Interactions of the effector ExoU from *Pseudomonas aeruginosa* with short-chain phosphatidylinositides provide insights into ExoU targeting to host membranes

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Tzvia I. Springer, Terry-Elinor Reid, Samantha L. Gies, and Jimmy B. Feix

From the Department of Pharmaceutical Sciences, Concordia University Wisconsin, Mequon, Wisconsin 53097 and the Department of Biophysics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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**Pseudomonas aeruginosa** is an opportunistic multidrug-resistant pathogen and a common cause of infection in cystic fibrosis and ventilator-associated pneumonia and in burn and wound patients. *P. aeruginosa* uses its type III secretion system to secrete various effector proteins directly into mammalian host cells. ExoU is a potent type III secretion system effector that, after secretion, localizes to the inner cytoplasmic membrane of eukaryotic cells, where it exerts its phospholipase A2 activity upon interacting with ubiquitin and/or ubiquitinated proteins. In this study, we used site-directed spin-labeling electron paramagnetic resonance spectroscopy to examine the interaction of ExoU with soluble analogs of phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P$_2$). We found that dioctanoyl PI(4,5)P$_2$ binds to and induces conformational changes in a C-terminal four-helix bundle (4HB) domain of ExoU implicated previously in membrane binding. Other soluble phosphoinositides also interacted with the 4HB but less effectively. Molecular modeling and ligand docking studies indicated the potential for numerous hydrogen bond interactions within and between interhelical loops of the 4HB and suggested several potential interaction sites for PI(4,5)P$_2$. Site-directed mutagenesis experiments confirmed that the side chains of Gln-623 and Arg-661 play important roles in mediating PI(4,5)P$_2$-induced conformational changes in ExoU. These results support a mechanism in which direct interactions with phosphatidylinositol-containing lipids play an essential role in targeting ExoU to host membrane bilayers. Molecules or peptides that block this interaction may prove useful in preventing the cytotoxic effects of ExoU to mitigate the virulence of *P. aeruginosa* strains that express this potent phospholipase toxin.

ExoU is a bacterial phospholipase and an important virulence factor produced by the opportunistic pathogen *Pseudomonas aeruginosa* (1, 2). In addition to its importance as a virulence factor, ExoU provides a valuable model for type IVA phospholipases. ExoU exhibits broad substrate specificity, cleaving a wide range of phospholipids and lysophospholipids (3, 4). In contrast to the broad substrate specificity of ExoU, lipid overlay (5) and solid-phase binding assays (6) suggest preferential binding to phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P$_2$). Further, ExoU phospholipase activity and membrane association are reportedly increased for vesicles (7) and lipid bilayers containing PI(4,5)P$_2$ (8, 9). Based on studies in a tissue culture model of *P. aeruginosa* infection, it has been proposed that selective association of ExoU at PI(4,5)P$_2$-enriched focal adhesion complexes is an initial event following intoxication of eukaryotic cells that results in the observed cytoskeletal collapse and cell death (6). In general, the presence of PI(4,5)P$_2$ in target membranes is known to enhance the activity of eukaryotic type IV phospholipases (11–13).

ExoU has been crystallized in the presence of its cognate type III chaperone, SpcU (5, 10). ExoU is a multidomain protein with an N-terminal chaperone-binding domain, a patatin-like phospholipase A2 (PLA2) catalytic domain, a bridging domain that appears to mediate binding of an obligate ubiquitin cofactor (14), and a C-terminal (C-term) four-helix bundle (4HB) domain that plays a role in targeting ExoU to the membrane (15–18). Although the ExoU–SpcU structure represents an inactive pre-export complex, our previous studies using site-directed spin labeling EPR spectroscopy have shown good agreement between structural parameters for the apo state of ExoU in solution and those expected based on the crystallographic models (18). In addition, ab initio Rosetta modeling based on structural constraints from double electron–electron resonance studies of ExoU in the apo state produced a model of the C-terminal four-helix bundle that was in excellent agreement with the crystal structures (19, 20).

