

Activation of ExoU Phospholipase Activity Requires Specific C-Terminal Regions[∇]

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***Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that utilizes a type III secretion system to subvert host innate immunity. Of the 4 known effector proteins injected into eukaryotic cells, ExoS and ExoU are cytotoxic. The cytotoxic phenotype of ExoU depends on the enzymatic activity of the patatin-like phospholipase A₂ domain localized to the N-terminal half of the protein. Amino acid residues located within the C-terminal region of ExoU are postulated to be required for trafficking or localization to the plasma membrane of eukaryotic cells. This report describes the characterization of a transposon-based linker insertion library in ExoU. Utilizing an unbiased screening approach and sensitive methods for measuring enzymatic activity, we identified regions of ExoU that are critical for activation of the phospholipase activity by the only known cofactor, SOD1. Insertions at D572 and L618 reduced the rate of substrate cleavage. Enzymatic activity could be restored to almost parental levels when SOD1 concentrations were increased, suggesting that the linker insertion disrupted the interaction between ExoU and SOD1. An enzyme-linked immunosorbent assay (ELISA)-based binding test was developed to measure ExoU-SOD1 binding. These experiments suggest that ExoU activation by SOD1 is hampered by linker insertion. ExoU derivatives harboring minimal phospholipase activity retained biological activity in tissue culture assays. These proteins affected primarily cellular architecture in a manner similar to that of ExoT. Our studies suggest that conformational changes in ExoU are facilitated by SOD1. Importantly, the level of phospholipase activity influences the biological outcome of ExoU intoxication.**

Pseudomonas aeruginosa is a Gram-negative bacterium responsible for severe and potentially fatal opportunistic infections. As a contributor to nosocomial infections, *P. aeruginosa* is a leading cause of hospital-acquired and ventilator-associated pneumonias (40). Furthermore, *P. aeruginosa* is responsible for ulcerative keratitis and ocular disease found in conjunction with the use of soft contact lenses (2, 10, 54). Infections with this pathogen are of critical concern for individuals admitted with severe burns, due to the bacterium's ability to colonize and persist in damaged tissues (35). Patients suffering from cystic fibrosis often succumb to severe lung infections and inflammation due to colonization with antibiotic-resistant, mucoid strains of *P. aeruginosa* (3). The expression of multiple efflux pumps and the ability to inactivate and modify antibiotics make *P. aeruginosa* dangerous and difficult to treat (27). Several investigators are exploring ways, as adjuncts or alternatives to antibiotic treatment, to neutralize virulence factors that contribute to the ability of *P. aeruginosa* to suppress host innate and adaptive immune responses (17, 21, 22, 52).

Many Gram-negative bacteria, including *P. aeruginosa*, encode one or more type III secretion systems (T3SS), which are thought to aid in pathogenesis and increase disease severity (19, 32, 39). Four effectors are translocated by the T3SS of *P. aeruginosa* and include ExoS, ExoT, ExoU, and ExoY (8, 23,

56, 57). The activity of each effector is dependent upon interaction with a cofactor present in eukaryotic but not prokaryotic cells. ExoS and ExoT are bifunctional enzymes that possess both Rho GTPase-activating protein and ADP-ribosyltransferase activities (23, 25, 51). The ADP ribosylation of eukaryotic proteins by ExoS and ExoT requires activation by members of the 14-3-3 family of scaffolding proteins (13). ExoY is an adenyl cyclase that causes the accumulation of cyclic AMP (cAMP) in intoxicated cells. The eukaryotic cofactor required for ExoY activity has not been identified (57). ExoU, a potent A₂ phospholipase responsible for membrane disruption and cellular lysis, requires superoxide dismutase 1 (SOD1) for the detection of enzymatic activity (43, 46).

ExoU is an important virulence factor of *P. aeruginosa*, as it causes rapid cell death during *in vitro* infections and is associated with poor clinical outcomes (19, 39, 44). Several studies have used truncation analyses, linker mutagenesis, and site-specific amino acid substitutions to define regions of ExoU important for various functions (7, 36). ExoU is a 74-kDa, hydrophilic, and slightly acidic protein with a pI of 5.9 (8). The first 52 amino acids are required for interaction with the chaperone SpcU and may be important for translocation through the T3SS (7, 9). Enzymatic activity is attributed to the patatin-like phospholipase domain located between residues 107 and 357 (34, 46). Two catalytic residues, S142 and D344, and a sequence encoding an oxyanion hole (₁₁₂GGAK₁₁₅) are located within this domain (34, 46). The oxyanion hole is thought to stabilize the negative charge of the intermediate structure during substrate cleavage (5). C-terminal residues of ExoU, specifically the last 137 amino acids, have been implicated in

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or properties	Reference or source
Strains		
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	28
<i>E. coli</i> BL21(DE3) pLysS	F ⁻ <i>ompT</i> <i>hsdS_B</i> (<i>r_B⁻</i> <i>m_B⁻</i>) <i>gal</i> <i>dcm</i> (DE3) pLysS (Cam ^r)	50
<i>P. aeruginosa</i> PA103 Δ <i>exoU</i> <i>exoT</i> ::Tc	Functional secretion apparatus, no effectors	53
Plasmids		
pUCP19	Wild type, <i>E. coli</i> - <i>P. aeruginosa</i> shuttle vector, Amp ^r	48
pUCP <i>exoT</i>	ADP-ribosyl transferase and Rho-GAP activities	56
pUCP <i>exoU</i>	Catalytically active phospholipase	8
pUCP <i>exoU</i> -S142A	Catalytically inactive phospholipase	46
pUCPL618-S142A	Catalytically inactive phospholipase	This study
pUCPL651-S142A	Catalytically inactive phospholipase	This study
pet15b	N-terminal His-tag fusion expression vector with thrombin cleavage site, Amp ^r	Novagen
pet16b	N-terminal His-tag fusion expression vector, Amp ^r	Novagen
pet16b <i>pcrV</i>	<i>Pseudomonas</i> V antigen, 294 aa	47
pet15 <i>bexoU</i>	Catalytically active phospholipase, 687 aa	1
pet16 <i>bexoU</i>	Catalytically active phospholipase, 687 aa	9
pet16 <i>bexoU</i> -S142A	Catalytically inactive phospholipase, 687 aa	46
pet16 <i>bexoU</i> -D344A	Catalytically inactive phospholipase, 687 aa	46
pet16 <i>bexoU</i> -P56	Linker insertion after P56, 692 aa	This study
pet16 <i>bexoU</i> -L109	Linker insertion after L109, 692 aa	This study
pet16 <i>bexoU</i> -A117	Linker insertion after A117, 692 aa	This study
pet16 <i>bexoU</i> -P365	Linker insertion after P365, 692 aa	This study
pet16 <i>bexoU</i> -A393	Linker insertion after A393, 692 aa	This study
pet16 <i>bexoU</i> -D572	Linker insertion after D572, 692 aa	This study
pet16 <i>bexoU</i> -D572*	Linker insertion after D572, 692 aa	This study
pet16 <i>bexoU</i> -L618	Linker insertion after L618, 692 aa	This study

membrane localization after translocation into mammalian cells (37). The domain or region(s) required for the activation of ExoU by SOD1 have not been identified.

In this study, linker-scanning mutagenesis (the insertion of 15 nucleotides randomly throughout the coding sequence) was used to identify regions of *exoU* that impair activation of phospholipase activity by SOD1. Our data support the model that SOD1 may be facilitating the activation of ExoU by altering the conformational properties of the enzyme. Understanding the molecular mechanisms mediating SOD1 and ExoU interaction may contribute to the design of therapeutics for the treatment of acute *P. aeruginosa* infections.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Pseudomonas* strains were grown at 37°C in Luria-Bertani (LB) broth and plated on Vogel Bonner medium (55). To produce recombinant proteins from linker-containing genes, open reading frames were subcloned from pUCP*exoU*-LINKER into pET16b and transformed into *Escherichia coli* BL21(DE3) pLysS. Point mutations in pUCP*exoU*-LINKER were generated using the Change-IT mutation site-directed mutagenesis kit (USB Corporation). Bacterial strains containing plasmids were grown in the presence of the appropriate antibiotic(s) at a final concentration of 100 μ g/ml of ampicillin, 30 μ g/ml of chloramphenicol, or 50 μ g/ml of kanamycin for *E. coli* and 400 μ g/ml of carbenicillin for *P. aeruginosa*.

