A Novel Phosphatidylinositol 4,5-Bisphosphate Binding Domain Mediates Plasma Membrane Localization of ExoU and Other Patatin-like Phospholipases

Background: The Pseudomonas aeruginosa cytotoxin ExoU localizes to the plasma membrane in eukaryotic cells.

Results: ExoU and related proteins utilize a conserved four-helical bundle to bind the lipid phosphatidylinositol 4,5-bisphosphate for localization.

Conclusion: The membrane localization domain of ExoU represents a novel phosphoinositide binding domain.

Significance: This is the first report of a four-helical bundle with specificity for phosphatidylinositol 4,5-bisphosphate.

Bacterial toxins require localization to specific intracellular compartments following injection into host cells. In this study, we examined the membrane targeting of a broad family of bacterial proteins, the patatin-like phospholipases. The best characterized member of this family is ExoU, an effector of the Pseudomonas aeruginosa type III secretion system. Upon injection into host cells, ExoU localizes to the plasma membrane, where it uses its phospholipase A2 activity to lyse infected cells. The targeting mechanism of ExoU is poorly characterized, but it was recently found to bind to the phospholipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), a marker for the plasma membrane of eukaryotic cells. We confirmed that the membrane localization domain (MLD) of ExoU had a direct affinity for PI(4,5)P2, and we determined that this binding was required for ExoU localization. Previously uncharacterized ExoU homologs from Pseudomonas fluorescens and Photobacteroides asymbiotica also localized to the plasma membrane and required PI(4,5)P2 for this localization. A conserved arginine within the MLD was critical for interaction of each protein with PI(4,5)P2 and for localization. Furthermore, we determined the crystal structure of the full-length P. fluorescens ExoU and found that it was similar to that of P. aeruginosa ExoU. Each MLD contains a four-helical bundle, with the conserved arginine exposed at its cap to allow for interaction with the negatively charged PI(4,5)P2. Overall, these findings provide a structural explanation for the targeting of patatin-like phospholipases to the plasma membrane and define the MLD of ExoU as a member of a new class of PI(4,5)P2 binding domains.

Bacteria use a number of mechanisms to inject toxins into eukaryotic cells (1). Once inside these cells, the toxins perform a variety of functions to disrupt host cell physiology (2–4). To adequately perform these functions, it is critical that toxins are localized to the appropriate intracellular compartment. A number of bacterial toxins have dedicated membrane localization domains (MLDs)4 that vary considerably in their targeting specificities and mechanisms (5). For instance, the botulinum neurotoxin A concentrates at the plasma membrane of neurons by direct affinity to the protein SNAP-25 (6). Other proteins localize to different membrane compartments by undergoing covalent lipid modification, such as the Salmonella effector SifA, which targets the plasma membrane following farnesylation (7). Still other proteins, such as the Pasteurella multocida toxin, have direct affinity for lipids (8). The many sophisticated and resourceful mechanisms by which bacterial toxins undergo intracellular targeting underscore the importance of proper localization to toxin function (7, 9). Although the examples listed above illustrate several well characterized mechanisms of toxin localization, it remains unclear how the majority of bacterial toxins are targeted to the appropriate intracellular compartment.

Patatin-like phospholipases are an important and intriguing family of microbial proteins. They are defined by the presence
of a patatin domain, which encodes for phospholipase A$_2$ (PLA$_2$) activity that cleaves phospholipids at the sn-2 position (4). Patatin-like phospholipases are closely related to eukaryotic group IV cytosolic PLA$_2$ (cPLA$_2$) and group VI calcium-independent PLA$_2$ enzymes, which share a defined serine-aspartate catalytic dyad (10, 11). While only recently identified, this family of proteins is quite large: ~4400 potential proteins containing typical patatin domains are encoded in sequenced bacterial genomes (12, 13). Only a few of these proteins have been characterized, but these limited studies have found that patatin-like phospholipases can be delivered by type III, type IV, or type V secretion systems into host cells (11, 14, 15). Once in the eukaryotic intracellular environment, the PLA$_2$ activities are associated with host cell death, disruption of signaling pathways, and bacterial internalization (15, 16, 18).

Arguably, the best characterized member of the family of patatin-like phospholipases is ExoU of _Pseudomonas aeruginosa_. This protein was initially discovered based upon its ability to confer cytotoxicity to _P. aeruginosa_ strains and its secretion by the type III secretion system (19–21). Subsequent studies have defined it as one of the predominant virulence determinants of _P. aeruginosa_, as the secretion of ExoU is associated with poor clinical outcomes in human patients and with more severe infection in animal models (22, 23). ExoU does not act as a PLA$_2$ enzyme on its own but instead requires eukaryotic host cofactors to be activated (24). Ubiquitin and ubiquitinated proteins have been identified as important for the activation of ExoU (25), as has the lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) (26). Together, ubiquitin and PI(4,5)P$_2$ activate ExoU synergistically, with each being important to the cytotoxic action of ExoU (26).

The C-terminal MLD of ExoU is necessary and sufficient for targeting of this effector protein to the plasma membrane (27). The crystal structure of ExoU in complex with its bacterial chaperone SpcU revealed that the MLD region encompasses residues 503–687 (28, 31). Furthermore, the C-terminal half (residues 604–687) of the MLD forms a four-helical bundle, a structural motif used by other toxins to target the plasma membrane (8, 29). Several key residues within the four-helical bundle have been identified as being critical to ExoU localization (27, 30), but the mechanism by which the MLD specifically recognizes the plasma membrane is unclear. Interestingly, it was recently shown that purified recombinant ExoU bound to PI(4,5)P$_2$, immobilized on a solid support and that this binding required the MLD (31). Subsequent work has established that ExoU has a high affinity for PI(4,5)P$_2$ and can use PI(4,5)P$_2$ as a substrate (32). PI(4,5)P$_2$ is a phospholipid found in the inner leaflet of the plasma membrane of eukaryotic cells (but not bacteria) (33), where it is recognized as a “signpost” to appropriately direct a number of endogenous proteins to this intracellular compartment (34). To dock at the plasma membrane, these proteins use a number of well characterized PI(4,5)P$_2$ binding domains (e.g. pleckstrin homology, PSD-95/Dlg/ZO-1, and FERM domains), which play important roles in numerous cellular processes (35, 36). Although ExoU does not contain a known PI(4,5)P$_2$ binding domain, its high affinity for PI(4,5)P$_2$ has led others to hypothesize that it may localize to the plasma membrane by binding PI(4,5)P$_2$ (31, 32). Thus, it is conceivable that the MLD of ExoU contains a novel PI(4,5)P$_2$ binding domain.

