acyl lyso-PE acyltransferase/acyl carrier protein synthetase (Aas), using the acyl-acyl carrier protein as acyl donor to regenerate PE on the inner leaflet of the membrane (see Fig. 1A). LplT consists of 12 predicted transmembrane helices and belongs to the major facilitator superfamily. In some bacteria, including Escherichia coli and Klebsiella pneumoniae, LplT and Aas are adjacent encoded by the same bicistronic operon, whereas in many other bacteria the two enzymes are physically connected as an LplT-Aas fusion protein.

Both PLAs and lipoprotein acyltransferase apparently utilize all three major membrane phospholipids as substrates. (14)–(16). Thus far, no recycling mechanism has been identified for PG and CL in bacteria. Available evidence indicates that LplT- and Aas-mediated transport/acylation is limited to only 2-acyl lyso-PE (13). Because LplT is the only transporter system for lysophospholipids characterized thus far, functional assessment of other lysophospholipids is necessary to determine whether this 2-acyl lyso-PE recycling system is a general phospholipid repair system in bacteria. Bacterial membranes also contain lysophosphatidic acid (lyso-PA) (3). Whether LplT transport of this membrane precursor is involved in de novo phospholipid biosynthesis is still unknown. LplT from E. coli transports 2-acyl lyso-PE with an apparent 7.5 μM binding affinity (13). It remains unclear whether LplT also translocates diacylphospholipids as do other flippases or scramblases. (17). This unknown function is predicted to be a critical factor in LplT substrate recognition and transport because a mechanism discriminating lyso substrates from the diacylphospholipid-condensed membrane bilayer should exist.

To address these fundamental questions, we studied the LplT protein from K. pneumoniae (LplT-Kp), which shares 81% amino acid sequence identity with its E. coli LplT counterpart. In this study, we comprehensively examined binding, transport, and remodeling of several lysophospholipids and their derivatives. We utilized in vitro substrate binding and in vivo lysolipid transport assays to demonstrate the essential role of the LplT/Aas system in membrane phospholipid repair in bacteria. We discovered a novel pathway for PLA2-mediated CL degeneration and remodeling. Our study also determined specific chemical requirements for LplT substrate selectivity in the cell membrane.

Experimental Procedures

Materials—H3[32P]PO4 was from MP Biomedicals. 1-Oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (18:1 lyso-PC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 1-oleoyl-2-hydroxy-sn-glycero-3-phosphoglycerol (18:1 lyso-PC), 1,2-dioleoyl-sn-glycero-3-phospho-(1’-racemic glycerol) (sodium salt; 18:1 lyso-PC), and 1,2-dioleoyl-sn-glycero-3-phospho-(1’-racemic glycerol) (sodium salt) from Avanti Polar Lipids. Bovine heart phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylycerol (PG) were purchased from Avanti Polar Lipids. Thin-layer chromatography (TLC) plates were from MP Biomedicals. Purified PLAs from Corynebacterium adamanteum venom was purchased from Worthington. Restriction endonucleases, T4 DNA ligase, and Phusion DNA polymerase were from either Thermo Scientific or New England Biolabs. Oligodeoxynucleotides were custom synthesized by Sigma Genosys. n-Dodecyl β-D-maltoside (DDM) was from Anatrace. All of the other chemicals were of reagent grade or better from commercial sources.

Protein Expression and Purification LplT-Kp from K. pneumoniae—To overexpress the LplT-Kp protein, its gene was cloned from chromosomal DNA of K. pneumoniae subsp. pneumoniae obtained from ATCC into E. coli expression vector pET28a using PCR with the forward primer GGAATTCC-ATATGATGAGTGAGTCAGTACATACTAACC (NdeI site underlined) and the reverse primer CGGCTCGATTTACGCCGCCCCCAGACCCCAAC (XhoI site underlined). The resulting construct, pET28a-LplT-Kp, encodes the full-length LplT-Kp protein with a His6 tag at its N terminus and was verified by DNA sequencing.

The LplT-Kp protein was expressed in E. coli BL21(DE3) strain in autoinduction medium (18) for 3 h at 37 °C followed by overnight incubation at 25 °C. Cells were harvested by centrifugation and resuspended in lysis buffer containing 500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM imidazole, 10% glycerol. The cell membrane was disrupted by passing through an Avestin H3 homogenizer at 15,000 p.s.i. Cell debris was removed by centrifugation at 16,000 rpm for 30 min using an S34 rotor (Beckman Coulter). The supernatant was collected and ultra centrifuged at 40,000 rpm for 1 h using a Ti45 rotor (Beckman Coulter). The pellet containing the membrane fraction was resuspended in lysis buffer and then incubated with 1% (w/v) DDM for 1 h at 4 °C. After another ultracentrifugation step at 40,000 rpm for 30 min, the supernatant was incubated with Nickel II–NTA affinity resin (Thermo Scientific) at 4 °C for 1 h in an orbital shaker. The Nickel II–NTA affinity resin was washed with lysis buffer containing 0.3% DDM and 40 mM imidazole. The LplT-Kp protein was eluted in lysis buffer with 400 mM imidazole and further purified by size exclusion chromatography in a buffer containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.03% DDM. The purity of the protein was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing the LplT-Kp protein were pooled and frozen at −80 °C until use.