ExoU secretion and detection by Western blot analysis. For ExoU secretion assays, *P. aeruginosa* strains were grown in deferrated and dialyzed Trypticase soy broth with 10 mM nitrilotriacetic acid (secretion medium) as previously described for ExoS expression and secretion (23). Secretion medium was inoculated with an emulsion of bacteria at a starting optical density at 540 nm (OD₅₄₀) of 0.2 and cultured at 32°C until the OD₅₄₀ reached 3 or 4. The culture supernatant was separated from bacteria by centrifugation at 5,000 \times g for 10 min at 4°C and concentrated 20-fold by 55% ammonium sulfate precipitation. Proteins were analyzed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and then by Western blotting. Blots were probed with a monoclo-

nal anti-ExoU antibody, U29F8 (1:20,000), and a secondary mouse anti-immunoglobulin G (IgG) antibody (1:10,000) conjugated to horseradish peroxidase (HRP; Roche). Bound IgG-HRP was detected with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Linker-scanning mutagenesis of *exoU*. Nucleotide insertions (encoding five amino acids) were generated by transposon mutagenesis of pUCP*exoU* using the GPS-LS linker scanning system (New England Biolabs, Inc.). The pUCP*exoU* transposon mutants were transformed into DH5 α , selected for transposon-encoded kanamycin resistance (Kan^r), and subsequently screened for plasmid-encoded ampicillin resistance (Amp^r). Approximately 498 colonies were picked after transformation; of these, 101 colonies were resistant to both kanamycin and ampicillin. Plasmids were isolated (Montage plasmid miniprep96 kit) and digested with PmeI (Fermentas) to remove the transposon, and the vector ends were ligated together using standard cloning techniques. The ligated plasmid was transformed into *E. coli* DH5 α , and colonies were picked onto LB supplemented with kanamycin (LB_{kan}) and ampicillin (LB_{amp}) to screen for loss of the transposon. Plasmids were isolated from mutants that were Amp^r and Kan^s and transformed (33) into PA103 Δ *exoU**exoT*::Tc, abbreviated throughout as PA103 Δ UT. Insertions within the *exoU* open reading frame will be referred to as ExoU-LINKER molecules. Linker sequences and locations were identified by DNA sequencing of each ExoU-LINKER gene using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). For clarification, the L109 gene encodes an in-frame 5-amino acid linker after the leucine residue at position 109 in wild-type (WT) ExoU.

Recombinant protein expression, purification, and quantitation. Cultures were grown at 37°C until the OD₆₀₀ reached 0.5 or 0.6, and isopropyl- β -D-thiogalactopyranoside (0.5 mM) was added to induce protein expression. The temperature was shifted to either 16°C or 30°C, and the cultures were incubated for 2 or 3 h until the OD₆₀₀ was greater than 1.0. Cells were harvested by centrifugation at 5,000 \times g for 10 min at 4°C. The bacterial pellet was washed with binding buffer (50 mM Na₂HPO₄, 300 mM NaCl, 5 mM imidazole, pH 7.0) in the presence of protease inhibitors (128 μ M benzamide, 84 μ M leupeptin, 2.92 μ M pepstatin A, and 6.8 μ M phosphoramidon) and subjected to centrifugation at 5,000 \times g at 4°C for 10 min. Bacterial pellets were suspended in binding buffer in the presence of protease inhibitors, 0.65 μ M DNase (Sigma), and 1.5 μ M RNase (Sigma). Cells were lysed by probe-tip sonication (Sonifier cell disrupter; Heat Systems, Inc.), and between pulses the samples were cooled on ice for 5 min. The lysate was subjected to centrifugation at 16,000 \times g for 15 min

at 4°C to remove membrane debris and unlysed cells. The supernatant was then subjected to ultracentrifugation at $100,000 \times g$ for 1 h at 4°C to remove insoluble material. The soluble supernatant was collected, and histidine-tagged proteins were purified by Talon cobalt chromatography per the manufacturer's instructions (Clontech). Eluted fractions were analyzed by SDS-PAGE, pooled, and dialyzed extensively in the following buffer: 50 mM MOPS (morpholinepropane-sulfonic acid) (pH 6.3) and 50 mM NaCl. The protein concentration was determined by bicinchoninic acid (BCA) analysis (Thermo Scientific). Proteins were concentrated in Amicon (Millipore) units that retain molecules above 10 kDa. Untagged rExoU was made as previously described and dialyzed against 50 mM MOPS (pH 6.3) and 50 mM NaCl (1). Purified recombinant protein preparations were stored at -80°C .

In vitro fluorescence-based ExoU activity assay. The fluorescence-based ExoU activity assay utilizes the fluorogenic substrate 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, triethylammonium salt) (Invitrogen). Cleavage of PED6 at the *sn*-2 position relieves the intramolecular quenching effect of the dinitrophenyl group, and upon excitation, the BODIPY-FL fluorescent dye releases a photon at 511 nm. Fluorescence intensity was measured at an excitation wavelength of 488 nm and emission wavelength of 511 nm (495-nm cutoff filter, Spectramax M5 microplate reader; Molecular Devices). The assay conditions were modeled after those used by Sato et al. and Benson et al., with modifications (1, 43).

Supernatants from *P. aeruginosa* cultures grown overnight at 32°C containing secreted ExoU-LINKER molecules were tested for phospholipase activity using the fluorescence-based ExoU activity assay. Briefly, 20 μl of culture supernatant was added to a mixture of 50 mM MOPS (pH 6.3), 50 mM NaCl, 3.125 μM bovine superoxide dismutase 1 (bSOD1; Sigma), 250 mM monosodium glutamate (MSG) (pH 6.3; Sigma), and 30 μM PED6 in a final volume of 50 μl . Background fluorescence was measured under reaction conditions lacking bSOD1. Background relative fluorescence units (RFU) were subtracted from each sample at each time point. Fluorescence intensity was measured every 15 min for 2 h and normalized to the OD_{540} at the time of harvest. Positive phospholipase activity was judged as at least 150 RFU and possession of an increasing catalytic rate over the duration of the assay.

When activity was measured from recombinant proteins, the reaction conditions included 50 mM MOPS (pH 6.3), 750 mM MSG (pH 6.3), 30 μM PED6, 0.16 μM ExoU/ExoU-LINKER, and 6.3 μM bSOD1. Background fluorescence, the fluorescence intensity recorded from the reaction conditions lacking bSOD1, was subtracted from each sample. For the bSOD1 titration (6.3 to 75.0 μM) analysis, 0.2 μM ExoU/ExoU-LINKER was added to the assay. Fluorescence intensity was recorded every 15 min for 6 h. The data for phospholipase activity and the bSOD1 titration were plotted as the mean RFU \pm standard error of 3 independent experiments.

Assessment of cytotoxicity mediated by *P. aeruginosa* ExoU-LINKER strains. To determine if the linker insertion in *exoU* disrupted cytotoxicity of mammalian cells, HeLa cell monolayers were infected with the *P. aeruginosa* ExoU-LINKER strains. HeLa cells were seeded in a 24-well plate in Dulbecco's modified Eagle medium (DMEM; Invitrogen) with 10% newborn calf serum (Invitrogen) for confluence (1.2×10^5 cells/well) the next day. Fresh streaks from freezer stocks of *P. aeruginosa* ExoU-LINKER strains were grown overnight at 37°C. Prior to infection, HeLa cell monolayers were rinsed with Hank's buffered salt solution (HBSS) containing calcium and magnesium. The HeLa cell monolayers were infected with a multiplicity of infection (MOI) of 20 and incubated for 4 h at 37°C with 5% CO_2 . Cells in an uninfected control well were incubated in serum-free DMEM. After infection, the inoculum was removed, and the monolayers were stained with a 0.3% crystal violet solution in 5% isopropanol/ethanol/methanol for 5 min. Stained cells were rinsed three times with water. After the wells were dry, the retained crystal violet was solubilized in 95% ethanol, and the absorbance at 570 nm was measured. The results were interpreted as positive or negative for monolayer (crystal violet) retention. This procedure was also performed to characterize the monolayer retention with human lung epithelial A549 cells, incorporating the following changes. The A549 cells were seeded in a 48-well plate in DMEM with 10% fetal calf serum (Invitrogen) for confluence (2.4×10^5 cells/well) the next day. When the monolayer was intact, cells were not affected by ExoU intoxication and the crystal violet stain was retained (positive retention). Conversely, loss of the cells corresponded to little or no stain retention (negative retention) and indicated that ExoU cytotoxic activity was expressed.