In this study, we demonstrate that ExoU localizes to the plasma membrane through direct binding of the MLD to PI(4,5)P$_2$. We extended these findings to patatin-like phospholipases of _Pseudomonas fluorescens_ and _Photorhabdus asymbiotica_, demonstrating that the MLD of ExoU is a domain used by proteins of other bacterial genera and species to target the plasma membrane. To gain more information about this family of MLDs, we determined the crystal structure of the ExoU homolog of _P. fluorescens_. This structure was used along with the previously determined structure of _P. aeruginosa_ ExoU to show that the four-helical bundle is conserved within the MLD and is positioned to expose a conserved positively charged arginine residue to interact with the negatively charged phosphate groups of PI(4,5)P$_2$ in the plasma membrane. These results define a novel PI(4,5)P$_2$ binding domain used by bacterial proteins to target the plasma membrane of host cells.

**EXPERIMENTAL PROCEDURES**

*Cell Lines, Bacterial and Yeast Strains, and Media—*Bacterial and yeast strains are listed in Tables 1 and 2, respectively. _Escherichia coli_ strains were grown in Luria-Bertani (LB) broth, and when appropriate, media were supplemented with 100 µg/ml ampicillin. _P. fluorescens_ strain A506 was a generous gift from Joyce Loper. _P. asymbiotica_ strain ATCC 43949 was acquired from the ATCC. _Saccharomyces cerevisiae_ strains SEY6210 (wild-type) and Δinp54/Δsac1, which were generous gifts from Peter Mayinger, were grown in yeast extract peptone dextrose (YPD) medium. Yeast strains expressing glucose-repressible, galactose-inducible pYc vectors were grown in synthetic complete medium lacking uracil and supplemented with 2% glucose (SC-ura + glu) or 2% galactose (SC-ura + gal) for varied expression of proteins (26). HeLa cells were cultured in modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen).

*Purification of ExoU and Its Homologs—*_P. fluorescens_ and _P. asymbiotica_ were grown overnight in LB broth, and genomic DNA was isolated with the DNeasy blood and tissue kit (Qiagen). Primers containing HindIII and NotI sites were used to amplify the _exoU_ gene homolog from each species. (All primers used in this study are listed in Table 3.) PCR amplification products and the HN-C expression vector (Clontech) were digested with HindIII and NotI restriction enzymes, and the products were ligated and transformed into BL21 (DE3) Star competent cells. (All plasmids used in this study are listed in Tables 1 and 2.) ExoU proteins were purified as described previously using a HisTrap FF nickel column and a HiPrep 26/10 desalting column (GE Healthcare) (26). For purification of MLD proteins, corresponding primers were used to amplify the portion of the gene encoding the MLD, and amplification products were digested with HindIII and NotI. The digested products were ligated into a similarly digested HN-N vector (Clontech), and the ligated construct was transformed into BL21 (DE3) Star competent cells. Purification of these proteins was performed as described above.

*HeLa Cell Cytotoxicity Assays—*ExoU proteins were expressed in HeLa cells by transfection of expression vectors. Constructs
expressing wild-type ExoU from *P. aeruginosa* (hereafter referred to as ExoU<sub>P.aer</sub>) and variants containing amino acid substitutions had previously been generated using plasmid pcDNA3.1 NT-GFP (30). To generate similar constructs expressing ExoU homologs from *P. fluorescens* and *P. asymbiotica* (ExoU<sub>P.flu</sub> and ExoU<sub>P.asy</sub>, respectively) primers containing flanking Agel and NotI sites were used to amplify the ExoU-encoding genes from the respective purification vectors. Then the pcDNA3.1 NT-GFP vector (Invitrogen) and the PCR products were digested with Agel and NotI. The digested products were purified, ligated, and transformed into *E. coli* Top10 cells. The catalytic site substitutions were engineered by site-directed mutagenesis of exoU<sub>P.flu</sub> and exoU<sub>P.asy</sub> using the QuikChange site-directed mutagenesis kit (Agilent) (30) and the primers listed in Table 3. The constructs encoding the R661L substitution in ExoU<sub>P.aer</sub> and the corresponding substitutions in ExoU<sub>P.flu</sub> and ExoU<sub>P.asy</sub> were similarly engineered in the pcDNA plasmids by site-directed mutagenesis using primers listed in Table 3. Plasmids were purified using the QiAprep spin miniprep kit (Qiagen). X-treme gene transfection reagent (Roche Applied Science) was coupled with the DNA transfection constructs in serum-free medium to perform transfections with HeLa cells as described previously (30). Medium was collected after 24 h and measured for lactate dehydrogenase activity using the Cytotox 96 nonradioactive cytotoxicity assay (Promega).

**Immunoblot Analysis for Detection of GFP-tagged Proteins**—The same constructs used in the HeLa cell cytotoxicity assays were used for expression of GFP-tagged ExoU proteins from the pcDNA3.1 NT-GFP vector. Lipofectamine 2000 (Invitrogen) was coupled with DNA in serum-free medium and trans-
fected onto 10-cm dishes of HeLa cells. After 24 h, cells were washed, collected, and lysed as described previously (26). Samples were run on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated in blocking buffer for 2 h at room temperature, as described previously (26). The membrane was incubated overnight at 4 °C with gentle shaking. The membrane was washed and incubated with goat anti-mouse secondary antibody IR dye 800 (Li-Cor Biosciences) diluted 1:10,000 in blocking buffer with 0.1% Tween 20, for 1 h at room temperature with gentle shaking. The membrane was incubated overnight with gentle shaking. The membrane was washed again, and blots were imaged using the Li-Cor Odyssey system.

HeLa Cell Fluorescence Microscopy—As described for the cytotoxicity assays, the MLD-encoding portions of exoU genes (both wild-type alleles and those encoding arginine-to-leucine substitutions) were amplified by PCR from the pcDNA3.1 NT-GFP vectors. The exoU<sub>p.assy</sub> MLD was similarly amplified. A pYC2/NT A vector (Invitrogen), which contains a glucose-repressible, galactose-inducible promoter (37), was digested with HindIII and XbaI. Using the LiAc heat shock method (37), 15 μl of PCR product and 1 μl of digested plasmid DNA were ligated within Δ<i>imp54</i>/Δ<i>scs1</i> strains, and appropriate colonies were selected on synthetic complete medium lacking uracil.

Visualization of Yeast Strains—Yeast strains were grown in SC-ura + Gal medium to induce expression of the GFP-tagged constructs. Yeast were immobilized in 1% agarose solution on coverslips and visualized using a Nikon C2+ multispectral laser scanning confocal microscope.

Liposome Binding Assays—Liposome binding assays were performed as described previously (38). Briefly, a neutral lipid backbone was prepared with 30% phosphatidylcholine (PC), 20% phosphatidylethanolamine (PE), and 20% cholesterol (Avanti Polar Lipids). The remaining lipids were 30% PC, 30% PE, 30% phosphatidylserine (PS), 30% phosphatidylinositol, or a combination of 25% PC with 5% phosphatidylinositol, PI(4)P, PI(4,5)P<sub>2</sub>, or PI(3,4,5)P<sub>3</sub>. These lipids were mixed as chloroform stocks that were dried under nitrogen gas and then by vacuum. Lipids were resuspended in liposome buffer (100 mM KCl, 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> in 20 mM HEPES (pH 7.5)) to a final concentration of 3 mM and sonicated. ExoU pro-
tein was added to a final concentration of 3 μM (38). Protein and liposomes were coincubated at 37 °C for 5 min. Ultracentrifugation was then performed at 200,000 × g for 2 h at 25 °C. The supernatants were removed, and the pellets were resuspended in an equal volume of liposome buffer. Samples were run on 4–15% gradient SDS-polyacrylamide gels, and proteins were visualized by Coomassie staining. Band intensity was analyzed using Image J version 1.43 software.