Fluorescent Substrate Binding and Competitive Binding Assays—Fluorescence was measured at room temperature in a 1-cm quartz cuvette with a QuantaMaster™ spectrophotometer and processed with Felix 32 software (Photon Technology International).
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International). A fluorescent substrate binding assay was performed in 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.03% DDM with TopFluor lyso-PE, which was added at a concentration of 750 nM. TopFluor lyso-PE was excited at 450 nm, and the fluorescence signal was traced at 508 nm. At 30-s intervals, LpIT-Kp was added stepwise until no change in fluorescence signal was observed. To eliminate the effect of volume change on fluorescence intensity, we performed the same assay except that LpIT-Kp was replaced by the same volume of 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.03% DDM. By subtracting the effect of volume change, the fluorescence variation was plotted as a function of LpIT protein concentration. The apparent K_
 was determined by fitting the data with a hyperbolic equation using Origin 7.0. Competitive binding assays were carried out with the purified LpIT-Kp protein in 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.03% DDM. Variations in the fluorescence intensity of TopFluor lyso-PE, added at a concentration of 750 nM, were traced at 508 nm by exciting at 450 nm after lyso-PE, lyso-PC, lyso-PG, lyso-PA, or CL derivatives were added stepwise as described (19).

Diacyl Lipid-LpIT Pulldown Assay—250 µg of LpIT-Kp protein was incubated with 50 µl of Ni²⁺-NTA affinity resin for 1 h. Unbound proteins were removed by washing with 20 mM Tris-HCl, pH 7.5 buffer containing 100 mM NaCl, 0.03% DDM, and 40 mM imidazole. 3²P-Labeled PE prepared as described below was dried, solubilized in 0.3% DDM, then diluted 10× as in the binding assay, and then mixed with the resin for 10 min. The resulting resin was washed with 500 µl of the washing buffer three times and then eluted with a buffer containing 400 mM imidazole. Radioactivity of the eluate was measured by liquid scintillation spectrometry to assess the efficiency of lipid transfer between donor lipid-detergent micelles and recipient LpIT-protein micelles. An “empty” resin without LpIT-Kp protein was used as control.

Preparation of ³²P-Labeled Phospholipids—³²P-Labeled phospholipids were prepared by growing various E. coli strains in LB medium containing 5 µCi/ml [³²P]orthophosphate. ³²P-Labeled PE was synthesized in E. coli strain UE54 (MG1655 ppp-2Aara714 rcsF::mini-Tn10 cam pgsA::FRT-kan-FRT) (21). This strain lacks the major anionic phospholipids PG and CL, accumulating a PE level of 95% in deep stationary phase cells. ³²P-Labeled PG and CL were made in the E. coli AL95 strain (pbs 93::kan lacY::Tn9), which lacks the ability to synthesize PE (22). Strain AL95 carrying plasmid pAC-PCSpSp-Gm was used to prepare ³²P-labeled PC (23). This strain is capable of making PC instead of PE due to expression of PC synthase encoded by the Legionella pneumophila pcsA gene under control of an arabinose-inducible promoter (ParaB). To label PE, PG, and CL, E. coli strains were grown in LB medium overnight. To generate PC, an overnight culture was diluted to an A₆₀₀ of 0.025 into fresh LB broth supplemented with 0.2% arabinose, 2 mM choline, 5 µCi/ml [³²P]orthophosphate and further grown to a final A₆₀₀ of 0.5–0.7. The cells from 50 ml of culture were harvested, and the lipids were extracted as described (24). The total extracted lipids were loaded onto Silica Gel G thin-layer plates and developed with chloroform/methanol/ammonia/H₂O (60:37.5:1:3, v/v). The dried plate was exposed to an x-ray film for 2 h. The phospholipid bands were visualized by developing the film, and bands corresponding ³²P-labeled PE, PC, PG, and CL on the TLC plate were scraped and extracted using chloroform.

Preparation of ³²P-Labeled 1-Acyl-lyso phospholipids—Radiolabeled lyso-PE, lyso-PC, lyso-PG, and CL derivatives were prepared by digestion of the corresponding purified ³²P-labeled phospholipids with venom PLA₂ essentially as described (25) with the following modifications. Purified phospholipids from a 50-ml culture mixed with cold PC at 9:1 (w/w) were dispersed after evaporation by sonication in 0.5 ml of 100 mM KCl, 0.1 mM HEPES, pH 7.5, 10 mM CaCl₂, and 20 units of PLA₂, and the reactions were incubated at 37 °C for appropriate times with shaking. After incubation, lipids were extracted and purified by TLC as described above. The radioactive bands were marked, scraped, and extracted using chloroform. ³²P-Labeled lyso-PA was directly purified from a TLC-developed lipid extract from a thermosensitive mutant of E. coli SM2-1 defective in 1-acyl-sn-glycerol-3-phosphate acyltransferase. This strain accumulates 1-acyl-sn-glycerol-3-phosphate when the temperature of the culture is shifted to 42 °C (26). All purified radiolabeled lysophospholipids were kept at −20 °C prior to assay.