In this study, we examined the interaction of ExoU with short-alkyl-chain, soluble phosphatidylinositol analogs (Fig. S1). We asked whether short-chain phosphatidylinositides

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The abbreviations used are: PI(4,5)P$_2$, phosphatidylinositol (4,5)-bisphosphate; C-term, C-terminal; 4HB, four-helix bundle; EPR, electron paramagnetic resonance; diC$_8$, dioctanoyl; PI(3)P, phosphatidylinositol 3-mono-phosphate; PI(3,4)P$_2$, phosphatidylinositol 3,4-bisphosphate; PI(3,4,5)P$_3$, phosphatidylinositol 3,4,5-trisphosphate; MTSL, 5-[(1-oxyl-2,2,5,5-tetramethylpyrrolyl-2-y1)methyl methanethiosulfonate]; diUb, diubiquitin; diC$_4$, dibutyryl; PED6, N-[(2,4-dini trophenyl) amino]-hexanoyl)-2-(4,4-difuorou-5,7-dimethy1-4-bora-3a,4a-diaza-s-indacene-3- pentanoyl)-1-hexadecanoyl-sy-glycerol-3-phosphoethanolamine, triethyl ammonium salt; ligprep, ligand preparation.
were able to induce similar conformational changes in ExoU as observed previously for PI(4,5)P2-containing lipid bilayers (18) and whether the reported effects on catalytic activity noted above were dependent on membrane association. We show that a PI(4,5)P2-containing analog with octanoyl acyl chains (diC8-PI(4,5)P2) induces conformational changes throughout the C-term 4HB domain of ExoU even in the absence of a ubiquitin cofactor or membrane bilayer. In contrast, other soluble phosphoinositides, including diC4-PI(4,5)P2, diC8-phosphatidylinositol 3-monophosphate (diC8-PI(3)P), diC8–phosphatidylinositol 3,4-bisphosphate (diC8-PI(3,4)P2), and phosphatidylinositol 3,4,5-trisphosphate (diC8-PI(3,4,5)P3), had either a diminished or no effect, indicating at least partial specificity for the PI(4,5)P2 headgroup and alkyl chain length. Computational modeling studies were employed to examine potential docking poses of diC8-PI(4,5)P2 and to gain insights into possible binding modes of PI(4,5)P2 in the C-term 4HB.

Results

To investigate the effects of diC8-PI(4,5)P2 binding, we utilized a collection of ExoU variants containing single-cysteine substitutions spin-labeled with MTSL to monitor structural perturbations in ExoU’s C-terminal 4HB domain (18, 20). These spin-labeled constructs have been used previously to study interaction of the ExoU C-term 4HB with model lipid bilayers mimicking the inner surface of a eukaryotic plasma membrane (18). EPR spectra of spin labels are highly sensitive to the rotational mobility of the nitroxide, in turn reflecting the local conformational environment and backbone dynamics at the site of attachment (21–24). We refer to the four helices and three interhelical connecting loops of the C-term 4HB observed in the ExoU–SpcU crystal structures as H1–H4 and L1–L3 (Fig. 1), respectively (5, 10). Labeling sites were selected for analysis along the entire lengths of H1, L1, and L3, along with representative sites for each of the other secondary structural elements (H2, L2, and H3), thus providing reporter sites positioned throughout the entire 4HB domain.

Addition of diC8-PI(4,5)P2 to ExoU alone in solution induced conformational changes at sites in H1, H4, L1, and L3 (Fig. 2). This is in contrast to our previous studies using PI(4,5)P2-containing liposomes, in which both membrane bilayers and a ubiquitin cofactor were required to elicit conformational changes (18, 20). For H1 sites 611R1 and 615R1, there was a shift to a slightly more mobile conformation upon addi-
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The motion of the spin label became slightly more restricted (Fig. 2 and Fig. S2). A more substantial increase in mobility was observed at H4 site 673R1 (Fig. 2). Conversely, addition of diC8-PI(4,5)P2 caused a dramatic decrease in spin label mobility at labeling sites in the interhelical loops, as shown for L1 site 622R1 and L3 site 664R1 (Fig. 2). Indeed, for L3, a decrease in mobility was observed at each site examined (Fig. 3), suggesting that diC8-PI(4,5)P2 binding induces a conformational rearrangement of the entire L3 interhelical loop.

Unlike in our previous studies with model membranes (18), the observed diC8-PI(4,5)P2-induced changes were not influenced by the presence of a ubiquitin cofactor. We tested the effect of adding linear diubiquitin (diUb), a potent activator of ExoU catalysis (8), and found that no additional changes occurred when diC8-PI(4,5)P2 was added in combination with diUb compared with diC8-PI(4,5)P2 alone (Figs. 4, A and B). In addition, diC8-PI(4,5)P2-induced changes were independent of ExoU phospholipase activity. The effects of diC8-PI(4,5)P2 on spin labeled variants prepared in a catalytically inactive background (25), where the catalytic serine 142 had been mutated to alanine, were identical to those observed in a WT background (Fig. 4, C and D).