**PLA<sub>2</sub> Assays—**PLA<sub>2</sub> assays were performed using the Cayman Chemical cPLA<sub>2</sub> kit as described previously (26). Briefly, a total of 65 pmol of ExoU was added to 200 μl of 1.5 mM arachidonoyl thiophosphatidylcholine substrate for each assay condition. When indicated, 65 pmol of PI(4,5)P<sub>2</sub> (Avanti Polar Lipids) or 1.3 nmol of 55% PC, 20% PE, 20% cholesterol, and 5% PI(4,5)P<sub>2</sub>-containing liposomes were added. In addition, 65 pmol or 65 nmol of ubiquitin (Sigma) were added when indicated. Absorbance was measured at 405 nm at the times indicated after the addition of 10 μl of 25 mM 5,5’-dithiobis(2-dinitrobenzoic acid). The PLA<sub>2</sub> activity of ExoU was calculated using the following formula: A<sub>405</sub>/10.00 × 1/(nmol of ExoU), where 10.00 is the extinction coefficient for 5,5’-dithiobis(2-dinitrobenzoic acid).

**Protein BLAST Analysis—**Nonredundant protein sequences were analyzed for similarity to ExoU<sub>P. aer</sub> (UniProtKB accession number O34208) by performing protein-protein BLAST searches (39). MLD similarity was based upon ExoU<sub>P. aer</sub> residues 503–687, and homologs from different bacterial species were analyzed for alignment of key residues.

**Surface Plasmon Resonance (SPR) Analysis—**SPR analysis was performed on a BIAcore X instrument with a lipid-coated L1 chip as described previously (40). Lipids were prepared by mixing chloroform stocks and then drying them under nitrogen gas. Lipids were resuspended in 160 mM NaCl in 20 mM Tris (pH 7.4) to a final concentration of 400 μg/ml, sonicated, and extruded for uniform 100 nm liposome size. Kinetic and equilibrium SPR measurements were performed with the flow rate set at 30 and 10 μl/ml, respectively. Experiments determining <i>K<sub>d</sub></i> values were performed in triplicate. PC vesicles, to which ExoU has no detectable affinity, were used for the control surface. The active surface was coated with liposomes containing 77% PC, 20% PS, and 3% phosphoinositides or with 97% PC and 3% phosphoinositides. Assuming a Langmuir-type binding between the protein and protein-binding sites on vesicles, <i>R<sub>eq</sub></i> values were then plotted versus the total protein concentration (<i>[P]<sub>tot</sub></i>), and <i>K<sub>d</sub></i> values were determined by nonlinear least squares analysis of the binding isotherm using the equation: 

\[
R_{eq} = R_{max}/(1 + K_d/[P]_{tot})
\]

**Crystallization of ExoU<sub>P. flu</sub>—**Crystallization of ExoU<sub>P. flu</sub> was performed at a protein concentration of 5.8 mg/ml in 10 mM Tris-HCl (pH 8.3) buffer containing 500 mM NaCl and 5 mM 2-mercaptoethanol by the sitting-drop vapor diffusion method at 295 K. Crystals suitable for data collection were obtained from the Classics II Suite (Qiagen Inc., Valencia, CA) condition consisting of 0.2 M ammonium acetate, 25% (w/v) PEG 3350 in 0.1 M BisTris (pH 5.5/6.5) buffer. Crystals were soaked in the well/crystallization solutions for cryoprotection and flash-frozen in liquid nitrogen for x-ray data collection.

**Table 4**

| Data collection and structure-refinement statistics | Values in parentheses are for the highest resolution shell.
|----------------------------------------------------|---------------------------------------------------------------|
| Data collection | P<sub>2</sub>, |
| Space group |  |
| Cell dimensions | a, b, c (Å) | 67.2, 115.3, 88.4 |
| | β (°) | 102.9 |
| | No. of reflections | 43,226 (1,696) |
| | Resolution (Å) | 30.00-2.50 (2.54-2.50) |
| | R<sub>merge</sub> (%)<sup>a</sup> | 7.0 (44.1) |
| | Completeness (%) | 15.7 (2.1) |
| | Average redundancy | 95.2 (75.7) |
| Refinement | |
| Resolution (Å) | 29.86-2.50 (2.56-2.50) |
| Completeness (%) | 94.8 (74.1) |
| No. of reflections | 41,015 (2,344) |
| R<sub>work</sub>/R<sub>free</sub> (%) | 20.6/26.9 (32.4/42.1) |
| No. of atoms |  |
| Protein (chain A/B) | 4,060/3,895 |
| Water (oxygen atoms) | 207 |
| B-factor (Å<sup>2</sup>) |  |
| Overall | 56.7 |
| Protein (chain A/B) | 47.9/66.5 |
| Water | 47.5 |
| Coordinate deviation |  |
| r.m.s.d. bond lengths (Å) | 0.014 |
| r.m.s.d. bond angles (°) | 1.609 |
| Ramachandran statistics<sup>b</sup> |  |
| Most favorable (%) | 94.6 |
| Allowed (%) | 5.0 |
| Generously allowed (%) | 0.5 |
| Outside allowed (%) | 0.0 |

<sup>a</sup> <i>R<sub>merge</sub></i> = \( \sum_{hkl} f_{o} - \sum_{hkl} f_{c} \)/\( \sum_{hkl} f_{o} \)

<sup>b</sup> Statistics are based on PROCHECK (17).

<sup>c</sup> r.m.s.d. is root mean square deviation.

**Data Collection and Structure Determination—**A single-wavelength (λ = 0.97856) oscillation x-ray data set was collected on the Life Science Collaborative Access Team (LS-CAT) 21-ID-G. Diffraction images were indexed and scaled with HKL-2000 (41) to 2.5 Å resolution. Cell content analysis indicated the presence of two molecules of ExoU<sub>P. flu</sub> (V<sub>m</sub> (Matthews coefficient) = 24; versus (solvent content) = 49.5%) within the P<sub>2</sub><sub>1</sub> space group at 2.5 Å resolution. A partial molecular replacement solution of ExoU<sub>P. flu</sub> was obtained running Phaser (42) from the CCP4 package (43) and the ExoU<sub>P. aer</sub> structure (Protein Data Bank 3TU3 (28)) as a search model. The structure solution was rebuilt, and water molecules were added using ARP/wARP (44) from the CCP4 package. The structure was refined with REFMAC version 5.7 (45) from the CCP4 package with further manual building and alteration in Coot (46, 47). The quality of the structure was checked with the Protein Data Bank validation server (ADIT validation server) and MolProbity (48, 49). Total buried surface area of the ExoU<sub>P. flu</sub> dimer was determined by the “Protein interfaces, surfaces, and assemblies” service PISA at the European Bioinformatics Institute (50). Structure figures were generated with PyMOL (51). Data collection and structure-refinement statistics are given in Table 4. The final model was deposited in the Protein Data Bank under accession code 4QMK. Diffraction images for the deposited structure are available at the Center for Structural Genomics of Infectious Diseases (CSGID) website.
Statistical Methods—Student’s *t* test was used to compare means for cytotoxicity assays and yeast viability experiments. Significance was defined as a *p* value of <0.05. One-way analysis of variance was used for all other assays with multiple comparisons with the Bonferroni multiple comparisons test to determine significance.