Mass Spectroscopy—The products of CL digestion by PLA₂ were analyzed using normal phase liquid chromatography-tandem mass spectrometry (LC/MS/MS), which allowed the separation and identification of CL (eluting at 13–14 min), triacylated CL (tri-CL), diacylated CL (di-CL), and monoacylated CL (mono-CL) (24). Normal phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column (5 µm, 25 cm × 2.1 mm; Sigma-Aldrich). Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v), mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v), and mobile phase C consisted of...
chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution program consisted of the following steps. 100% mobile phase A was held isocratically for 2 min, then linearly increased to 100% mobile phase B over 14 min, and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min, held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The LC eluent (with a total flow rate of 300 μl/min) was introduced into the ESI source of a high resolution TripleTOF5600 mass spectrometer (AB-Sciex, Foster City, CA). Instrumental settings for negative ion ESI and MS/MS analysis of lipid species were as follows: ion spray, −4500 V; curtain gas, 20 p.s.i.; gas I, 20 psi; declustering potential, −55 V; and focusing potential, −150 V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (AB-Sciex). The product of overnight hydrolysis of CL by PLA2 was kindly analyzed by using the ESI mass spectrometry service provided by the Mass Spectroscopy Facility of Rice University.

Lysophospholipid Transport and Remodeling Assay in Spheroplasts—Transport assays were performed in freshly prepared spheroplasts prepared according to published protocols with slight modification (27). *E. coli* BL21(DE3) wild type or knock-out mutants were grown in LB broth at 37 °C until a final A600nm of 0.5. Cells were pelleted, washed twice, and resuspended in 10 mM HEPES, pH 7.5, 0.75 m sucrose, 10 mM MgSO4, 2.5% glucose with slight modification (27). E. coli BL21(DE3) wild type or knock-out mutants were grown in LB broth at 37 °C until a final A600nm of 0.5. Cells were pelleted, washed twice, and resuspended in 10 mM HEPES, pH 7.5, 0.75 m sucrose, 10 mM MgSO4, 2.5% glucose. After addition of 1 mg/ml lysozyme, cell suspensions were ice-chilled, warmed to room temperature, and subsequently incubated with gentle shaking at 30 °C for 30 min. Intact spheroplasts were collected by centrifugation (3,000 × g for 10 min) at room temperature and resuspended at 10 mg/ml total protein in the above buffer without LiCl. Spheroplast formation and stability were thoroughly monitored nephelometrically by comparing the A600 of a 100-μl spheroplast solution with 2 ml of either plain water or a solution of 10 mM MgCl2, 0.75 m sucrose, respectively.

Prior to the transport assay, radioactive lysophospholipids were mixed with cold counterparts and resuspended in ethanol to a final concentration of 200 μM. Transport assays were initiated by adding 10 μM (final concentration) substrates into spheroplast solutions. At the indicated time, reactions were terminated by adding chloroform/methanol (1:2), and total lipids were extracted and separated on Silica Gel G TLC plates. The dry plate was exposed to a Storage Phosphor Screen (Eastman Kodak Co.) overnight. The individual phospholipids were visualized and quantified using a Molecular Imager™ FX (Bio-Rad). Stored images were processed and quantified using Quantity One software (Bio-Rad) for scanning and analysis of the captured phosphorimages. Phospholipid content is expressed as mol % of total phospholipid based on the intensity of the captured signal generating a latent image of the radiolabeled spot on the Phosphor Screen.

Michaelis-Menten Transport Kinetic Assay—Centrifugation through a layer of silicone oil (28) was utilized for the kinetic analysis of LplT-mediated lysophospholipid transport into *E. coli* spheroplasts. The maintenance of structural integrity of spheroplasts during the procedure and their fast separation from the assay mixture make this technique advantageous for the analysis of lysophospholipid transport. Lysophospholipids (premixed “cold” and “hot”) were added to the spheroplasts to a desired final concentration as indicated and incubated at 37 °C for 30 min. After incubation, 0.5-ml samples were removed from the reaction mixtures, layered onto 0.15 ml of 22% perchloric acid and 0.50 ml of silicone oil (d = 1.05; Aldrich) in microcentrifuge tubes, and centrifuged through the silicone oil in an Eppendorf microcentrifuge at 14,000 × g for 5 min at room temperature to stop the reaction and separate spheroplasts from non-transported lysophospholipids. After centrifugation, 0.05-ml samples of the perchloric acid phase were removed, and the radioactivity was measured by an LKB-Wallac liquid scintillation counter (Model 1209). The excess amount of non-reacted substrate was confirmed in each transport reaction by comparing radioactivity with total input to ensure proper kinetic analysis. The data were normalized to nmol/g of total protein/h based on the specific radioactivity of the lipids.

Results

Purification of LplT Protein—Members of the LplT protein family share a conserved topology of 12 transmembrane helices with both N and C termini predicted to be on the cytoplasmic surface (Fig. 1A). No signal peptide sequence was identified using the SignalP server (data not shown) (29). Thus, to purify...
the LplT protein in vitro, we cloned the LplT gene from *K. pneumoniae* into the pET28a vector to fuse with a histidine tag at its N terminus. The LplT protein was expressed in *E. coli* BL21(DE3) cells and then purified using the non-ionic detergent DDM from Ni²⁺-NTA resin followed by size exclusion chromatography. The eluted protein showed one band at ~30 kDa on SDS-PAGE that was confirmed by Western blotting using anti-His6 antibody (Fig. 2A). The molecular mass of LplT-*Kp* is ~42 kDa. The faster migration on SDS-PAGE may be due to its partial unfolding by SDS, which is a common effect for membrane proteins (30). The homogeneity of the protein was further exhibited as one single peak eluted at ~14 ml from a Superdex 200 column (GE Healthcare), and no protein aggregation was observed (Fig. 2B). To the best of our knowledge, this is the first purification result of an LplT family protein.