Figure 2. diC8-PI(4,5)P2-mediated conformational changes in the C-terminal four-helix bundle. A–D, EPR spectra for ExoU alone in solution (apo, black) and in the presence of diC8-PI(4,5)P2 (red) for residues in H1 (A, 615R1), L1 (B, 622R1), L3 (C, 664R1), and H4 (D, 673R1). Spectra are normalized to represent an equal number of spins. Scan widths, 100 G.

Figure 3. diC8-PI(4,5)P2-mediated conformational changes in L3. Shown are EPR spectra for ExoU variants located within interhelical loop 3 in the C-terminal 4HB alone in solution (apo, black) and in the presence of diC8-PI(4,5)P2 (red). Changes in the EPR spectra reflecting perturbation of the spin label environment are evident throughout L3. Spectra are normalized to represent an equal number of spins. Scan widths, 100 G.

Figure 4. Structural changes induced by diC8-PI(4,5)P2 do not depend on ubiquitin binding or catalytic activity. A and B, EPR spectra for ExoU alone in solution (apo, black) and in the presence of diC8-PI(4,5)P2 without (red) or with (blue) addition of excess diUb for L1 site 622R1 (A) and H4 site 677R1 (B). C and D, EPR spectra for the same sites in a catalytically inactive background in the absence (apo, black) and presence of diC8-PI(4,5)P2 (red). C, 622R1/S142A. D, 677R1/S142A.

Phosphatidylinositol analogs

To evaluate the specificity of the interaction between ExoU and the phosphorylated inositol headgroup, we examined a series of soluble phosphoinositide analogs. For L1 site 622R1, no changes were observed upon addition of either diC8-PI(3)P or diC8-PI(3,4,5)P3 (Fig. S4). A reduction in spin label mobility at 622R1 was observed upon addition of diC8-PI(3,4)P2, although to a much lesser extent than observed with diC8-PI(4,5)P2 (Fig. S4). For L3 site 664R1, a slight decrease in motion was observed with either diC8-PI(3,4)P2 or diC8-PI(3,4,5)P3, although again, in each case, the extent of change was diminished relative to that observed with diC8-PI(4,5)P2 (Fig. S4). In addition, no changes were observed with dibutyryl (diC4) PI(4,5)P2 at any of the sites examined, including 622R1, 624R1, 665R1, 668R1, and 677R1 (data not shown), indicating that the interaction with diC8-PI(4,5)P2 is at least partly dependent on
alkyl chain length. These results indicate a preference for the PI(4,5)P₂ headgroup, in agreement with previous studies using phosphatidylinositol lipids in lipid blot (5) or solid-phase (6) binding assays or in vesicles or lipid bilayers (7–9).

**Molecular modeling of loops 1 and 3 with diC8-PI(4,5)P₂**

To gain insight into which residues in ExoU’s C-terminal 4HB might be responsible for the diC8-PI(4,5)P₂ binding events resulting in the structural effects described above, we carried out molecular modeling studies of the L1 and L3 interhelical loops, which are missing in the ExoU–SpcU crystal structures (5, 10), and ligand docking with diC8-PI(4,5)P₂ using the Schrödinger protein preparation and ligand docking tools, respectively (see “Experimental procedures”). Although the absence of L1 and L3 in the crystal structures of ExoU indicate their dynamic nature in the ExoU–SpcU complex, computational modeling suggests the potential for H-bond interactions between L1 and L3 (Fig. 5A). These interactions include potential H-bonding between the side chain of Arg-620 of L1 and backbone residues Ser-672 and Thr-673 of L3 as well as a H-bond interaction between the side chains of Gln-623 (L1) and Tyr-658 (L3). Stabilizing interactions may also occur within L3, including H-bonds involving the Arg-661 and Ser-671 side chains and multiple H-bond interactions between the backbone atoms of Phe-663, Arg-665, Phe-666, Lys-668/Pro-669, and Ser-672/Thr-673 (Fig. 5, B and C).