RESULTS

**MLD of ExoU Binds to PI(4,5)P$_2$**—We sought to determine whether ExoU localization to the plasma membrane was mediated by direct affinity to phospholipids, as has been reported for several other toxins (8, 38). Although ExoU was known to bind to PI(4,5)P$_2$, it was unclear whether this was required for localization or whether the MLD of ExoU was sufficient to mediate this binding (32). To investigate this, we purified the recombinant MLD of ExoU (residues 503–687) and tested it for co-sedimentation with neutral liposomes supplemented with various phospholipids (38, 52). We used a final lipid concentration of 3 mM, which has previously been used to effectively assess lipid-protein interactions (38, 52). We found that liposomes supplemented with either PI(4,5)P$_2$ or PI(4)P bound to ExoU substantially more than other tested lipids (Fig. 1A). ExoU binding to PI(4,5)P$_2$ is consistent with its plasma membrane targeting, as PI(4,5)P$_2$ is found specifically in the plasma membrane of eukaryotic cells (27, 53). In contrast, PI(4)P is enriched in the Golgi, although a subpopulation of PI(4)P is in the plasma membrane and may contribute to plasma membrane targeting of some proteins (54). These findings are consistent with ExoU binding to PI(4,5)P$_2$ (and potentially PI(4)P) to achieve plasma membrane localization.

To obtain a more quantitative measure of ExoU lipid binding, we performed surface plasmon resonance (SPR) binding analysis using full-length recombinant ExoU protein and liposomes supported on a solid sensor surface (55). ExoU did not appreciably bind to phosphatidylcholine liposomes but did bind with high affinity to PI(4,5)P$_2$ liposomes (*K*$_d$ 110 ± 30 nM; Fig. 2A). ExoU also bound to PI(4)P but with lower affinity (*K*$_d$ 290 ± 30 nM; Fig. 2B). These data confirm the liposome co-sedimentation assay results and demonstrate high affinity interactions between ExoU and PI(4,5)P$_2$ and somewhat weaker interactions between ExoU and PI(4)P, which was not known previously. Liposome binding and SPR assays are more physiological methods of measuring protein-lipid binding than the lipid strips used previously by Gendrin *et al.* (56), potentially explaining why they did not observe ExoU-PI(4)P binding (31). Interestingly, although not required for PI(4,5)P$_2$ binding, the presence of phosphatidylinerine significantly increased ExoU-PI(4,5)P$_2$ affinity (Fig. 2C). The addition of PS mimics its presence at the inner leaflet of the plasma membrane; this phospholipid contributes to the plasma membrane-targeting specificity of other phosphoinositide binding domains (57).
In an earlier study, we had identified several residues of the MLD that were critical for the proper intracellular localization of ExoU but that did not disrupt the overall secondary structure of ExoU, as measured by circular dichroism (30). We reasoned that if MLD binding to PI(4,5)P₂ truly was responsible for MLD localization to the plasma membrane, then these mislocalized ExoU variants should fail to bind PI(4,5)P₂. We therefore purified recombinant MLD proteins with one of six single amino acid substitutions or with one five-amino acid insertion that had resulted in mislocalization inside host cells (30). These purified proteins were tested for their ability to bind liposomes containing PI(4,5)P₂. Each was deficient in PI(4,5)P₂ binding (Fig. 1B), demonstrating that ExoU residues necessary for localization to the plasma membrane of host cells were also critical for binding to PI(4,5)P₂. One variant, R661L, was also tested for binding to PI(4)P. As was the case with PI(4,5)P₂, it was deficient in PI(4)P binding, suggesting that the same mechanism may be used to bind both phosphoinositides (Fig. 1C).

ExoU-like MLDs from Other Patatin-like Proteins Also Bind to PI(4,5)P₂—PI(4,5)P₂ binding domains play important roles in intracellular trafficking of eukaryotic proteins, and each distinct binding domain is often utilized by a number of different proteins (33). For this reason, we sought to determine whether other bacterial proteins also used an ExoU MLD-like motif to bind PI(4,5)P₂. Accordingly, BLAST analysis with the MLD of ExoU was used to identify several proteins containing similar MLD sequences (Fig. 3A). Genes encoding these proteins were found in the genomes of other Pseudomonas species such as P. fluorescens and Pseudomonas syringae but also in other genera, including Photobacterium asymbiotica and Burkholderia thailandensis. Alignments revealed that a number of residues within these sequences were 100% conserved among this group of proteins, including some residues that had previously been shown to be important for ExoU localization, such as Gln-623, Asn-627, Arg-661, and Ala-678 (Fig. 3A) (30). Of special interest was Arg-661 of ExoU, a highly conserved and positively charged residue in a loop at the exposed cap of the four-helical bundle in the MLD (28). This residue could potentially directly interact with the negatively charged phosphate groups of PI(4,5)P₂. In support of this model, substitution of Arg-661 with a leucine (R661L) had previously been shown to cause loss of activation of P. aeruginosa ExoU by PI(4,5)P₂ (26). Likewise, recombinant MLD protein with this substitution did not bind.
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PI(4,5)P₂ in liposome binding assays (Fig. 1B). These results suggest that this conserved arginine residue might be critical for direct MLD binding to PI(4,5)P₂.