**LplT Interaction with Lysophospholipids but Not with Diacylphospholipids—**To study LplT substrate binding, interaction was initiated by the addition of 0.75 μM TopFluor lyso-PE as a binding component, and fluorescence intensity change was monitored at 480-nm wavelength as a function of the amount of purified protein. As shown in Fig. 2C, titration of the LplT protein in the solution resulted in progressive fluorescence quenching, which was completely saturated at 10 μM protein, indicating specific interaction of the LplT protein with the ligand. In contrast, no significant change was observed with buffer containing only DDM detergent. The fluorescence intensity changes were fit to a single exponential function by nonlinear regression to obtain the amplitude and rate of the fluorescence change, yielding a calculated *K₅ₐ* of 1.15 ± 0.18 μM (Fig. 2D). This *in vitro* binding value is consistent with a previous value for the *E. coli* LplT homolog obtained using crude membrane vesicles (13), confirming that *in vitro* data obtained using detergent-solubilized protein represents substrate binding of the LplT transporter on the membrane bilayer. We also used this assay to assess the binding of other lysolipids. LplT-*Kp* protein (0.44 μM) was first mixed with 0.75 μM fluorescent lyso-PE, and then 5 or 10 μM non-fluorescent lyso-PE was added to the solution as indicated to monitor any reversal of fluorescence change. As expected, the addition of lyso-PE increased the fluorescence intensity in a concentration-dependent manner due to its competitive displacement of fluorescent ligand from the LplT protein (Fig. 3A). In contrast, no response was detected by titrating detergent-containing buffer. In addition, no fluorescence change was observed after adding lyso-PC (Fig. 3B) despite the fact that both lyso-PC and lyso-PE share a similar zwitterionic lipid chemical structure. However, either lyso-PG or di-CL was able to evoke a fluorescence intensity change similar to that seen with lyso-PE (Fig. 3, C and D), and therefore these act as competing ligands, displacing fluorescent substrate from the specific LplT binding site. These results strongly indicate specific interactions of the LplT protein with the lyso forms of all three bacterial major membrane phospho-
lipids (PE, PG, and CL). Binding of these lipids is strictly limited to their sn-1 lyso configurations because any diacyl form of palmitoyloleoylphosphatidylethanolamine, palmitoyloleoylphosphatidylglycerol, or tetra-acylated CL induced no fluorescence change at the same concentrations (Fig. 3, A, C, and D, blue curves).

Intermicellar Lipid Transfer—To rule out a potential effect of intermicellar exchange of diacyl phospholipids, we performed a pulldown assay of 32P-labeled PE with the LplT-Kp protein on Ni2+/H11001-NTA resin to confirm that transfer of diacyl lipid indeed exists under these experimental conditions. Based on radioactivity, 15% (8,000 cpm) from the added [32P]PE (total, 53,000 cpm) was associated with the protein-detergent micelles compared with only 0.15% (80 cpm) on the control empty resin lacking LplT. These results clearly demonstrate that [32P]PE was transferred from lipid-detergent micelles and stayed associated with LplT-protein micelles, confirming that non-competitive binding of diacyl lipids is not due to the absence of intermicellar transfer. Therefore, the absence of the sn-2 acyl chain is clearly crucial for substrate binding to the LplT protein. It should be noted that an excess amount of the competing ligands did not restore fluorescence back to the original level. This may be related to the complexity of lyso-phospholipid association/dissociation with detergent vesicles in solution. One possible explanation is that the fluorescent ligand was partially retained in the protein-detergent vesicles after displacement from the binding site.

FIGURE 3. In vitro interaction of LplT protein with lysophospholipids and derivatives. TopFluor lyso-PE was premixed with LplT protein followed by adding 5 or 10 μM (final concentrations) non-fluorescent lipids as indicated by arrows. A, lyso-PE (red), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (blue), or buffer (black); B, lyso-PC (red) or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (blue); C, lyso-PG (red) or 1,2-dioleoyl-sn-glycero-3-phospho-(1′-racemic glycerol) (DOPG) (blue); D, di-CL (red), CL-hg (green), or CL (blue); E, lyso-PA (red) or 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) (blue); F, 1 or 10 mM glycerol 3-phosphate (G-3-P; olive), phosphate (cyan), or vanadate (magenta). Specific interaction of lyso-PE, lyso-PG, di-CL, or CL-hg with LplT induced fluorescence increase due to competitive displacement of fluorescent ligand. a.u., arbitrary units.

Design of LplT-based Transport Assays—The above in vitro assays implied that the LplT protein is involved in remodeling of all three bacterial major membrane phospholipids. To verify the broader substrate selectivity of LplT, we established two in vivo assays in spheroplasts: 1) a remodeling assay to demonstrate lysophospholipid translocation and remodeling by measuring the Aas-dependent acylation of transported lysophos-
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FIGURE 4. Membrane destabilization caused by lplt or aas deletion. A, 1 × 10^9 E. coli cells were used to generate spheroplasts as described under “Experimental Procedures” followed by regeneration in LB medium and subsequent plating on LB agar plates. Colonies were counted to calculate the recovery rate (%) compared with wild type. B, spheroplasts generated from [32P]-labeled cells were treated with venom PLA2, as a function of time. The total lipid exact was separated on a TLC plate and visualized by a phosphorimaging system.