Ligand docking of diC8-PI(4,5)P₂ to the modeled interhelical loops indicated two potential binding poses: one between L1 and L3 (Fig. 6) and the other at the major turn of L3 (Fig. 7) with G scores of −7.989 kcal/mol and −6.484 kcal/mol, respectively. The observation of changes in spin label mobility at all sites examined in L3 (Figs. 2 and 3) are consistent with the potential for multiple interactions between Arg-661 and the C3 phosphate of diC8-PI(4,5)P₂, as suggested by pose 1 (Fig. 6), as well as the potential for multiple interactions between diC8-PI(4,5)P₂ and other L3 residues, as suggested by pose 2 (Fig. 7). In addition, the strong immobilization induced by diC8-PI(4,5)P₂ at 622R1 (Fig. 2) may reflect interaction of the ligand with Arg-620 (Fig. 6). Note that no significant change was observed for the 620R1 variant (Fig. S3), in which the arginine side chain had been replaced by the spin label, again consistent with a role of the Arg-620 side chain in diC8-PI(4,5)P₂ binding.

**Mutational analysis of predicted diC8-PI(4,5)P₂ interactions**

To test the potential roles of the Arg-620, Gln-623, and Arg-661 side chains in diC8-PI(4,5)P₂–induced conformational changes, we constructed the double mutants G622C/R620A, G622C/Q623A, L664C/Q623A, and T673C/R661A, each comprised of an alanine substitution at the site of interest and a nearby cysteine for attachment of the spin label reporter. Mutation sites were selected based on results of the ligand docking experiments as described above as well as sequence conservation among ExoU and ExoU-like proteins from other *Pseudomonas* species (8, 9). Cysteine residues were spin-labeled with MTSL to give 622R1/R620A, 622R1/Q623A, 664R1/Q623A, and 673R1/R661A. In these constructs, 622R1 serves as...
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a reporter group for changes in the structure of L1, 664R1 serves as a reporter for changes in L3, and 673R1 serves as a reporter for changes in H4 (Fig. 2).

As shown in Fig. 8, the diC8-PI(4,5)P2–induced conformational change observed in L1 at 622R1 was substantially diminished by the R620A substitution (compare with 622R1 in Fig. 2) and completely eliminated for the 622R1/Q623A construct. The Q623A substitution also reduced the effects of diC8-PI(4,5)P2 binding on L3, as indicated by the diminished response of the 664R1/Q623A construct (Fig. 8, compare with 664R1 in Fig. 2). These results indicate essential roles of Arg-620 and Gln-623 in the interaction with diC8-PI(4,5)P2. Although the lowest-energy complexes identified by ligand docking did not indicate a direct interaction with diC8-PI(4,5)P2, the Gln-623 side chain is proposed to form an H-bond with Tyr-658 adjacent to L3 (Fig. 5).

Ligand docking to the modeled ExoU structure suggests a prominent role of Arg-661 in maintaining L3 structure (Fig. 5C) and in the interaction with diC8-PI(4,5)P2 (Fig. 6). Experimentally, the introduction of an R661A mutation substantially altered the EPR spectrum of 673R1, even in the absence of diC8-PI(4,5)P2 (compare Figs. 8D and 2D), supporting a role of the Arg-661 side chain in maintaining the structure of the 4HB. In a WT background, the spin label side chain of 673R1 is strongly immobilized (Fig. 2), consistent with its location on the inside surface of H4. In contrast, the spin label side chain of 673R1/R661A was much more mobile (Fig. 8D), indicating a modification of the local structure surrounding 673R1. In addition, 673R1 was no longer responsive to diC8-PI(4,5)P2 binding in the R661A background (Fig. 8D).

Binding of diC8-PI(4,5)P2 in solution did not affect the catalytic activity of ExoU when measured using a soluble fluorescent lipid analog substrate, PED6 (Fig. S5). Thus, diC8-PI(4,5)P2 did not enhance ubiquitin-stimulated ExoU activity, nor did it competitively inhibit the enzyme. These results suggest that the enhanced activity of ExoU in the presence of membranes containing or enriched in PI(4,5)P2 (6–9) likely reflects increased membrane association rather than an independent effect of PI(4,5)P2 on catalytic efficiency. Consistent with this, it has been reported previously that two highly nonconservative mutations at sites critical for PI(4,5)P2 binding, Q623G and R661E, retained catalytic activity against PED6 (26), further indicating the independence of PI(4,5)P2 binding per se and catalytic activity.

Discussion

Several studies have suggested that the C-terminal 4HB domain of ExoU plays an important role in trafficking the protein to the membrane (9, 15–18) and that PI(4,5)P2 facilitates membrane binding (8, 9) and ExoU catalytic activity (6–9). In this study, we utilized soluble analogs of PI(4,5)P2 to address the question of whether PI(4,5)P2 interacts directly with the C-term 4HB in the absence of a bilayer interface and to evaluate its effect on ExoU activity in solution. Soluble analogs have proven valuable in defining phosphoinositide binding sites for a variety of integral membrane proteins (27–30) but, to our knowledge, have not been used previously in structural analysis of phospholipases.