To investigate whether ExoU MLD-like domains from other bacteria also bound PI(4,5)P₂, we tested the recombinant MLDs from P. fluorescens and P. asymbiotica in the liposome binding assay. P. fluorescens is an environmental bacterium (58), and P. asymbiotica infects insects and is an emerging human pathogen (59). The MLD-containing proteins from these bacteria had not been previously studied (59), but their homologies to ExoU (59). The MLD-containing proteins from these bacteria had not been previously studied (59). The MLD-containing proteins from these bacteria had not been previously studied (59). The MLD-containing proteins from these bacteria had not been previously studied (59). The MLD-containing proteins from these bacteria had not been previously studied (59). The MLD-containing proteins from these bacteria had not been previously studied (59). The MLD-containing proteins from these bacteria had not been previously studied (59). 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that was amenable to manipulation of PI(4,5)P₂ localization. Yeast are an established model system for the study of ExoU activity and localization and have yielded results similar to those observed in mammalian cells (26, 37). We reasoned that if ExoU localization was truly dependent on binding to PI(4,5)P₂, then mislocalization of PI(4,5)P₂ should cause a corresponding mislocalization of ExoU. In these experiments, PI(4,5)P₂ was visualized in yeast using a construct that expressed a GFP-tagged pleckstrin homology (PH) domain from mammalian phospholipase Cδ, which is a natural ligand of PI(4,5)P₂ (Fig. 4).
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5A) (61). In wild-type yeast, PI(4,5)P₂ exhibited a peripheral distribution, consistent with plasma membrane localization. However, in a Δinp54/Δsac1 yeast mutant, which lacks two phosphatases important for appropriate PI(4,5)P₂ distribution, this phospholipid was mislocalized to the vacuole and cytosol (Fig. 5A), as described previously (61). Prior reports demonstrated that PI(4)P localization is unchanged in these yeast and that proteins that localize to the plasma membrane independently of PI(4,5)P₂ are also unaffected (61). Full-length ExoUₚₐₑᵦ tagged with GFP localized to the plasma membrane in wild-type yeast but was largely associated with intracellular structures in Δinp54/Δsac1 mutant yeast (Fig. 5B). Thus, mislocalization of PI(4,5)P₂ resulted in a similar mislocalization of ExoUₚₐₑᵦ consistent with the model that ExoUₚₐₑᵦ binds to PI(4,5)P₂ to target the plasma membrane.

We next assessed the localization of ExoUₚₐₑᵦ and ExoUₚₐₛᵧ in yeast. In wild-type yeast, full-length ExoUₚₐₑᵦ localized to the plasma membrane similarly to ExoUₚₐₑᵦ (Fig. 5B). In the Δinp54/Δsac1 mutant yeast, ExoUₚₐₑᵦ displayed mislocalization away from the plasma membrane, indicating PI(4,5)P₂-dependent localization. ExoUₚₛᵧ also localized to the plasma membrane in wild-type yeast but formed more punctate structures at or adjacent to the membrane (Fig. 5B). In the Δinp54/Δsac1 mutant yeast, the distribution of ExoUₚₛᵧ shifted somewhat to the interior of the yeast, although substantial amounts remained at the periphery. Because the localization of full-length ExoUₚₛᵧ in yeast differed from that of the ExoUₚₛᵧ MLD in HeLa cells, we examined the localization of the ExoUₚₛᵧ MLD in yeast. As observed in HeLa cells, the MLD alone localized specifically to the plasma membrane of yeast (Fig. 5B). Importantly, the ExoUₚₛᵧ MLD was mislocalized in Δinp54/Δsac1 yeast, suggesting that localization of the ExoUₚₛᵧ MLD is dependent on PI(4,5)P₂. These results suggest that the MLD of ExoUₚₛᵧ requires PI(4,5)P₂ for localization but that full-length ExoUₚₛᵧ may have residues outside the MLD that contribute to an altered intracellular distribution. Together these data indicate that ExoU and its homologs localize to the plasma membrane in a PI(4,5)P₂-dependent manner, either as full-length proteins or as isolated MLD domains.

ExoUₚₐₑᵦ and ExoUₚₛᵧ Are Also Phospholipases—The proteins listed in Fig. 3A contained regions with similarity to not only the MLD of ExoUₚₐₑᵦ but also to its catalytic domain. In particular, both ExoUₚₐₑᵦ and ExoUₚₛᵧ have a putative PLA₂ serine-aspartate catalytic dyad and glycine-rich oxyanion hole characteristic of patatin-like phospholipases (Fig. 6A). We therefore examined whether these proteins have PLA₂ activity. We first confirmed that ExoUₚₐₑᵦ by itself does not have PLA₂ activity but is synergistically activated by a combination of ubiquitin and PI(4,5)P₂ (Fig. 6B) (25, 26). To test whether ExoUₚₐₑᵦ and ExoUₚₛᵧ act as phospholipases, recombinant versions of both proteins were purified and assessed in vitro for evidence of PLA₂ activity. The enzymatic characteristics of ExoUₚₛᵧ were similar to those of ExoUₚₐₑᵦ, with no activity on its own and a synergistic activation with the addition of ubiquitin and PI(4,5)P₂ (Fig. 6B). However, the overall activity of ExoUₚₛᵧ when combined with ubiquitin and PI(4,5)P₂ was low, only 22% that of ExoUₚₐₑᵦ under the conditions of these assays. In contrast, ExoUₚₐₑᵦ alone had considerable PLA₂ activity, and the addition of ubiquitin substantially increased this activity (Fig. 6B). In fact, addition of only 0.1% as much ubiquitin as ExoUₚₐₑᵦ resulted in significantly increased PLA₂ activity. Supplementation with PI(4,5)P₂, however, caused only a slight additional increase in catalytic activity, suggesting that ubiquitin plays a more predominant role in the activation of ExoUₚₐₑᵦ. These results demonstrate that MLD-containing patatin-like proteins from other bacterial species and genera also have PLA₂ activity but differ...
from ExoU<sub>P. aer</sub> in how active they are by themselves or with ubiquitin and PI(4,5)P<sub>2</sub>. ExoU<sub>P. aer</sub> was also activated by PI(4,5)P<sub>2</sub>-containing liposomes, indicating that the liposome binding assay conditions are suitable for ExoU activation in the presence of ubiquitin (Fig. 6C).

We next examined whether ExoU<sub>P. flu</sub> and ExoU<sub>P. asy</sub>, like ExoU<sub>P. aer</sub>, could kill eukaryotic cells. Each of these proteins was expressed in HeLa cells by transfection, and cytotoxicity was assessed by measuring lactate dehydrogenase release. ExoU<sub>P. aer</sub>, as shown previously, caused substantial cytotoxicity by 24 h, and this cytotoxicity was diminished by substituting an alanine for the catalytic serine at position 142 within the PLA<sub>2</sub> domain (Fig. 7A) (30). Consistent with its high PLA<sub>2</sub> activity, ExoU<sub>P. flu</sub> was also highly cytotoxic, and substitution of its putative catalytic serine also resulted in decreased cytotoxicity. Because of the rapid lysis of cells following expression of ExoU<sub>P. aer</sub> and ExoU<sub>P. flu</sub>, these proteins could not be detected within transfected cells, whereas expression of the proteins with catalytic site substitutions could be detected (Fig. 7B). Despite repeated attempts, we were not able to detect stable GFP-tagged protein in cells transfected with ExoU<sub>P. asy</sub>-expressing constructs and were therefore unable to assess the cytotoxicity of this protein (Fig. 7B).