Membrane Destabilization of lplt- or aas- Deletion Strains—Three knock-out mutants, Δlplt, Δaas, and Δlplt/Δaas, were created in an E. coli BL21(DE3) strain background using homologous recombination. These deletion mutants exhibited no difference in cell growth in LB medium (data not shown). However, noticeable alterations were detected in their phospholipid compositions. As illustrated by TLC analysis (Fig. 4B and Table 1), their PE and PG levels were both reduced by 5–8% from 72 (PE) and 20% (PG), and accordingly an additional band representing 7–8% of total phospholipids appeared at a position below the PG spot. This extra spot was mostly represented by lysolipids. Interestingly, the CL amount increased by 3-fold and reached 12–13% in both strains. These changes apparently resulted in dramatic membrane destabilization as follows. 1) Spheroplasts generated from these mutants were extremely unstable in hypotonic LB medium. Only 5 or 12% of the intact cells were recovered from “regenerated” spheroplasts prepared from lplt- or aas- mutant cells, respectively (Fig. 4A), although their integrity could be maintained in isonic solution for 1 h. 2) These spheroplasts displayed high susceptibility to venom PLA2. As seen in Fig. 4B, a massive amount of lysophospholipids was accumulated after incubation with PLA2 for 30 min, reaching more than 70% of total phospholipid content, whereas the amounts of all three major phospholipids (PE, PG, and CL) were reduced by 4–5-fold simultaneously (Table 1). In sharp contrast, spheroplasts from BL21(DE3) wild type exhibited strong resistance to PLA2, and no significant change was observed after 60-min treatment. These disruptive effects revealed that the combination of LplT and Aas plays a primary role in maintaining the native phospholipid composition and membrane integrity of E. coli cells upon PLA2 treatment. The appearance of multiple bands of lysophospholipid further supports the broader substrate selectivity of the LplT/Aas system.

Remodeling of Lyso-PE and Lyso-PG by Lplt/Aas—To perform a lipid transport assay in vivo, we prepared [32P]-labeled lyso-PE, lyso-PG, and lyso-PG using PLA3 from the corresponding radioactive diacyl forms followed by purification on TLC plates. Different E. coli strains, as described under “Experimental Procedures,” were used to improve the yield and purification efficiency of individual [32P]-labeled lipids. Lipid remodeling assays were initiated by mixing radioactive substrates with Δlplt spheroplasts expressing the LplT-Kp protein. LplT-Kp catalyzes flipping of lysophospholipids from the outer surface of the spheroplast onto the inner surface where they are further acylated by endogenous Aas protein. All transport assays were performed in isotonic buffer where spheroplast stability was preserved and carefully monitored. As analyzed on TLC plates (Figs. 5A and 6A), both PE and PG were generated from lyso-PE or lyso-PG, and about 80% of the conversion was complete in 30 min (Figs. 5B and 6B). Catalysis was clearly dependent on the presence of LplT-Kp and Aas in the spheroplasts as no PE or PG was accumulated in either Δlplt or Δaas control samples (Figs. 5A and 6A). The 20% activity observed in these control experiments may be due to spontaneous lipid flipping or activity of other lipid flipase(s) in the cells (13). Remarkably, no activity was observed with lyso-PC (Fig. 5, D and E). These transport assays confirmed that lyso-PE and lyso-PG, but not lyso-PC, are substrates for the LplT and Aas systems.

Kinetics of LplT-mediated Lyso-PE and Lyso-PG Transport—Aas-dependent formation of diacyl forms from the corresponding lysophospholipids was carried out to examine substrate specificity of the LplT/Aas transport/acylation regeneration system. Limitations of this method prevent its use to characterize LplT transport activity per se due to two facts. 1) Substrates flipped into the spheroplasts are indistinguishable from nontransported substrates monitored by TLC because to maintain stability the spheroplasts were not collected by centrifugation before lipid extraction. 2) Whether LplT transport activity is facilitated by downstream Aas-mediated continuous reacylation of transported lysophospholipids driving their downhill uptake is unknown. To overcome this problem, the transport reaction was separated from the assay mixture by centrifugation through a layer of silicon oil. Due to their different sedimentation rates and miscibility with oil, non-transported lipids are retained in silicon oil and the upper layer, whereas the pelleted spheroplasts containing transported substrates are assessed by scintillation counting to measure LplT transport kinetics. To eliminate any coupling effect of Aas, the transport

TABLE 1

Lipid composition of E. coli spheroplasts treated with venom PLA2

| PLA2 digestion time (min) | WT | Δlplt | Δaas |
|--------------------------|----|-------|------|
| CL (%)                   | 4  | 9     | 13   |
| PE (%)                   | 72 | 73    | 78   |
| PG (%)                   | 20 | 14    | 15   |
| Lysolipids (%)           | 4  | 4     | 8    |