To confirm the cytotoxic/noncytotoxic phenotype of the *P. aeruginosa* ExoU-LINKER strains, lactate dehydrogenase (LDH) release from A549 cells was measured. A549 cells were seeded in a 24-well plate in DMEM with 10% fetal calf serum for confluence (2.4×10^5 cells/well) the next day. The A549 cell monolayers were infected with an MOI of 20 and incubated for 4 h at 37°C with

5% CO_2 . Culture medium from infected cells was harvested and subjected to centrifugation at $2,000 \times g$ for 5 min at 4°C to remove cellular debris and bacteria. The supernatant was transferred to a fresh tube, and LDH activity was measured per the manufacturer's instructions (CytoTox96 nonradioactive assay; Promega). These data were compared to those for a detergent-lysed monolayer (100% lysis) and plotted as the mean \pm standard error of 3 independent experiments.

Immunofluorescence microscopy. To determine the biological activity of *P. aeruginosa* strains expressing ExoU-LINKER molecules possessing minimal phospholipase activity, infected A549 cells were examined by fluorescence microscopy. A549 cells were seeded for confluence (4.8×10^5 cells/well) in a 6-well plate containing one fibronectin (10 $\mu\text{g}/\text{ml}$; Sigma)-coated coverslip per well. Prior to infection, A549 cell monolayers were rinsed once with HBSS containing calcium and magnesium. The A549 cell monolayers were infected with an MOI of 10 for 4 h at 37°C with 5% CO_2 . Cells in a control well were incubated in serum-free DMEM. After infection, cells were rinsed with PBS and fixed with 3.7% paraformaldehyde for 10 min at room temperature. After fixation, the cells were washed three times with PBS and permeabilized by incubation with 0.5% saponin in PBS buffer (BP) containing 1% BSA for 15 min at room temperature. The A549 cell monolayers were incubated in BP buffer containing the polyclonal anti-LPS antibody T2WB (1:400,000; a kind gift of Gerald Pier) for 30 min at room temperature. A549 cell monolayers were washed three times with BP buffer and incubated with a goat anti-rabbit Alexa488-labeled secondary antibody (1:1,000; Invitrogen) in BP buffer for 30 min at room temperature. The stained cells were rinsed three times with PBS before exposure to Texas Red-X phalloidin in PBS containing 1% BSA per the manufacturer's instructions (Molecular Probes). Cells were rinsed three times with PBS, and the coverslips were mounted on slides with ProLong Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole; Invitrogen). After 24 h, the coverslips were sealed with nail polish and viewed under a 20 \times or 60 \times (oil immersion) objective lens using a Nikon Eclipse Ti inverted microscope.

Binding assay for rExoU association with immobilized SOD1. To determine if the interaction between ExoU and SOD1 was disrupted in different linker-containing derivatives, a plate-binding assay using recombinant proteins was developed. Briefly, bSOD1 (3 μg in 100 μl of 50 mM sodium carbonate buffer [pH 9.6]) was used to coat a 96-well polyvinyl chloride plate (Falcon; BD Bioscience). The immobilization of bSOD1 was performed at room temperature for 2 h. Bovine SOD1 was aspirated, and the wells were washed three times with 10 mM Tris and 0.9% NaCl (pH 8.0) and blocked overnight at 4°C using a solution of 1% gelatin (BD Bioscience) in sodium carbonate buffer (200 μl). Blocking agent was aspirated, and the wells were washed three times with 50 mM MOPS (pH 6.3), 50 mM NaCl (ExoU assay buffer) with 1.3 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate (CHAPS; Sigma). All subsequent steps for protein binding, detecting with antibodies, or washing were performed in assay buffer containing CHAPS. Reagents were allowed to bind for 2 h at room temperature. Primary antibodies were used at a 1:2,000 dilution. Histidine-tagged ExoU or rPcrV protein was detected using monoclonal antibody (Mab) U29F8 or Mab 166 (12), respectively. A secondary mouse anti-IgG antibody conjugated to HRP (1:5,000; Roche) was used to detect the monoclonal antibody binding. Bound IgG-HRP was detected after incubation for 55 min with Quant-aBlu substrate solution per the manufacturer's protocol (Pierce). The fluorescence intensity was measured at an excitation wavelength of 325 nm and an emission wavelength of 420 nm. Nonspecific binding was assessed with two types of wells. One set of wells was coated with gelatin, exposed to recombinant ExoU (rExoU), rExoU derivatives, or rPcrV at identical protein concentrations used to assess specific binding, washed, and processed with primary and secondary antisera (nonspecific binding of test protein controls). Other control wells were coated with SOD1 and blocked with gelatin but were not exposed to rExoUs or rPcrV. These wells were processed with primary and secondary antisera and served as a control for nonspecific antibody binding. The cumulative background from both wells was subtracted from values obtained with wells with SOD1, rExoUs, or rPcrV and the appropriate primary and secondary antibodies. Data were plotted as the mean \pm standard error of 3 independent experiments.

Statistics and software. Statistical analysis and graphs were assembled using Prism 5.0 (GraphPad Software, Inc.) and Microsoft Excel. All figures were generated using Adobe Photoshop CS3, and microscopy images were analyzed using NIS-Elements AR 3.0 (Nikon).

RESULTS

ExoU-LINKER library construction. To define regions of ExoU required for cytotoxicity, a previous study used linker-

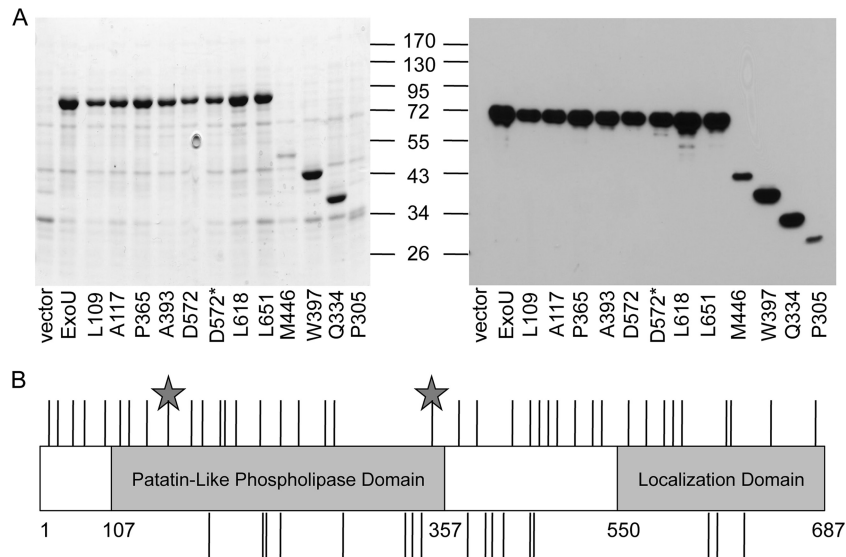


FIG. 1. Summary of the ExoU-LINKER molecules. (A) Supernatants from strains were screened by SDS-PAGE and Western blot analyses to determine whether the linker insertion encoded a full-length or truncated ExoU derivative. Shown are a Coomassie-stained gel showing the representative full-length and truncated ExoU-LINKER molecules (left) and a Western blot probed with a monoclonal antibody to ExoU (right). pUCP $exoU$ is a control for full-length molecules, and vector is a negative control for ExoU expression. All ExoU-LINKER molecules were competent for secretion from *P. aeruginosa* under inducing conditions. Each ExoU-LINKER insertion is labeled according to the site of insertion. For example, L109 represents an insertion after amino acid 109 and before amino acid 110. D572* is a second insertion at position 572, with a linker sequence different than that of D572. The sizes of the molecular weight markers (in thousands) are indicated in the middle. (B) The location of each insertion is depicted on a diagram of *exoU*. The lines above and below the diagram represent the unique full-length and truncated ExoU-LINKER molecules, respectively. The gray stars depict the two catalytic amino acids required for phospholipase activity, S142 and D344.