Detection following substitution of other tags in place of GFP was also not successful (data not shown). Interestingly, the MLD of ExoU<sub>P. asy</sub> was stable when expressed by itself in mammalian cells (Fig. 4), and the full-length GFP-tagged ExoU<sub>P. asy</sub>-S137A construct could be visualized in yeast (Fig. 5B). It is possible that the full-length ExoU<sub>P. asy</sub> protein may not fold properly in the mammalian cell cytosol or it may be destabilized or degraded. Overall, these experiments demonstrated that ExoU<sub>P. flu</sub> is cytotoxic to HeLa cells, but we could not assess the cytotoxicity of ExoU<sub>P. asy</sub> in this cell type.

**ExoU<sub>P. flu</sub> Crystal Structure and Its Comparison with the Structure of ExoU<sub>P. aer</sub>**—As mentioned, the crystal structure of ExoU<sub>P. aer</sub> was recently determined and suggested that the four-helical bundle region of the MLD might bind to the plasma membrane (28, 31), similar to the four-helical bundles of other toxins (8, 29). This structure suffered from two limitations. First, ExoU<sub>P. aer</sub> could only be crystallized in complex with its cognate chaperone SpcU, a bacterial protein that is not present...
inside eukaryotic cells. Thus, the relevance of this structure to ExoU interaction with eukaryotic membranes is unclear. Second, the loop containing the conserved Arg-661 was disordered in this structure, preventing an assessment of how it may promote membrane localization. In the hopes of overcoming these limitations and to further investigate MLD-PI(4,5)P2 interactions, we attempted to crystallize full-length ExoU.

We successfully crystallized this protein in the absence of any chaperone proteins and determined its structure to 2.5 Å resolution (Fig. 8A). Surprisingly, ExoU crystallized as a dimer, with a total buried surface area of ∼2000 Å² (Fig. 8B). Residues 130–169 (helices α4 and α5) of each patatin-like PLA2 domain constitute the primary interaction interface within the dimer. This dimeric structure is mainly stabilized by stretches of hydrophobic amino acids buried at the interface (Fig. 8C).

Comparison with the domain architecture of ExoU (28) allowed the ExoU structure to be broadly divided into four domains as follows: a putative chaperone binding domain (residues 21–51 and 421–451), a patatin-like PLA2 domain (residues 52–420), and an MLD region, which encompasses domains three and four (residues 452–559 and 560–639, respectively) (Fig. 8A). ExoU and ExoU are quite similar in their overall tertiary structures, overlapping with root-mean-square deviation of 2.2 Å (based on the DaliLite server (62–64)) over 473 Ca atoms (Fig. 9). The two ExoU structures also have comparable disordered regions (Fig. 9). The catalytic region of the two proteins are similar as well, with the glycine-rich oxyanion hole (Gly-61–63 in ExoU and Gly-111–113 in ExoU) and the serine hydrolase motif containing catalytic Ser-92 (Ser-142 in ExoU) each aligning well structurally (Fig. 10A) (65). Gly-286, an additional glycine important for ExoU activity, is also conserved structurally (Fig. 10A, Gly-235 in ExoU) (65). The catalytic Asp-294 was not modeled due to disorder, as was previously the case for ExoU (28).

Notably, the chaperone binding domains of these two proteins are less similar, suggesting that ExoU lacks a structurally distinct chaperone, lacks a chaperone, or that crystal packing has affected the domain’s orientation in ExoU (Fig. 9). It is

FIGURE 7. Cytotoxicity of ExoU homologs. A, HeLa cells were transfected with ExoU expression constructs and analyzed for release of lactate dehydrogenase as a measure of cell death. Results were normalized to treatment with Triton X-100 (100% cell death) and mock-transfected cells (0% cell death). Vector indicates transfection of pcDNA3.1 NT-GFP vector without insert. Each assay was performed in triplicate; values are means, and error bars represent S.E. *, p < 0.001. B, same GFP-tagged constructs shown in A were transfected into HeLa cells, which were subsequently analyzed for expression of the resulting proteins with anti-GFP antibody. MW denotes the molecular weight markers.
unclear whether this may explain potential PLA2 activity differences between the two proteins.

The MLD regions (domains three and four) of ExoU <i>P. flu</i> and ExoU <i>P. aer</i> are very similar structurally, with domain four of each protein forming a four-helical bundle that overlaps with root-mean-square deviation of just 0.7 Å (Fig. 11A). The conserved Arg-616 of ExoU <i>P. flu</i> is part of the L3 loop that protrudes from the four-helical bundle, similarly located to Arg-661 of ExoU <i>P. aer</i>. As mentioned, the conserved nature of this arginine, its location at the cap of the four-helical bundle, its critical role in membrane localization (Fig. 4), and its positive charge makes it an attractive candidate for binding to the negatively charged phosphates of PI(4,5)P2. Whereas the portion of the ExoU <i>P. aer</i> L3 loop containing Arg-661 was disordered, this loop was modeled in the ExoU <i>P. flu</i> structure (Fig. 11A). One of the ExoU <i>P. flu</i> monomers showed the L3 loop protruding and well positioned to interact with membranes (Fig. 8B). Importantly, the ExoU <i>P. flu</i> monomers have both the four-helical bundle and the PLA2 catalytic residues oriented to the same face of the protein (Fig. 8B). This arrangement is consistent with the four-helical bundle positioning ExoU at the plasma membrane in an orientation that facilitates placement of substrate membrane phospholipids into the catalytic pocket. The caps of the four-helical bundles of both ExoU <i>P. flu</i> and ExoU <i>P. aer</i> form positively charged surface-exposed pockets, suggesting potential additional contacts with negatively charged PI(4,5)P2 molecules (Fig. 10B). Interestingly, this surface charge distribution differs from structurally similar four-helical bundle domains of other toxins such as <i>P. multocida</i> toxin (PMT) and <i>Clostridium difficile</i> toxin B (TcdB) that do not have specificity for PI(4,5)P2 (Fig. 10B).

Additional conserved residues important for the localization of ExoU <i>P. aer</i> (30) are also structurally conserved in ExoU <i>P. flu</i> (Figs. 3A and 11A). Furthermore, the conserved polar or charged residues Arg-616, Gln-578, and Asn-582 of ExoU <i>P. flu</i> (Arg-661, Gln-623, and Asn-627 in ExoU <i>P. aer</i>) were all located in the cap of the four-helical bundle, suggesting a role in binding to PI(4,5)P2. In contrast, the conserved hydrophobic residues Phe-564, Ile-609, and Ala-632 of ExoU <i>P. flu</i> (Ile-609, Ile-654, and Ala-678 in ExoU <i>P. aer</i>) were located within the helices themselves, signifying a role in maintaining the overall four-helical bundle configuration (Fig. 11A). In summary, structural data suggest a model whereby the four-helical bundle structural motif is used by many bacterial proteins to interact with a variety of membranes, but the caps of the four-helical bundles of patatin-like proteins have been “customized” to bind PI(4,5)P2 and target these proteins to the plasma membrane of eukaryotic cells.