*Table data are from Table 1 in the original text.*
assay was performed in an $\Delta lplt/\Delta aas$ mutant-expressing LplT-Kp. Transport kinetic analysis revealed that both lyso-PE and lyso-PG exhibit similar apparent binding constants: $K_{1/2} = 1.43 \pm 0.07 \mu M$ for lyso-PE (Fig. 5C) and $2.99 \pm 0.15 \mu M$ for lyso-PG (Fig. 6C). The maximal rate of uptake ($V_{\text{max}}$) was $0.097 \pm 0.006 \mu mol/g$ dry weight/h for lyso-PE and $0.081 \pm 0.007 \mu mol/g$ dry weight/h for lyso-PG. No activity was detected with lyso-PC (Fig. 5F), confirming the feasibility of this approach. It is challenging to study lipid transporter kinetics because of the nature of their substrates. In our assays, LplT-independent lipid insertion in the control experiments remains minimal after spheroplast separation through silicon oil. Therefore, this simple approach may be generally applicable to study transport kinetics of other lipid flippases.

Novel CL Deacylation Activity of Venom PLA$_2$—CL remodeling is unknown in bacterial cells. To explore any involvement of LplT/Aas in cardiolipin recycling, we generated E. coli CL derivatives using venom PLA$_2$. Previous studies by other groups have shown a stepwise process of CL deacylation by exogenous PLA$_2$ with tri-CL and di-CL as products (16, 25). These intermediates can be easily identified by their different migration rates on TLC (Fig. 7B). However, we observed a distinct CL in
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Figure 6. Transport and remodeling of lyso-PG, not lyso-PA, by LplT/Aas.

A and D, lyso-PG or lyso-PA was mixed with spheroplasts prepared from E. coli Δplt cells expressing LplT-Kp or harboring the empty vector or from Δaas mutant cells. The reactions were stopped by chloroform at the indicated times, and total lipids were extracted and then separated by thin-layer chromatography. B and E, quantification of substrates and acylated products shown in A and D using a phosphorimaging system. C and F, transport assays of lyso-PG or lyso-PA by LplT-Kp in spheroplasts from Δplt/aas mutant cells. Spheroplasts from E. coli Δplt/aas mutant cells harboring the empty vector were used as control. Error bars represent S.E.

vitro deacylation profile. As seen in Fig. 7A, venom PLA2 directly generated di-CL, and no tri-CL was detected in the reaction. The reaction was complete after 2 h at 37 °C. As verified by mass spectroscopy analysis, the reaction generated 80% di-CL (18:1/18:1; molecular weight [M – H]−, 927.8) and 20% mono-CL (18:1; molecular weight [M – H]−, 663.5) (Fig. 7C). Interestingly, continuing this reaction overnight further digested di-CL and mono-CL to a new product that remained largely in the aqueous phase during lipid chloroform extraction (Fig. 7A). Surprisingly, this polar product was identified by ESI-MS as bis(glycero-3-phospho)glycerol (molecular weight, 398.2) and directly corresponds to the CL headgroup (referred to as CL-hg), indicating complete deacylation of cardiolipin by PLA2 (Fig. 7D). This novel PLA2 reaction is independent of the CL source because similar results were observed with both 32P-labeled CL extracted from the AL95 strain and synthetic CL purchased from Avanti Polar Lipids.

Remodeling of CL Derivatives by LplT/Aas—This pair of CL derivatives provides a great opportunity to study CL remodeling in bacteria, in particular to assess the contribution of sterically distinct fatty acid chains to the transport mechanism of LplT. Because both di-CL and CL-hg were capable of direct interaction with the LplT protein (Fig. 3D), we further studied their remodeling in spheroplasts. Unexpectedly, spheroplasts expressing LplT-Kp actively imported both CL derivatives, simultaneously generating a unique product migrating slower than CL on TLC plates (Fig. 8, A and D). No other intermediates were found in the reaction. This regenerated product was identified as tri-CL based on 1) its migrating position on TLC plates and 2) the fact that its formation was exclusively dependent on the presence of Aas. LplT/Aas apparently utilized CL-hg more efficiently than di-CL; i.e. more than 80% of CL-hg conversion was complete in 40 min, whereas catalysis reached the plateau phase with 50% of di-CL remaining in the reaction (Fig. 8, B and E). Interestingly, both di-CL and CL-hg exhibited similar transport kinetics: $K_{1/2}$ = 1.55 ± 0.12 μM for di-CL (Fig. 8C) and 2.56 ± 0.22 μM for CL-hg (Fig. 8F), and $V_{max}$ = 0.066 ± 0.005 μmol/g dry weight/h for di-CL and 0.062 ± 0.011 μmol/g dry weight/h for CL-hg. Therefore, we concluded that the lower remodeling efficiency of di-CL is not limited by LplT function but rather is due to the different reacetylation activities of Aas for these substrates. Nevertheless, these experiments offer the first evidence of CL remodeling in bacterial cells. No observed difference in transport kinetics between di-CL and CL-hg strongly suggests that the fatty acid chain is not involved in LplT substrate binding.

To further examine substrate selectivity requirements, we next focused on the headgroup of lysophospholipids. To determine whether the headgroup is required for LplT substrate recognition, we tested lyso-PA and synthetic 1,2-dioleoyl-sn-glycero-3-phosphate. Elimination of further PA headgroup modification clearly disrupted substrate binding and uptake in all three types of LplT assays (Figs. 3E and 6, D and F). Therefore, most likely LplT is not coupled to de novo phospholipid biosynthesis. Because the CL-hg experiment showed that the acyl chain is not required for substrate binding, we also tested orthophosphate, orthovanadate, and glycerol 3-phosphate. No inhibition of competitive ligand binding was found (Fig. 3F), suggesting that these phospholipid structural components or analogs are not essential, at least solely, for substrate binding.