Addition of diC8-PI(4,5)P2 resulted in distinct conformational changes throughout ExoU’s C-terminal 4HB. In contrast to our previous study (18), these changes occurred in the absence of a bilayer interface and without addition of a ubiquitin cofactor. Structural changes in the C-term 4HB in response to PI(4,5)P2 may facilitate further integration into the membrane as part of the transition of ExoU to a catalytically active state. These results also indicate the presence of PI(4,5)P2 binding sites within the C-term 4HB, as suggested previously by the crystal structure of an ExoU homolog from Pseudomonas fluorescens (9). In principle, the observed changes in spin label mobility could be due to direct binding of PI(4,5)P2 or to allosteric changes arising from PI(4,5)P2 binding at a distal site. However, abrogation of the PI(4,5)P2–induced conformational changes by the Q623A and R661A mutations demonstrates that these C-term 4HB residues are either involved directly in PI(4,5)P2 binding or participate in maintaining the structure of the PI(4,5)P2 binding site.

Although the absence of L1 and L3 residues from crystallographic electron density maps (5, 10) indicates that these loops are structurally dynamic, our computational modeling studies suggest the potential for multiple stabilizing interactions within and between these loops. Such interactions may facilitate PI(4,5)P2 binding. Ligand docking to the computational model further suggests important roles for select side chains, including Gln-623 and the positively charged residues Arg-620 and Arg-661. Sequence alignments show that Arg-620, Gln-623, and Arg-661 are strongly conserved in the C-term 4HB domain of ExoU homologs from a variety of Pseudomonas species (8, 9), and the importance of Gln-623 and Arg-661 in mediating PI(4,5)P2 interaction with liposomal membranes has been described previously (9). Our modeling results and experimental data (described above) are consistent with participation of these sites in PI(4,5)P2 binding, either through direct interaction or in maintaining the 4HB conformation.

Selectivity for a specific phosphoinositide headgroup is thought to be the basis for targeting membrane-associated proteins to specific organelles in eukaryotic systems (6, 31, 32), and previous studies have established preferential binding of ExoU to PI(4,5)P2 lipids immobilized on a solid support (5, 9). In agreement with these studies, we found that ExoU preferentially interacts with a PI(4,5)P2 phosphoinositide in solution. Although we also observed structural perturbation at some...
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Figure 7. Molecular docking of diC8-PI(4,5)P2 highlighting a second pose. A, electrostatic surface representation ExoU with diC8-PI(4,5)P2 (orange sticks) docked between modeled L1 and L3. The dashed yellow lines represent hydrogen bonds interactions between diC8-PI(4,5)P2 and modeled loops of ExoU. The second pose maintains hydrogen bond interactions; however, salt bridge interactions with the phosphate headgroup of diC8-PI(4,5)P2 are lost. B, 2D interaction diagram between diC8-PI(4,5)P2 and the modeled L3 loop of ExoU. Potential hydrogen bonds are indicated in purple, and multicolored lines (red/blue) are potential salt bridge interactions. Gray halos indicate that the ligand is solvent-exposed. Hydrophobic interactions are indicated in green. Color scheme as described in Fig. 5.
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Figure 8. Validation of diC8-PI(4,5)P2 interactions by site-directed mutagenesis. A–D, EPR spectra for ExoU variants in the apo state (black) and in the presence of diC8-PI(4,5)P2 (red) for 622R1/620A (A), 622R1/623A (B), 664R1/623A (C), and 673R1/661A (D). In each case, the secondary alanine mutation diminishes (A and C) or eliminates (B and D) the PI(4,5)P2-induced conformational change observed in the WT background (see Fig. 2).

Experimental procedures

Recombinant protein expression, spin labeling, and purification

All ExoU samples were expressed, spin-labeled, and purified according to a protocol described previously (18). In brief, cells were treated with lysozyme (Sigma Aldrich) prior to lysis using a French press, and His6-tagged ExoU was purified by cobalt affinity chromatography on a HiTrap TALON Crude column (GE Healthcare) with elution buffer containing 50 mM sodium phosphate (pH 7.4), 300 mM NaCl, and 100 imidazole (18). Cysteine substitutions were prepared using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing (Retrogen).