**DISCUSSION**

We describe the mechanism by which three members of the patatin-like phospholipases bind PI(4,5)P2 to localize to the plasma membrane of host cells. Using liposome binding assays, we found that the MLD of ExoU <i>P. aer</i> has specific affinity for PI(4,5)P2 and that MLD residues important for plasma membrane localization are also critical for PI(4,5)P2 binding. Although the ability of ExoU to bind PI(4,5)P2 was already known (31, 32), we found that PI(4,5)P2 mislocalization dis-

**FIGURE 8. Crystal structure of ExoU from P. fluorescens.** **A,** ribbon diagram of a single ExoU <i>P. flu</i> molecule showing subunit domain 1 (the putative chaperone binding domain, green), domain 2 (the patatin PLA2 domain, red), domain 3 (light blue), and domain 4 (dark blue). Domains 3 and 4 together constitute the MLD. B, ribbon and surface representation of the asymmetric unit composition of the structure showing the dimeric arrangement of ExoU <i>P. flu</i> L1, L2, and L3 are loops of domain 4 that connect the helices 19–20, 20–21, and 21–22, respectively, of the four-helical bundle. C, major dimerization interface generated by helices 4 and 5 of the patatin PLA2 domain. Buried hydrophobic residues (white and labeled with one-letter code) within the helices are displayed.
ruptured targeting of ExoU<sub>P. aer</sub> to the plasma membrane. The use of this novel PI(4,5)P<sub>2</sub> binding domain is not restricted to ExoU<sub>P. aer</sub> but was also utilized by patatin-like proteins from <i>P. fluorescens</i> and <i>P. asymbiotica</i>. The crystal structures of the full-length ExoU<sub>P. flu</sub> and ExoU<sub>P. aer</sub> demonstrated that their MLDs likely use a four-helical bundle structural motif to present a positively charged pocket containing a conserved arginine residue important for PI(4,5)P<sub>2</sub> binding. Binding of PI(4,5)P<sub>2</sub> by this motif may orient the ExoU patatin-like catalytic site toward phospholipid substrates in the plasma membrane. Together, these findings define a new PI(4,5)P<sub>2</sub>-binding motif used by bacterial proteins to target the plasma membrane of eukaryotic cells, where they are well positioned to access phospholipid substrates within the plasma membrane and to cause cell lysis.

Phosphoinositides (phosphatidylinositol lipids with phosphates attached to the 3-, 4-, or 5-positions of the inositol ring) such as PI(4,5)P<sub>2</sub> are critical for the function of eukaryotic cells, often serving as signposts for defined membrane compartments (34). PI(4,5)P<sub>2</sub> is predominantly found in the plasma membrane, PI(4)P in the Golgi (66), and PI(3)P in endosomes (67). This allows proteins with particular lipid-binding specificities to be targeted to the corresponding membrane compartments. PI(4,5)P<sub>2</sub> binding domains in particular are critical for the function of eukaryotic cells. For example, PH domains, epsin N-terminal homology domains, and FERM domains each target endogenous proteins to the plasma membrane (35, 36, 68). These and other domains play critical roles in cell processes such as endocytosis, motility, and cytoskeletal anchoring (35, 69, 70). They use different mechanisms for PI(4,5)P<sub>2</sub> recognition, but a common theme is the presence of basic residues that bind to the negatively charged PI(4,5)P<sub>2</sub> (71). Our results add further evidence to the notion that bacteria have evolved mechanisms to co-opt this elaborate localization system.

**FIGURE 9. Comparison of the ExoU<sub>P. flu</sub> and ExoU<sub>P. aer</sub> structures.** A, linear depiction of ExoU<sub>P. flu</sub> and ExoU<sub>P. aer</sub> domains. The putative chaperone binding domain (domain 1) is shown in green, the patatin PLA<sub>2</sub> domain (domain 2) in red, and the MLD in blue. The MLD is composed of domain 3 (light blue) and domain 4 (dark blue). Disordered regions in both structures are shown in white. B, domain-to-domain comparisons of the least squares superimposed ExoU<sub>P. flu</sub> (colored as in A) and ExoU<sub>P. aer</sub> (gray) structures.
Although PI(4,5)P₂ binding domains are common among eukaryotic proteins, the ExoU MLD is only the second report of a bacterial PI(4,5)P₂ binding domain (72). Orth and co-workers (72) recently identified a bacterial phosphoinositide binding domain (BPD) in four effectors of the type III secretion systems of *Vibrio parahaemolyticus*, *Yersinia* spp., and *P. syringae*. The BPD localized these effectors to the plasma membrane of eukaryotic cells following injection. Unlike the MLD of ExoU, however, BPDs are at the N termini and overlap with the chaperone binding domains of these proteins. Structurally, BPDs have two β-strands and two α-helices instead of a four-helical bundle. These differences indicate that the ExoU MLD is quite distinct from the BPD and acts by a different mechanism. Perhaps this is because the PLA₂ activities of ExoU and other patatin-like proteins impose more stringent constraints, requiring that the MLDs not only localize to membranes but also provide access to phospholipid substrates in the membrane. In support of this, ExoU phospholipases appear to more closely mimic the strategy employed by some eukaryotic phospholipases, which use PI(4,5)P₂ to regulate not only their localization but also their activity. For instance, phospholipase D contains both a dedicated PH domain and an additional PI(4,5)P₂-binding site responsible for activation of its phospholipase activity (73). cPLA₂ is also localized to and activated by PI(4,5)P₂, further illustrating the important role of PI(4,5)P₂ in both intracellular targeting and activation of eukaryotic phospholipases (74). ExoU strongly binds to PI(4,5)P₂ with a $K_d$ of just 110 nM, comparable with the PH domain of phospholipase C, a proto-
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FIGURE 11. Four-helical bundle structures of ExoU MLDs. A, domain 4 structures of ExoU<sub>P. aer</sub> and ExoU<sub>P. flu</sub>. Residues critical to PI(4,5)P<sub>2</sub> binding by ExoU<sub>P. aer</sub> (Fig. 18) are indicated, as are the homologous residues present in ExoU<sub>P. flu</sub>. The L3 of ExoU<sub>P. aer</sub> which contains Arg-661 (blue dashed line), was disordered, but the corresponding L3 of ExoU<sub>P. flu</sub> was ordered. B, depictions of the four-helical bundle structures of ExoU<sub>P. aer</sub>, PMT, and TcdB. The ExoU<sub>P. aer</sub> structure is a model derived from the ExoU<sub>P. aer</sub> MLD (rainbow colors; blue, N terminus and red, C terminus). C, ExoU<sub>P. aer</sub> (colored as in Fig. 8B) with modeled PI(4)P molecule (ball-and-stick model with carbon atoms in green) based on the L. pneumophila effector DrrA/SidM structure (DrrA/SidM omitted for clarity; Protein Data Bank code 4MXP).

Type mammalian PI(4,5)P<sub>2</sub> binding domain (75). It will be interesting to see whether eukaryotic PI(4,5)P<sub>2</sub> binding domains can substitute for the MLD of ExoU or whether the structural uniqueness of the MLD confers specific PI(4,5)P<sub>2</sub>-binding properties that facilitate cell lysis by ExoU. In this regard, it should be noted that artificial targeting to eukaryotic membranes by farnesylation was insufficient to restore cell lysis to an ExoU variant with a disrupted MLD (30).