Discussion

In this study, we demonstrated for the first time the important role of tandem LplT/Aas transport and acylation in the maintenance of membrane stability by counteracting disruptive effects of endogenous lysophospholipids in the bacterial envelope (Fig. 4). We demonstrated, using in vitro and in vivo assays, that LplT facilitates uptake of lyso forms of all three major bacterial membrane phospholipids into the cell for Aas-mediated acylation on the cytosolic membrane surface (Fig. 1B). The transport kinetics of LplT for lyso-PE, lyso-PG, di-CL, and CL-hg are nearly indistinguishable, suggesting its role as a general facilitator for bacterial membrane remodeling. Aas was previously described as 2-acyl lyso-PE acyl-
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A

AL95 strain total lipids

PLA2 digestion time (min)

0 5 10 30 40 o/n

B

DOPG CL Tri-CL Di-CL CL-hg

C

Di-CL

Mono-CL

Di-CL (18:1/18:1) [M-H]

927.820

928.824

928.826

961.790

D

Mono-CL (18:1) [M-H]

663.494

664.497

665.499

685.481

E

cardiolipin

Mw: 1405

PLA2

Di-CL

Mw: 928

PLA2

Mono-CL

Mw: 664

PLA2

CL-hg

Mw: 398

Tri-CL

Mw: 1190

LpiT/Aas

LpiT/Aas
**FIGURE 7.** PLA₂-catalyzed CL deacylation. A, ³²P-labeled CL from the AL95 strain digested by venom PLA₂ as a function of time was separated by TLC and detected by a phosphorimaging system. B, PG, CL, and CL derivatives show different migration rates on TLC developed by iodine vapor. C, LC/MS/MS analysis shows 80% di-CL and 20% mono-CL after 2-h PLA₂ digestion of CL. D, ESI-MS analysis of CL digestion after an overnight reaction shows complete deacylation and liberation of the CL headgroup. E, chemical scheme of PLA₂-mediated CL deacylation and LplT/Aas-mediated remodeling reactions in *E. coli*. CL is deacylated by venom PLA₂, to di-CL, then to mono-CL, and eventually to CL-hg. Both di-CL and CL-hg are reacylated to tri-CL. DOPG, 1,2-dioleoyl-sn-glycero-3-phospho-(1'racemic glycerol).

**FIGURE 8.** Transport and remodeling of two CL derivatives by LplT/Aas. A and C, di-CL or CL-hg was mixed with spheroplasts prepared with *E. coli* Δiplt cells expressing LplT-Kp or harboring the empty vector or from Δaas cells as indicated. The reactions were stopped by chloroform/methanol (1:2) at the indicated times, and then extracted lipids were separated by TLC. B and E, quantification of substrates and acylated products shown in A and D using a phosphorimaging system. C and F, transport assays of di-CL and CL-hg by LplT-Kp in spheroplasts from Δiplt/Δaas mutant cells. Spheroplasts from the *E. coli* Δiplt/Δaas mutant strain harboring the empty vector were used as control. Error bars represent S.E.
transferase. Given its ability to reacylate multiple substrates demonstrated here, Aas should be renamed lysosphospholipid acyltransferase.

We also characterized a novel reaction of PLA2 that can completely deacylate CL to form a bald headgroup compound (Fig. 7). To our knowledge, complete hydrolysis of a CL molecule by PLA2 has not been reported in the literature. In previous PLA2 studies, di-CL is the major product of secreted PLA2 from Naja naja venom and human non-pancreatic secreted PLA2 (16). Many lipases also possess PLA1 activity. Human calcium-dependent PLA2 was suggested to hydrolyze both 1-acyl- and 2-acyl-lysophospholipids (16). All these reported assays utilized a relatively short reaction time (minutes). However, we found that CL hydrolysis can proceed via two major stages. The first stage of the reaction was paused at di-CL for at least 2 h before proceeding to the next stage to form the fully deacylated product CL-hg. It is unlikely that this is due to a less efficient catalytic digestion of di-CL as substrate because purified heart di-CL (Avanti Polar Lipids) at the same enzyme/substrate ratio was converted to CL-hg during the same time interval (data not shown). Therefore, one possibility is that the second stage hydrolysis was inhibited until all cardiolipin was used up in the hydrolytic reaction.

It remains unknown whether this CL-hg compound exists in living cells. However, both di-CL and CL-hg were taken up by LplT and reacylated to tri-CL. Tri-CL is the only detectable product in both remodeling assays. It is still uncertain why tri-CL is the final product of CL repair in living bacterial cells because our assays were performed in lysozyme-treated spheroplasts, and the reaction time was limited to 1 h due to the limited stability of spheroplasts. In fact, tri-CL remodeling is important for CL biosynthesis in mammalian cells. Tafazzin, a CL acyltransferase, modifies tri-CL by transferring linoleic acid from PC to generate CL on the mitochondrial membrane (31). Mutation of tafazzin affects CL remodeling and interrupts ATP synthesis (31). Di-CL and tri-CL have also been found in E. coli membrane fractions supplemented with lyso-PG and in an E. coli strain deficient in CL synthetase (clsA), suggesting involvement of ClsA in CL remodeling (32). Recently, two additional CL synthetases, ClsB and ClsC, have also been identified and characterized in E. coli (24, 33). Although all these Cls proteins belong to the phospholipase D superfamily, their diverse catalytic mechanisms may support an alternative synthesis of CL using different lyso derivatives as co-substrates.