scanning mutagenesis (36). Transposon mutagenesis was performed with a galactose-inducible yeast expression vector containing *exoU*, insertions within the coding region were resolved, and the resulting plasmids were transformed into a yeast host (36). Cytotoxicity was scored based on growth inhibition in the presence of galactose. As expected, linkers encoding stop codons or insertions disrupting the catalytic residues or oxyanion hole resulted in nontoxic derivatives. Additionally, a C-terminal region (amino acids [aa] 550 to 687) was identified as being required for cytotoxicity, and its functional role appeared to be involved in ExoU localization to the plasma membrane (37). We refined this experimental design to generate linker insertions within a plasmid used for ExoU expression in *P. aeruginosa* (pUCP $exoU$). This strategy allowed us to screen for secretion-competent ExoU-LINKER molecules, ensuring that the molecules were capable of passing through the type III needle. We isolated 498 insertions, of which 101 mapped to the *exoU* open reading frame by restriction endonuclease digestion. The transposons were resolved, and each plasmid was transformed into PA103 Δ UT. To screen for full-length molecules, PA103 Δ UT strains expressing each insertion were grown under conditions in which type III secretion is induced. Supernatants containing the secreted proteins were analyzed on Coomassie-stained polyacrylamide gels and by Western blot analysis with a primary antibody specific for ExoU to determine if insertion of the linker resulted in an in-frame insertion or a stop codon (Fig. 1A). Of the 101 insertions that mapped within the *exoU* open reading frame, 69 insertions were in frame and full length, 24 insertions resulted in a stop codon, and 8 insertions resulted in the absence of extracellular ExoU.

After removing duplicate insertion molecules, 38 full-length and 17 truncated ExoU-LINKER molecules were identified using this technique. All ExoU-LINKER molecules obtained were analyzed for *in vitro* phospholipase activity and for cytotoxicity in a HeLa cell infection model. Figure 1B is a summary of the location of each ExoU-LINKER molecule, with full-length ExoU-LINKER molecules above the diagram and truncated molecules below the diagram. D572 and D572* are two ExoU-LINKER molecules with insertions at the same position but with different amino acid sequences encoded by the linker. The two stars in Fig. 1B represent the two catalytic residues of *exoU*, S142 and D344. Each ExoU-LINKER molecule is identified as the last ExoU-specific amino acid before the linker insertion.

ExoU phospholipase activity and cytotoxicity screens. To determine the effect of the linker insertions on ExoU activity, each ExoU-LINKER protein was screened for *in vitro* phospholipase activity present in bacterial culture supernatants and for cytotoxicity during cellular infections. An optimized activity assay was utilized that measures phospholipase activity by the cleavage of the fluorogenic substrate PED6 (1, 43). To normalize for the phospholipase activity relative to the growth of the culture, RFU values were divided by the OD₅₄₀ value at the time of harvest. Supernatants of each ExoU-LINKER molecule were scored as active, based on an increase in fluorescence intensity as a function of time, or inactive if no increase in fluorescence intensity was observed within 2 h. An initial increase in fluorescence intensity was observed for a few ExoU-LINKER supernatants, but the rate associated with substrate cleavage did not increase by the 2-h time point. These ExoU-

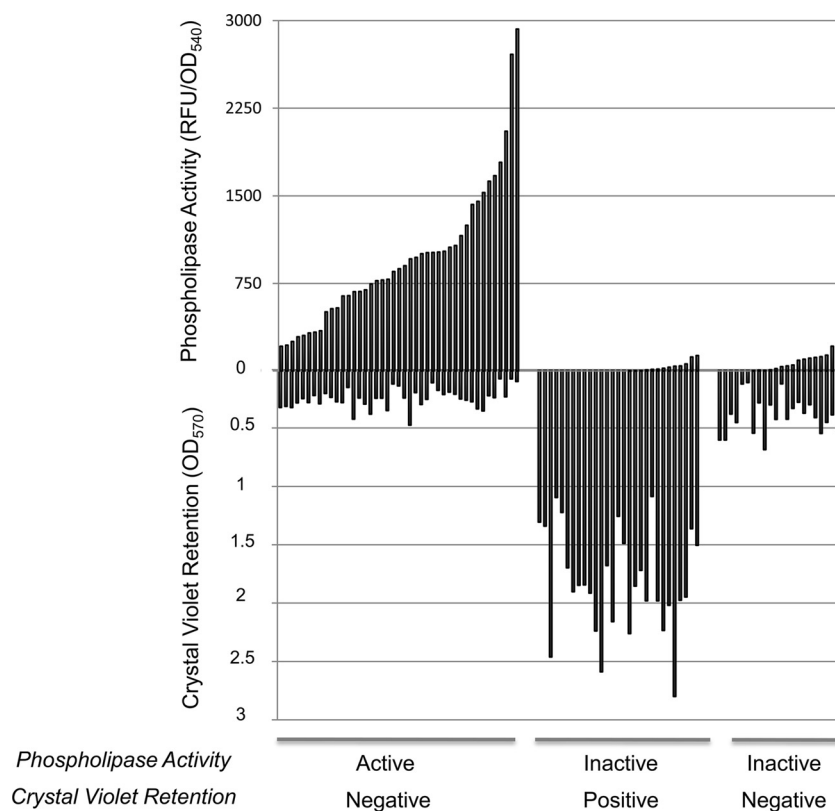


FIG. 2. Compilation of phospholipase and cytotoxic activities for all ExoU-LINKER molecules. Supernatants containing ExoU-LINKER molecules secreted from *P. aeruginosa* were used to determine phospholipase activity in a fluorescence-based ExoU activity assay. ExoU-LINKER molecules are categorized as either active or inactive based on PED6 cleavage after 2 h (top half). Every ExoU-LINKER strain was tested for cytotoxicity as determined by loss of a crystal violet-stained HeLa cell monolayer. After a 4-h infection, the HeLa cell monolayers were fixed and stained as outlined in Materials and Methods. The stain was solubilized with 95% ethanol, and the absorbance was recorded at 570 nm. Each ExoU-LINKER molecule is categorized as being positive or negative for crystal violet retention (bottom half).

LINKER molecules were considered inactive. A supernatant containing either no ExoU or WT ExoU were the negative or positive control for the assay, respectively. Figure 2 (top) shows the distribution of active or inactive ExoU-LINKER molecules from this high-throughput screen.

To determine the activity of ExoU-LINKER molecules in the context of a *P. aeruginosa* infection, confluent HeLa cell monolayers were infected with each *P. aeruginosa* ExoU-LINKER strain and stained with crystal violet. The retention of crystal violet (positive retention) indicates that the monolayer is not lysed after intoxication with ExoU, while the absence of staining (negative retention) indicates a fully cytotoxic strain. The negative and positive controls (PA103ΔUT vector only and pUCP_{exoU}, respectively) were incorporated into each assay set. ExoU-LINKER strains possessed either a cytotoxic or noncytotoxic phenotype (Fig. 2, bottom).

To understand the results from both high-throughput screenings of the ExoU-LINKER library, data from each screen were first plotted in the order of the lowest to highest values obtained (data not shown). Two trends were observed: ExoU-LINKER strains were either cytotoxic and had phospholipase activity or were noncytotoxic and had no phospholipase activity. However, upon aligning results from both screenings on the same graph, a unique set of ExoU derivatives that were cytotoxic to infected cells (low crystal violet retention) but

had no phospholipase activity were identified. These ExoU-LINKER molecules possessing unique phenotypes were investigated further.