By obtaining the full-length crystal structure of a second ExoU patatin-like phospholipase, we were able to compare these two members of this protein family (28). In particular, the MLDs of ExoU<sub>P. aer</sub> and ExoU<sub>P. flu</sub> were quite similar, illustrating a conserved four-helical bundle fold that is important for PI(4,5)P<sub>2</sub> binding. PI(4,5)P<sub>2</sub> binding domains frequently contain positively charged residues that interact with the negatively charged phosphates of PI(4,5)P<sub>2</sub> (71). Our findings suggest that Arg-661 of ExoU<sub>P. aer</sub> is such a residue. In a previous examination of ExoU<sub>P. aer</sub> for important MLD residues, a substitution in Arg-661 significantly attenuated ExoU localization and cytotoxicity (30). In this study, the homologous Arg-616 residue of ExoU<sub>P. flu</sub> was also important for localization, and both residues were contained within the L3 loop of the four-helical bundle. Additionally, modeling of the ExoU<sub>P. asy</sub> MLD structure based on the previously characterized ExoU<sub>P. aer</sub> structure indicated that it too could form a four-helical bundle containing the conserved arginine in the L3 loop (Fig. 11B). This is consistent with its similar localization pattern, as well as the importance of its conserved arginine residue for localization. Interestingly, other unrelated bacterial toxins, such as P. multocida PMT and C. difficile TcdB, also contain four-helical bundles (Fig. 11B) (8, 76), but these proteins have different lipid-binding specificities.

Unlike ExoU<sub>P. aer</sub> and ExoU<sub>P. flu</sub>, PMT and TcdB bind with high affinity to a variety of negatively charged phospholipids such as phosphatidylserine and phosphatidylinositol (8, 38). Substitutions of positively charged amino acids in both the L1 and L3 loops of PMT and TcdB resulted in disruption of localization to membranes rich in these phospholipids (38). These other proteins also have conserved hydrophobic residues in the L3 loops that help establish membrane binding (38). ExoU does have hydrophobic residues in its L3 loop (residues 660–672), but they are not well conserved, suggesting they may not play a major role in localization (Fig. 3A).

The similarities between the four-helical bundles of the ExoU homologs and the other bacterial toxins suggest that four-helical bundles are a conserved structural platform for binding phospholipids in membranes but that alterations within these domains (and in particular in their L1 and L3 loops) “customize” their lipid-binding specificities. It will be interesting to see whether other four-helical bundle proteins are tailored to recognize phosphoinositides characteristic of other intracellular membrane compartments.

A more detailed mechanism of MLD binding will require a crystal structure of ExoU in complex with PI(4,5)P<sub>2</sub>. Although such a structure is not yet available, the structure of the Legio-nella pneumophila toxin DrrA in complex with PI(4)P was recently solved (77). The membrane-binding portion of DrrA forms a three-helical bundle rather than a four-helical bundle, and the structure shows binding to PI(4)P rather than PI(4,5)P<sub>2</sub>, but we reasoned that the comparison may nevertheless be informative. For this reason, we used this structure to model ExoU<sub>P. flu</sub> binding to PI(4)P. Superposition of the helical bundle domains positioned PI(4)P within the immediate vicinity of the
L3 loop with the conserved Arg-616 of ExoU_{P.flu} as we had postulated (Fig. 11C). This supports the importance of the L3 loop and Arg-616 of ExoU_{P.flu} in direct PI(4,5)P_2 binding and plasma membrane localization. Additional contacts outside the MLD may contribute to this binding, as Arg-242 (Arg-293 of ExoU_{P.aer}) is also well positioned to bind PI(4,5)P_2 (Fig. 10B). However, the MLD alone is sufficient for PI(4,5)P_2 binding (Fig. 1A) and for localization (Fig. 4). A more comprehensive screen is necessary to determine other residues in the L3 loop and the four-helical bundle of ExoU necessary for PI(4,5)P_2 binding and the potential contribution of residues within the MLD but outside the four-helical bundle or outside the MLD (27). These studies could help inform the differences in apparent affinity for PI(4,5)P_2 by the ExoU homologs, despite their maintaining a conserved mechanism (Fig. 2D). Of the several other residues already demonstrated to be important for PI(4,5)P_2 binding by ExoU (Fig. 1B), most were buried within the four-helical bundle and not located near the postulated membrane-binding interface (28). It is possible that Arg-661 (of ExoU_{P.aer}) makes initial contact with PI(4,5)P_2, causing a conformational change in the four-helical bundle, which allows these buried residues to contact the membrane. Alternatively, these other residues may play a structural role in helical packing and maintenance of the overall four-helical conformation, as has been postulated for critical residues in other four-helical bundle MLDs (29).

ExoU_{P.aer} has been extensively investigated for its importance both clinically and in animal models of infection (23, 78). In contrast, the homologous ExoU proteins from the emerging human and insect pathogen P. fluorescens and the plant commensal P. aeruginosa have not been previously studied. Although these proteins need to be examined more thoroughly in the context of the bacteria that naturally produce them, several features suggest that they may be important for interaction with eukaryotic cells. First, they are postulated to be effector proteins of type III secretion systems, suggesting that they are injected into eukaryotic cells. Indeed, ExoU_{P.aer} is the sole identified putative effector in the P. fluorescens type III secretion system locus (59). Second, they localize by binding to PI(4,5)P_2, which is only found in eukaryotic cells. Third, they have PLA_2 activities that are modulated by eukaryotic factors, which, at least in the case of ExoU_{P.aer}, is used to subvert a broad spectrum of eukaryotic cells (79, 80). Interestingly, the enzymatic properties of these ExoU homologs differed substantially despite quite similar structures (Fig. 6). For example, compared with ExoU_{P.aer}, ExoU_{P.flu} was active in the absence of co-activators and highly active in the presence of ubiquitin. A more detailed and expanded comparative structural analysis of similar MLD-containing patatin-like proteins may identify mechanistic explanations for the different enzymatic properties of this family of proteins.

In summary, we have characterized a PI(4,5)P_2 binding domain used by proteins from several different bacteria, including human and insect pathogens and a plant saprophyte. The four-helical bundle of the ExoU MLD binds PI(4,5)P_2 through a mechanism that requires a conserved arginine in an exposed loop. It will be interesting to determine whether proteins other than patatin-like phospholipases use similar MLD domains to bind PI(4,5)P_2.

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Note Added in Proof—During the revision of this manuscript, Anderson et al. published an article (Anderson, D. M., Sato, H., Dirck, A. T., Feix, J. B., and Frank, D. W. (2014) J. Bacteriol. 10.1128/JB.02402-14) also demonstrating that some ExoU homologs bind PI(4,5)P_2 and are cytotoxic and that ubiquitin and PI(4,5)P_2 differentially activate these homologs.

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