Our results identified the PE or PG headgroup moieties as important for substrate binding and transport because lyso-PA was a non-efficient substrate in both types of assay (Fig. 1B). Di-CL is formed by two crosslinked lyso-PG molecules. The comparable activity of CL-hg as substrate completely eliminates any contribution of the fatty acid chain to the substrate binding mechanism. This was further confirmed by the in vitro binding assay in which the bulky fluorophore moiety attached at the end of the sn-1 acyl chain did not inhibit substrate binding. Substrate binding is not likely mediated by the phosphate group or glycerol backbone because no inhibition was found by orthophosphate, orthovanadate, or glycerol 3-phosphate (Fig. 3F). These results conclusively identify the ethanolamine or glycerol moiety as the crucial chemical determinant for substrate binding. This mechanism also excludes access of the choline group into the binding site as it is probably hindered by its bulky trimethylated group. PC is a major lipid in eukaryotic cells. Most bacteria including E. coli cannot synthesize PC. Exclusion of lyso-PC ensures efficient recycling of E. coli endogenous lipids and may also prevent any incorporation of foreign lipids into the bacterial membrane.

In this study, we used 1-acyl-lysolipids generated by PLA2 (Fig. 1B). LplT and Aas were previously proposed to have specificity for 2-acyl substrates based on the chemical mechanism of lipoprotein acyltransferase. Harvat et al. (13) using 2-acyl-lyso-PE generated by the Rhizopus arrhizus lipase determined a $K_d$ value for this E. coli homolog 5× lower than our result determined with the LplT-Kp protein and 1-acyl isomer. It may be difficult to determine the stereoselectivity of LplT because compelling evidence from different groups has shown that 2-acyl-lysophospholipids are extremely unstable in biological solution and are quickly converted to the 1-acyl-2-lyso form by a spontaneous intramolecular acyl migration, yielding a mixture mainly containing 1-acyl-2-lysoglycerophospholipids (15, 34). We cannot rule out utilization of both lysolipid isomers by LplT/Aas on the bacterial membrane.

All lipid molecules share a common asymmetric polarity with a polar headgroup and hydrophobic acyl tail(s). To achieve lipid flip-flopping across the cell membrane, its polar lipid headgroup has to pass through the hydrophobic membrane bilayer, which requires a large cost in free energy. A plausible hypothesis for a general flipase mechanism is that the substrate headgroup is tumbled through a pathway inside the transporter protein with its hydrophobic tail(s) sliding within the hydrophobic plane of the membrane bilayer during translocation (35). This rotation helps to overcome the free energy barrier for the polar headgroup to pass through the hydrophobic part of the lipid bilayer. Our data are consistent with this hypothesis, which can explain the LplT-mediated lysosphospholipid transport mechanism. However, in the recently described PglK (flipase) structure, one elongated groove on the protein membrane interface may serve as a pathway to facilitate diacylphospholipid translocation (36). The lipid translocating pathway in the PglK structure is largely open on the external protein surface. This model may not apply to LplT because its substrate specificity is highly selective for lysolipids, and no apparent inhibition by any diacyl forms was found in our competitive binding assay. Therefore, LplT must utilize a specific mechanism for lysolipid recognition and transport. This specificity would be critical for efficient lipid repair in an environment containing more than 90% diacyl lipids given that only 8% lysolipids are sufficient to destabilize the bacterial cell membrane.

To explore the specific mechanism of LplT catalysis, we generated a structural model of LplT-Kp using the I-TASSER server (37). Interestingly, among several structures available in the major facilitator superfamily, the structure of the glycerol 3-phosphate transporter GlpT was automatically chosen as template. As expected for a major facilitator superfamily mem-
ber (38–41), the structural model shows 12 transmembrane (TM) helices with pseudo-2-fold symmetry between two helical bundles, TMs 1–6 (green) and TMs 7–12 (gold). The groove between TMs 2 and 9 is highlighted in a dashed oval. The image was generated using PyMOL (43). B, evolutionary representation of the LplT space-filling model (variable residues are in blue, and conserved residues are in red) shows that the outer surface is highly varied except the highlighted groove formed by TM helices 2 and 9. C, wide distribution of the most conserved residues along the central cavity. Other residues are depicted as ribbons. D, the proposed "sideways sliding" mechanism of the LplT transporter for lysophospholipid remodeling in E. coli. Lyso-PE, lyso-PG, di-CL, or CL-hg is flipped through the narrow groove between the two domains of LplT (in green or gold) into the cytosolic surface for acylation by Aas to generate the corresponding product PE, PG, or tri-CL.

**Author Contributions**—Y. L., M. B., and L. Z. designed the study, performed experiments, and wrote the paper. S. T. generated the LplT expression vector, established the protein expression and purification protocol, and designed the fluorescent substrate binding assay. Z. Q. performed LC/MS/MS mass spectrometry analysis. All authors analyzed the results and approved the final version of the manuscript.

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