The data for each class of molecules identified are summarized in Tables 2 to 4. These tables include information identifying the location of each insertion, the amino acid sequence of the insertion, and the crystal violet retention value of the cytotoxicity assay. For comparison purposes, the crystal violet retention values are reported for the vector control strain and a strain complemented with parental ExoU. The mean phospholipase activity of the extracellular medium for each group is 800 ± 100 RFU for members that were cytotoxic and possessed activity (Table 2), 12 ± 6 RFU for candidates that were noncytotoxic and had no extracellular activity (Table 3), and 46 ± 13 RFU for isolates that were apparently cytotoxic but were below the threshold activity to be considered positive in our screens (Table 4). Insertions affecting the phospholipase activity were found throughout the molecule.

Phospholipase activity of recombinant ExoU-LINKER molecules. Although standardized by the OD₅₄₀ value at the time of harvest, supernatants are a crude source of enzyme for quantifying activity. Different amounts of ExoU-LINKER molecules could be present in the culture medium due to differences in production rate or protein degradation. We addressed this issue by expressing and purifying recombinant, histidine-

TABLE 2. Screen summary for cytotoxic ExoU-LINKER molecules with supernatant phospholipase activity

Designation	Sequence	Membrane retention (OD ₅₇₀)
Vector	NA	1.3
WT ExoU	NA	0.275
S5	LVFKQ	0.245
S22	QVFKQ	0.262
P56	EMFKQ	0.275
Q68	GCLNK	0.222
I135	RCLNI	0.324
L173	LFKHL	0.476
L194	MFKQL	0.243
K195	CLNMK	0.336
G263	CLNTG	0.245
F290	KQAMF	0.315
E430	LFKHE	0.529
Q498	LFKHQ	0.100
G530	CLNNG	0.248
R581	VFKQR	0.192
V585	QSLFK	0.283
S587	LFKQS	0.381
Y619	LFKQY	0.139
W681	CLNTW	0.204

NA, not applicable.

tagged ExoU-LINKER molecules. The locations of the ExoU-LINKER molecules chosen for recombinant protein analysis are shown in Fig. 3A. These derivatives were selected based on the apparent uncoupling of phospholipase activity and cytotoxicity as measured by crystal violet retention (P365, A393, D572, D572*, and L618) and represent a subset of the total number of strains with this phenotype. P56 was chosen as a linker control molecule that displayed parental extracellular phos-

TABLE 3. Screen summary for noncytotoxic ExoU-LINKER molecules without supernatant phospholipase activity

Designation	Sequence ^a	Membrane retention (OD ₅₇₀)
Vector	NA	1.3
WT ExoU	NA	0.275
L109	MFKHL	1.306
A117	AYCLN	2.461
I190	V*	1.719
P222	LDV*	1.914
L223	ERV*	1.980
D229	CLNSD	1.340
V251	TVV*	1.223
L272	SAV*	1.846
N300	V*	1.854
P305	DLV*	1.975
Q334	AGV*	1.086
A384	PDV*	1.044
W397	VVV*	1.697
V399	V*	1.902
L412	EGV*	2.588
T445	IV*	2.261
M446	V*	2.799
L458	QVFKH	1.504
G463	EHCLN	1.488
A606	V*	2.234
N608	V*	2.238
G622	GV*	1.678

^a * denotes a stop codon. NA, not applicable.

TABLE 4. Screen summary for cytotoxic ExoU-LINKER molecules without supernatant phospholipase activity

Designation	Sequence	Membrane retention (OD ₅₇₀)
Vector	NA	1.3
WT ExoU	NA	0.275
L104	TSCLN	0.332
K161	CLNIK	0.373
I254	CLNTI	0.301
P365	LLFKQ	0.279
A393	CLNSA	0.452
N443	FTCLN	0.453
A452	HLLFK	0.544
L537	VFKQL	0.425
W560	CLNTW	0.111
D572	HQCLN	0.122
D572*	CLNID	0.123
L618	CLNTL	0.302
L651	MFKQL	0.426

pholipase and cytotoxicity activities in the screen. Two insertions mapping adjacent to the oxyanion hole were selected (L109 and A117) to verify that phospholipase activity was eliminated (36). Histidine-tagged parental ExoU and ExoU-S142A were purified as positive and negative controls, respectively. All recombinant proteins were purified using affinity chromatography and analyzed by SDS-PAGE and Coomassie staining (Fig. 3B). Recombinant L651 appeared to be labile when expressed from *E. coli* and was excluded from *in vitro* phospholipase activity analyses (data not shown).

WT rExoU and rP56 displayed a rapid increase in fluorescence intensity and resulted in a substrate cleavage maximum of approximately 50,000 RFU by 2 h (Fig. 3C). The ExoU-LINKER proteins rP365, rA393, rD572, and rD572* exhibited phospholipase activity as the fluorescence intensity increased over time but at a rate significantly lower than that of WT rExoU. Recombinant L618 exhibited a significantly impaired, but measurable, increase in fluorescence intensity over the 5-h time period. The negative controls for the assay, rExoU-S142A and two linker molecules located close to the oxyanion hole, rL109 and rA117, displayed no phospholipase activity after 5 h. The position of the linkers and the phospholipase activity allowed grouping of the recombinant ExoU-LINKER proteins into two broad categories, enzymes with either intermediate activity or minimal activity. Proteins belonging to the first category display phospholipase activity at a level less than that of WT rExoU and include P365, A393, D572, and D572*. The second category includes L618 and possibly L651 (based on activity assays of *P. aeruginosa* culture supernatants), which possess a minimal increase in fluorescence intensity as a function of time. Together, these data suggest that of the insertions analyzed, those mapping within the C terminus impair the phospholipase activity of ExoU.

Crystal violet retention and LDH release in A549 cells. Results from the fluorescence-based ExoU activity assays suggested that the linker insertions in ExoU resulted in a collection of molecules with variable but detectable enzyme activity. Cytotoxicity assays using crystal violet staining, however, indicated that these molecules were still relatively potent. Based on these observations, we postulated that either a small