Recombinant ExoU was spin-labeled overnight with a 5-fold molar excess of (S-1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanethiosulfonate (MTSL) (Toronto Research Chemicals) in elution buffer with 20% glycerol and desalted using a HiPrep 26/10 desalting column (GE Healthcare) equilibrated with 50 mM sodium phosphate, 150 mM NaCl (pH 7.0). The His6 tag was removed by thrombin cleavage, followed by sequential passage through HiTrap benzamidine FF and HiTrap TALON Crude columns. Protein was concentrated using 30-kDa Amicon centrifugal filters, and concentration was determined by measuring absorbance at 280 nm using an extinction coefficient of 29,160 M⁻¹ cm⁻¹. Spin-labeled protein was aliquoted and stored at −80 °C.

Analysis of enzymatic activity

In vitro phospholipase activity assays were conducted at room temperature in a SpectraMax M2 plate reader using a soluble phospholipid mimic, PED6 (N-(6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine, triethyl ammonium salt, Invitrogen) as described previously (8, 18). Assays contained 10 nM ExoU, 10 μM PED6, and 20 μM recombinant diubiquitin and were titrated with 5–48 μM diC8-PI(4,5)P2 (Echelon Biosciences). Fluorescence (480 nm excitation/515 nm emission) was measured at 1-min intervals for 1 h. Conversion of relative fluorescence units to nanomoles of PED6 was made with an empirically determined factor of 59,300 RFU = 1 nmole PED6 (18).

Continuous-Wave Electron Paramagnetic Resonance and Phosphatidylinositol Analogs Binding Analyses

All experiments were conducted at ambient temperature (~22 °C) using an ELEXSY-S-II E500 spectrometer (Bruker) equipped with a high-Q cavity operating at X band. MTSL spin-labeled monocysteine substitutions in recombinant ExoU (18) were analyzed at 50–70 μM in the presence or absence of a 10- to 12-fold molar excess of soluble diC8-PI(4,5)P2 or another short-chain phosphoinositide (Echelon Biosciences) in buffer containing 20 mM MOPS (pH 6.8), 150 mM NaCl, and 20% glycerol. Spectrometer acquisition parameters were set as follows: time constant, 5.12 ms; conversion time, 40.96 s; sweep time, 41.94 s; 100-kHz field modulation amplitude, 1.6 G; microwave power, 10 milliwatt; and a sweep width of 100 G. Spectra were collected as an average of 25 scans. Samples containing ubiqui-
tin were taken in the presence of a 2-fold molar excess of linear diubiquitin (26). Spectra were baseline-corrected and normalized using the Labview Convert and Align program written by Christian Altenbach.

**Molecular modeling system preparation**

Prediction of ligand binding to ExoU was carried out using tools from Schrödinger Suite 2018-2. diC8-PI(4,5)P2 and its analogs were preprocessed using the ligand preparation (ligprep) tool. The ligprep tool generates 3D structures with accurate bond lengths and angles of ligands, providing a realistic representation of the conformation of the ligand as it would appear when naturally bound to the protein. Ligprep also adds hydrogen atoms to the ligands and assigns the appropriate protonation states as dictated by pH. The ExoU protein (PDB code 3TU3) was prepared using Protein Preparation Wizard, which includes, but is not limited to, assignment of H-bonds, ionization states (pH, 7.4), restrained minimization, and filling in missing loops and side chains with Prime (39). The receptor grid was generated from a centroid of selected residues, which included residues 620–624 and 661–677, with the inner receptor grid box adjusted to 20 × 20 × 20 Å. The ligprep structures were docked using Schrödinger’s Glide tool. Flexible ligand docking and extra precision docking were utilized to ensure a more accurate prediction of the binding pose of each analog. A scoring function, GlideScore (GScore), calculated in kilocalories per mole, was generated and used to rank the ligands according to their complementarity and contacts made to the protein binding site by exploring ligand conformation, position, and orientation within the cavity. Ligands ranked highly are assumed to have a higher binding affinity to the site, which infers improved activity. The GScore is an empirical formula represented as follows (40, 41): GScore = 0.065 × vdW + 0.130 × Coul + Lipo + Hbnd + Metal + BuryP + RotB + Site. vdW is the Van der Waals energy, Coul is the Coulomb energy, Lipo is the lipophilic term that rewards hydrophobic interactions, Hbnd is the hydrogen bond term, Metal is the metal binding term, BuryP is a penalty for buried polar groups, RotB is a penalty for freezing a rotatable bond, and Site denotes the polar but nonhydrogen-bonding interactions in the site.


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**References**

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