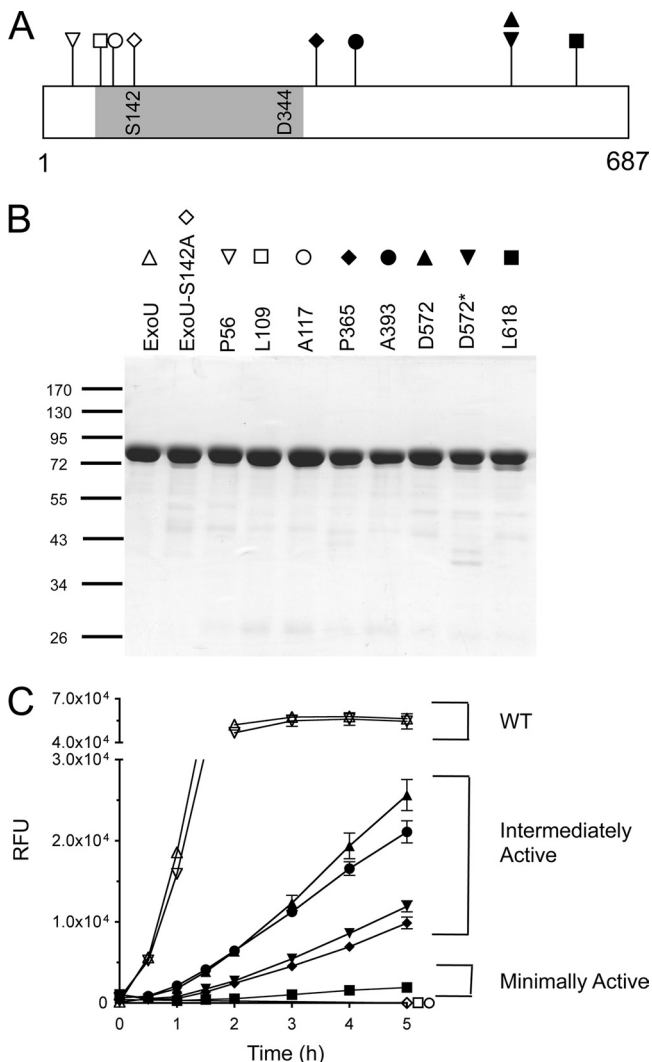


FIG. 3. Purified ExoU-LINKER proteins and phospholipase activity. (A) Location of ExoU-LINKER molecules chosen for analysis using recombinant proteins. The symbol above each location site in panel A corresponds to the symbol used for each ExoU derivative in panels B and C. The gray region represents the patatin-like phospholipase A₂ domain of ExoU. The catalytic sites are marked within the gray region of the diagram. The ExoU-LINKER molecules chosen for analysis include P56 (∇), L109 (\square), A117 (\circ), S142A (\diamond), P365 (\blacklozenge), A393 (\bullet), D572 (\blacktriangle), D572* (\blacktriangledown), and L618 (\blacksquare). Not depicted in the diagram is WT rExoU (Δ). Two insertions were obtained at D572, but with different sequences. (B) SDS-PAGE analysis of ExoU-LINKER recombinant proteins used in the fluorescence-based ExoU activity assay. Each well of the Coomassie-stained gel contains approximately 1.5 μ g of recombinant protein. The sizes of the molecular weight markers (in thousands) are indicated on the left. (C) Phospholipase activity of recombinant proteins was measured in the fluorescence-based ExoU activity assay. The assay reaction included 0.16 μ M ExoU/ExoU-LINKER, 6.3 μ M bSOD1, 50 mM MOPS (pH 6.3), 50 mM NaCl, 750 mM MSG (pH 6.3), and 30 μ M PED6. RFU were measured every 15 min for 2 h and every h thereafter for a total of 5 h. Recombinant L109 and rA117 possessed the same activity curve as a non-catalytic molecule, S142A. Each point represents the average of 3 independent experiments, with error bars for standard error.

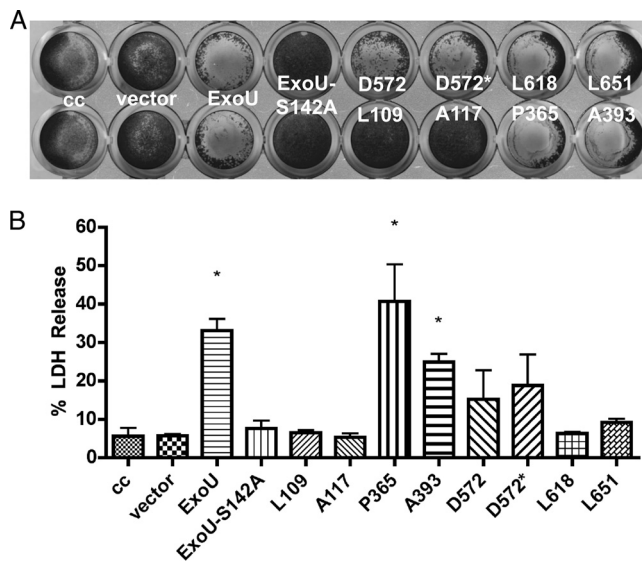


FIG. 4. ExoU-mediated cytotoxicity analysis in A549 cells. (A) Crystal violet retention was determined for selected ExoU-LINKER strains in A549 cells. A549 cell monolayers were infected with an MOI of 20 for 4 h and stained with crystal violet. The positive controls for crystal violet membrane retention include an uninfected cell control (cc), an empty vector control, and catalytically inactive ExoU-S142A. The negative control for crystal violet membrane retention is WT ExoU. (B) LDH release from A549 cells. The negative controls for LDH release include cell control (cc), vector, and catalytically inactive ExoU-S142A. A549 monolayers were infected with the same PA103 Δ UT strains, and LDH release was compared to a maximum lysis control to calculate a percent LDH release for each sample. The data are plotted as mean \pm standard error of 3 independent experiments. The asterisks represent values statistically significant compared to ExoU-S142A (Student's *t* test, *P* < 0.004).

amount of phospholipase activity was sufficient for cytotoxicity or that ExoU may express a second activity that is masked by membrane disruption. Alternatively, HeLa cells may be particularly susceptible to either the phospholipase or the postulated second activity. To determine if differences in the assay outcomes were due to different cell lines being tested, confluent A549 cell monolayers were infected with each ExoU-LINKER expression strain and stained with crystal violet (Fig. 4A). Infection with strains containing the vector control ExoU-S142A, L109, and A117 were no different than uninfected-cell controls. Infections with strains expressing ExoU or ExoU-LINKER molecules P365, A393, D573, D572*, L618, and L651 resulted in a loss of the monolayer and minimal crystal violet retention. These results were identical to those observed when HeLa cells were infected, suggesting that both cell lines are susceptible to the action(s) of ExoU.

To analyze cytotoxicity quantitatively, we infected A549 monolayers with the same strain set and measured LDH release (Fig. 4B). Infections with strains expressing ExoU, P365, or A393 resulted in LDH release and were clearly cytotoxic relative to negative controls (uninfected cells, vector control, and ExoU-S142A). LDH release from cells infected with strains expressing D572 and D572* trended toward being cytotoxic, but the values were variable between experiments, indicating that other factors may be dictating the susceptibility of cells to the intermediate phospholipase activity of these

variants. Cells infected with L109, A117, L618, and L651 released LDH at levels statistically identical to ExoU-S142A and were judged as noncytotoxic by this assay (Student's *t* test, $P < 0.004$). While the results of the LDH assay and crystal violet staining are consistent for L109 and A117, they are diametrically opposed for L618 and L651. These data suggest that the minimal phospholipase activities exhibited by these linker insertion molecules may result in changes in cell association with the tissue culture plate substrate and extracellular matrix rather than membrane permeability and cell death.

Minimal phospholipase activity affects the actin cytoskeleton. To determine the biological effect of minimal phospholipase activity, A549 cells were infected with the PA103 Δ UT ExoU-LINKER L618 and L651 mutant strains. These two strains have minimal phospholipase activity *in vitro* and result in loss of HeLa and A549 cell monolayers in the crystal violet retention assay but do not release LDH. A549 cell monolayers on coverslips were infected, and the actin cytoskeleton morphology was examined by phalloidin staining (Fig. 5). In addition to an uninfected control, A549 cell monolayers were also infected with PA103 Δ UT ExoU-S142A or the same host strain that is complemented with an ExoT expression construct. The actin cytoskeleton does not change shape after intoxication with a catalytically inactive phospholipase (ExoU-S142A). However, infection with an ExoT-expressing strain resulted in distinct changes to the cytoskeleton, as previously reported (15). Infection with the minimally active ExoU-LINKER molecules L618 and L651 also shows a rearrangement of the actin cytoskeleton similar to that of ExoT. To ensure that phospholipase activity was responsible for the cell-rounding phenotype, we introduced the S142A mutation into molecules L618 and L651 and repeated the *in vitro* infections. Introducing the catalytic mutation completely abrogated the effects on the actin cytoskeleton, confirming that minimal amounts of phospholipase activity are generating the observed cell rounding. In addition to crystal violet staining, these data confirm that both ExoU-LINKER molecules are translocated into A549 cells. Minimal phospholipase activity appears to have either specific or nonspecific effects on the cellular cytoskeleton that result in rounding and detachment.

Activation of ExoU-LINKER molecules with bSOD1. The C terminus of ExoU has previously been postulated to be important for the interaction with the cellular activator SOD1 (45). To determine if the activation of each molecule could be restored to a wild-type level with additional cofactor present, bSOD1 (6.3 to 75 μ M) was titrated into reaction mixtures containing purified rExoU, rP56, rD572, or rL618. Recombinant ExoU and rP56 exhibited dose-dependent increases in substrate cleavage, which reached a maximum fluorescence intensity of approximately 60,000 RFU (Fig. 6A and B). A dose-dependent increase in phospholipase activity was also observed for rD572 and rL618, but the level of substrate cleavage saturation was lower than that of WT rExoU or rP56 (Fig. 6C and D). Table 5 lists the enzymatic rates of rExoU, rP56, rD572, and rL618 upon activation by bSOD1. The rates were statistically different when rExoU or rP56 was compared to rD572 or rL618 (Student's *t* test, $P < 0.0394$). This further validates the assignment of D572 and L618 to intermediate and minimally active categories, respectively. These results

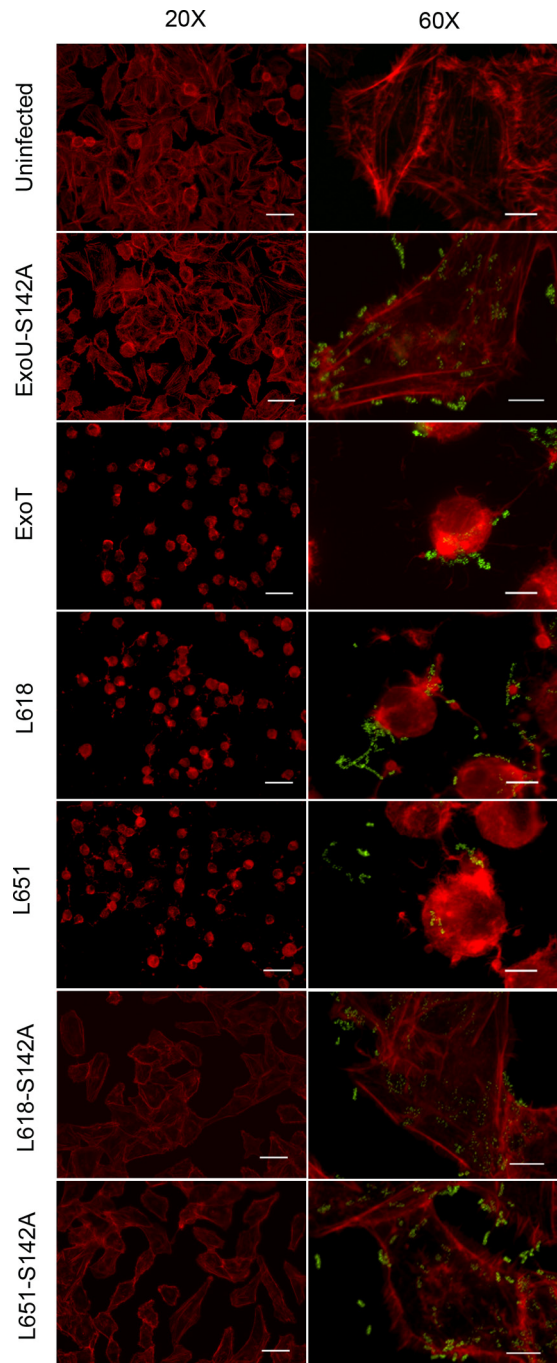


FIG. 5. Minimal activity of ExoU-LINKER molecules affect the actin cytoskeleton of infected cells. A549 cell monolayers were infected with PA103 Δ UT strains at an MOI of 10 for 4 h. After infection, samples were permeabilized and Texas Red-X phalloidin was used to visualize the actin cytoskeleton. Polyclonal anti-LPS antibody, followed by a secondary goat anti-rabbit Alexa488-labeled antibody, was used to detect *P. aeruginosa*. Slides were examined at $\times 20$ and $\times 60$ magnification of an objective lens to observe phalloidin staining of the actin cytoskeleton (left, $\times 20$), in conjunction with the presence of the bacteria (right, $\times 60$). The scale bars for $\times 20$ and $\times 60$ magnification are 50 and 10 μ m, respectively. A549 cells infected with PA103 Δ UT ExoT served as a positive control for translocation and cell rounding.

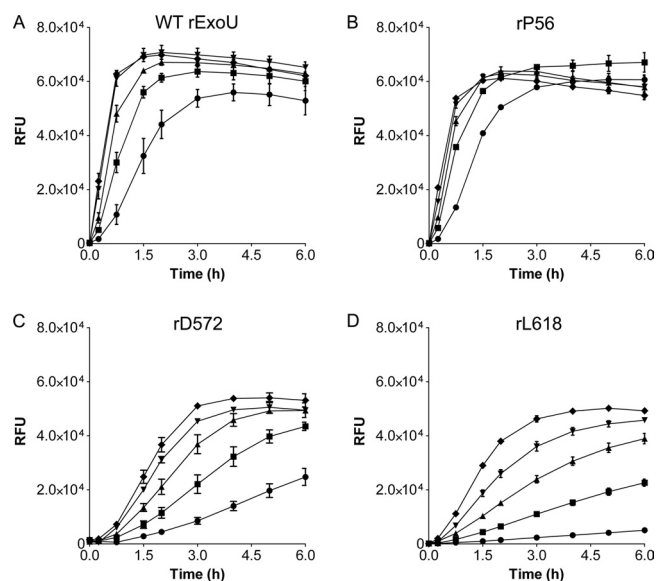


FIG. 6. SOD1 titration with ExoU-LINKER molecules. Phospholipase activity was measured in the presence of increasing concentrations of bSOD1. Progression curves for rExoU (A), rP56 (B), rD572 (C), and rL618 (D) show a dose-dependent increase in phospholipase activity over 6 h. The concentrations of bSOD1 used include 6.3 (●), 18.8 (■), 37.5 (▲), 56.3 (▼), and 75.0 (◆) μM . A blank lacking bSOD1 was measured for each recombinant protein to subtract background cleavage of PED6. Each point represents the average of 3 independent experiments, with error bars for standard error.

suggest that either the association with or activation by SOD1 is affected by the linker insertion within ExoU.

SOD1-ExoU association. To measure the association between bSOD1 and rExoU parental or derivative molecules, we developed an ELISA-based test. The highest specific binding was achieved when bSOD1 was immobilized to the solid phase. The inclusion of CHAPS, a zwitterionic detergent, in the wash and binding buffers increased the sensitivity of the assay. Finally, a highly sensitive horseradish peroxidase substrate was incorporated to detect potentially minor populations of interactants. Assay buffers, time, and incubation temperatures mimicked the *in vitro* conditions used to detect phospholipase activity. Since bSOD1 binds copper and zinc, assay conditions with and without 1 mM EDTA were tested to suppress the possible nonspecific binding of tagged molecules to the metals associated with bSOD1. The inclusion of EDTA in binding, wash, and assay buffers made no difference (data not shown). Various histidine-tagged proteins, including WT rExoU, rPcrV, rD344A, rD572, and rL618 and an untagged version of rExoU were tested.

The nonspecific binding control protein rPcrV associated with bSOD1 to a limited extent but was significantly different than WT rExoU and rD344A ($P < 0.0143$; Fig. 7A). All rExoU derivatives bound to immobilized bSOD1 similarly and were significantly different than rPcrV ($P < 0.0313$; Fig. 7B). The binding of untagged rExoU to bSOD1 was similar to that of WT rExoU but was more variable from experiment to experiment. These results indicate that the linker insertions within ExoU likely disrupt the ability of the molecule to be activated, perhaps by preventing ExoU from assuming an optimal conformation.

DISCUSSION

The long-term goal of our studies is to identify the mechanism of ExoU activation. Our strategy was to map regions important for cytotoxicity and enzyme activation. Previous biochemical analyses identified three regions encompassing an oxyanion hole and the two catalytic residues located within the patatin-like phospholipase domain (34, 46). Linker insertion mutagenesis of *exoU* indicated that in addition to these important regions, cytotoxic activity also required an uncharacterized region within the patatin-like phospholipase domain and a membrane recognition signal (36, 37). Although the techniques used in this study to generate linker insertion molecules in ExoU were identical to those of Rabin et al., our expression, screening, and catalytic analyses differed (36). Our initial screening for insertions was unbiased, in the sense that only clones with linkers within the *exoU* open reading frame were selected for study; expression or function was not assessed at this stage. Additionally, functional screens were performed with *P. aeruginosa* to eliminate molecules whose gross structure was disrupted to the extent that they could not be secreted by the *P. aeruginosa* T3SS. This approach allowed us to assay for activation by a eukaryotic cofactor after injection occurred in a biologically relevant manner. Finally, a highly sensitive and defined enzymatic assay was used that allowed characterization of marginally active molecules. Although no single screen can identify all the residues or regions required for the biologic activities of ExoU, the combined data from both studies represents a fairly saturated coverage of the molecule and reveals novel functional information.

The optimized enzymatic assay allowed characterization of ExoU molecules that differed in activity. To date, only fully enzymatic and catalytically inactive ExoU proteins have been examined for phospholipase activity (43, 46). However, upon linker insertion, two additional classes of enzymes were identified that had intermediate and minimal phospholipase activity. Possession of some enzymatic activity argues against a

TABLE 5. Enzymatic rates in response to bSOD1 concentration^a

μM bSOD1	rExoU	P56	D572	L618
6.3	24,950 \pm 2,654	32,992 \pm 617	4,978 \pm 559	839 \pm 41
18.8	40,287 \pm 1,339	41,418 \pm 826	7,863 \pm 386	3,948 \pm 222
37.5	65,740 \pm 3,914	59,393 \pm 1,710	12,124 \pm 735	6,977 \pm 275
56.3	81,178 \pm 3,378	71,781 \pm 1,018	15,884 \pm 373	12,797 \pm 684
75.0	82,780 \pm 1,686	91,102 \pm 143	17,792 \pm 517	19,871 \pm 456

^a Values (RFU/h) are reported as mean \pm standard error.

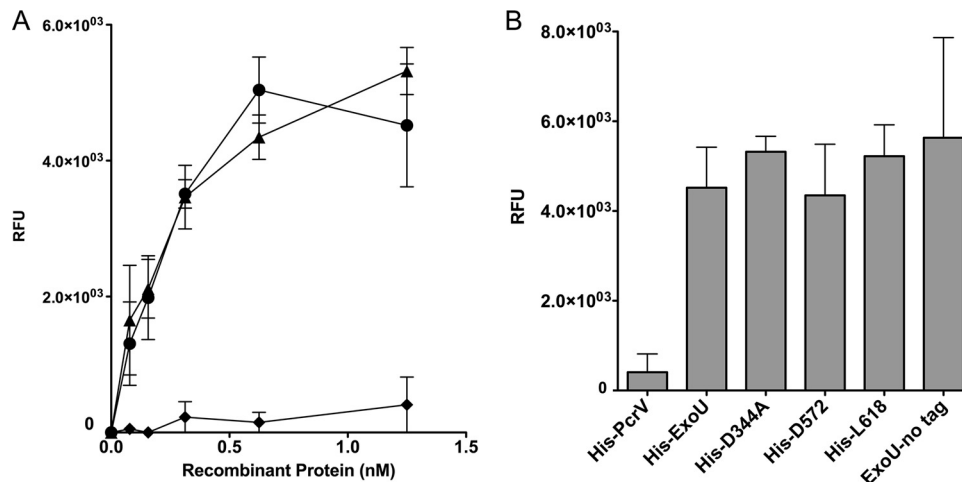


FIG. 7. The ExoU-SOD1 interaction is maintained for C-terminal linker insertion molecules. An ELISA was developed to study ExoU-SOD1 binding. Recombinant protein binding, wash conditions, and controls for nonspecific interactions are described in Materials and Methods. (A) Control histidine-tagged proteins tested include WT rExoU (●), rD344A (▲), and rPcrV (◆). (B) Binding of 1.25 nM recombinant protein to bSOD1, with cumulative background control wells subtracted. The data are plotted as the average of 3 independent experiments, with error bars for standard error.

general disruption of ExoU structure and indicates that the linker affects only a localized region of the molecule. The modulating properties of the identified residues in conjunction with SOD1 suggest that these regions may be involved in conformational changes that are perhaps required for ExoU to recognize its phospholipid substrates. This could be achieved by an interaction with SOD1 that removes an inhibitory domain or an interaction that opens a catalytic pocket. The linker insertions analyzed did not have a significant effect on interactions with immobilized SOD1 in an ELISA-based test, suggesting that SOD1 binding was not disrupted.

The impairment of the activation of ExoU by SOD1 is further supported by the SOD1 titration experiment. Wild-type levels of phospholipase activity were not achieved even when physiological concentrations of SOD1 were added to the reaction mixture. Under the conditions of our assay, we observed saturating kinetics for WT ExoU in the presence of 56.3 and 75 μ M bSOD1. These concentrations appear high, although biologically, they are similar to those present in mammalian cells (4, 38). The high concentrations of SOD1 required for activation may be related to the aggregation state of SOD1 or suggest that only a subpopulation of molecules is competent to interact and activate ExoU (1). Conversely, the intracellular aggregation state of ExoU may be important, and SOD1 may be functioning to oligomerize or disrupt oligomers of ExoU.

The ability to target the innate immune system prevents elimination of invading bacteria (29). Specifically, neutrophils and macrophages are targeted by type III secretion systems (6, 20, 29). As a result, bacterial replication can proceed beyond control of this first line of defense. During acute *P. aeruginosa* infections, bacterial burden is not the only issue worth consideration. *P. aeruginosa* strains possessing or not possessing a T3SS colonize the lung to the same extent; however, expression of T3SS effectors facilitates systemic dissemination (49). Neutralization of innate immunity as well as systemic dissemination appears to be promoted by the activities of the *P. aeruginosa* effectors secreted by the T3SS. Each enzyme utilizes eukary-

otic cofactors to potentiate their activity. ExoS, ExoT, and ExoY have profound effects on the cellular cytoskeleton and prevent phagocytosis (16, 53). ExoS is also cytotoxic and likely contributes to the tissue destruction that leads to systemic spread (11). Our analysis demonstrates that ExoU affects the cellular cytoskeleton as well. These effects are detectable only during *in vitro* infections in which strains express molecules with compromised activity. The effects do not appear to be related to another functional domain, as infection with strains expressing noncatalytic ExoU site-specific mutants (the S142A and D344A mutants) do not alter cellular architecture. Importantly, catalytic mutations introduced into the linker molecules abrogate the cell rounding activity, verifying that phospholipase activity itself is critical. The diminished activity of the mutants may provide a valuable clue to the primary activity of ExoU in a biological context. One might imagine that, in tissue, fully motile bacteria would be capable of injecting and moving to another location. Thus, the effects on the cytoskeleton by a low dose (single administration or small number of administrations) of ExoU may mimic *in vivo* events and suggest that ExoU's primary role may be to breach epithelial barriers rather than destroy them.

The phospholipase A₂ activity of ExoU has profound implications for lipid metabolism. The release of lysophospholipids and arachidonic acid from a membrane substrate results in the activation of several enzymes, including lipoxygenase and cyclooxygenase. Lipoxygenase and cyclooxygenase catalyze the conversion of arachidonic acid to leukotrienes and prostaglandins, respectively, which aid in generating an inflammatory response (26). ExoU and the T3SS have both been shown to be important for the release of arachidonic acid and the induction of cyclooxygenase 2 after infection with *P. aeruginosa* (41, 42). Furthermore, arachidonic acid has been shown to prime and activate NADPH oxidase, an enzyme that produces large amounts of superoxide radicals (18). SOD is responsible for catalyzing the conversion of the superoxide radical to molecular oxygen and hydrogen peroxide to prevent any further

oxidative damage to surrounding tissues (4). Thus, SOD1, which is generally in high concentrations within undamaged cells, is also in high concentration in inflammatory fluids in which *P. aeruginosa* replicates rapidly (24). Considering the activity of ExoU during tissue culture infections, it is conceivable that the inflammatory response coupled with necrosis could release a fair amount of extracellular SOD1 that might activate ExoU secreted by rapidly replicating bacteria. An extracellular phospholipase activity could contribute to tissue destruction and the systemic spread of *P. aeruginosa*. Additionally, SOD3 is abundant in the lungs of humans and mice and may also participate in activating extracellular ExoU (30, 31). Based on our analysis, the ExoU derivatives with reduced activity may be fully capable of affecting single cells but may lose extracellular properties due to the diminished activity. The analysis of these strains *in vivo* will be a worthwhile experiment.

Our data suggest that a domain that interacts with SOD1 has yet to be mapped. T3SS effectors are assumed to be unfolded during injection since the needle cannot accommodate folded molecules (14). We have considered the hypothesis that ExoU begins to fold within the cell in response to contact with SOD1. Folding may be a potential rate-limiting step in achieving an active conformation. In this model, SOD1 functions as a scaffold or protein chaperone for ExoU, perhaps explaining the high concentration of cofactor required for activation. Alternatively, SOD1 may alter the conformation of partially folded ExoU to remove an inhibitory domain. Finally, regions of the C terminus of ExoU may be involved in catalysis by positioning important residues within the N-terminal patatin domain. Clearly, future studies would benefit from structural analyses and a determination of the role of SOD1 or other molecules in ExoU activation.

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