Identification of the Major Ubiquitin-binding Domain of the Pseudomonas aeruginosa ExoU A2 Phospholipase*

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Background: Efficient activation of ExoU is dependent upon its interaction with ubiquitin.

Results: C-terminal regions, distal from the catalytic domain, mediate the highest affinity interaction with the hydrophobic patch of ubiquitin.

Conclusion: The C-terminal domain of ExoU has multiple functions critical to activity.

Significance: This is the first report characterizing the cofactor-binding domain of a bacterial toxin activated by ubiquitin.

Numerous Gram-negative bacterial pathogens use type III secretion systems to deliver effector molecules into the cytoplasm of a host cell. Many of these effectors have evolved to manipulate the host ubiquitin system to alter host cell physiology or the location, stability, or function of the effector itself. ExoU is a potent A2 phospholipase used by Pseudomonas aeruginosa to destroy membranes of infected cells. The enzyme is held in an inactive state inside of the bacterium due to the absence of a required eukaryotic activator, which was recently identified as ubiquitin. This study sought to identify the region of ExoU required to mediate this interaction and determine the properties of ubiquitin important for binding, ExoU activation, or both. Biochemical and biophysical approaches were used to map the ubiquitin-binding domain to a C-terminal four-helix bundle of ExoU. The hydrophobic patch of ubiquitin is required for full binding affinity and activation. Binding and activation were uncoupled by introducing an L8R substitution in ubiquitin. Purified L8R demonstrated a parental binding phenotype to ExoU but did not activate the phospholipase in vitro. Utilizing these new biochemical data and intermolecular distance measurements by double electron-electron resonance, we propose a model for an ExoU-monoubiquitin complex.

Pseudomonas aeruginosa is a ubiquitous Gram-negative soil organism and important opportunistic pathogen. It accounts for 11–14% of all nosocomial infections and is the third most common Gram-negative bacterium isolated from bloodstream infections (1). Cystic fibrosis patients are particularly susceptible to infection by P. aeruginosa with nearly an 80% prevalence rate among individuals greater than 18 years of age (2). A major virulence determinant used to establish infection and cause disease by this organism and other Gram-negative pathogens is the type III secretion system. Type III secretion structures resemble needle-like nanomachines and function to directly inject a diverse set of effector proteins into cells. The effectors generally manipulate host innate immunity or cellular physiology (3).

P. aeruginosa is known to encode four type III secretion effector proteins termed exoenzymes: ExoU, ExoT, ExoS, and ExoY. ExoT and ExoS are similar proteins, each possessing an N-terminal Rho-GTPase-activating protein domain and a C-terminal ADP-ribosyltransferase domain (4–6). ExoY exhibits adenylyl cyclase activity (7) but has been recently characterized as a broader nucleotidyl cyclase acting upon UTP, GTP, and CTP (8). ExoU is a potent phospholipase with A2 specificity (9, 10). Interestingly, the P. aeruginosa-encoded effector enzymatic activities are generally detectable in vitro only if a eukaryotic cellular factor is provided in the reaction (9–12). ExoS and ExoT are both activated by members of the 14-3-3 family of proteins (13). The cofactor for ExoY is currently unknown, and ubiquitin or ubiquitylated proteins activate ExoU (14).

Ubiquitin is a highly conserved 76-amino acid protein used as a post-translational modification essential for an array of cellular processes. The dynamic process of protein ubiquitylation is accomplished through the orchestrated activities of ubiquitin-activating (E1), ubiquitin-conjugating (E2), ubiquitin-ligating (E3), and deubiquitylating enzymes (15, 16). Modulation of the host ubiquitin system by effectors is a common strategy utilized by bacterial pathogens (17–19). Effectors mimicking multiple types of E3 ligases (20), deubiquitylases (21, 22), scaffolding proteins (23), and deamidating enzymes (24) have been described. Some effectors usurp the host ubiquitin system to modulate activity, localization, or temporal regulation of host and pathogen proteins (25). However, other effectors
modify host proteasome-dependent degradation to generate essential amino acids for intracellular growth (26). To our knowledge, ExoU is the first described enzyme that specifically requires an interaction with ubiquitin for enzymatic activation (14).

This work aimed to identify the ubiquitin-binding domain of ExoU and characterize the properties of ubiquitin leading to binding and activation. Sequence comparisons between regions of ExoU and other ubiquitin-binding proteins failed to yield clear results regarding a location of potential motifs as only partial homologies were identified (14). This result was not surprising as ubiquitin-binding domains are diverse in sequence and structure. Generally, these domains have binding affinities that range from 2 to 500 μM. Higher affinities are often associated with binding to polyubiquitin structures, usually of a particular linkage type (27–29). At present, no preference for a particular linkage type has been established for ExoU. However, it does exhibit higher affinity and activation constants with particular linkage type (27–29). At present, no preference for a particular linkage type has been established for ExoU. However, it does exhibit higher affinity and activation constants with ubiquitin chains of at least two monomers and appears to specifically utilize ubiquitin or ubiquitin-modified proteins as cofactors (14).

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of Recombinant Proteins—Glutathione S-transferase (GST) fusion proteins were constructed in pGEX4T2 vectors, the sequences were verified, and the confirmed plasmids were transformed into Escherichia coli BL21(DE3) cells for protein expression studies. Expression cultures were grown to an A600 of 0.5 at 37 °C in Luria-Bertani broth containing 100 μg/ml ampicillin followed by a 2-h induction at 30 °C with 0.5 mM isopropyl β-D-thiogalactopyranoside. Cells from 1-liter cultures were suspended in 10 mM phosphate-buffered saline (PBS) with protease inhibitors, DNase I, and RNase I as described previously (30). Suspensions were lysed by passage through a French pressure cell and subjected to centrifugation at 15,000 × g for 15 min (4 °C) to remove unbroken cells and debris. Supernatants from this fractionation procedure were subjected to a centrifugation step at 100,000 × g for 1 h (4 °C) to remove bacterial membranes. Soluble fractions containing the GST fusion proteins were purified by glutathione-Sepharose 4B affinity chromatography (GE Healthcare) on an ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare). Bound material was washed with PBS before elution in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM reduced glutathione. Eluted material was buffer-exchanged to Buffer A (50 mM sodium phosphate and 150 mM NaCl) using HiPrep 26/10 desalting columns. This material was concentrated using Amicon ultracentrifuge filtration, and the concentrations of the resulting purified protein preparations were determined using a bicinchoninic acid assay (BCA) assay with bovine serum albumin (BSA) as a standard. Hexahistidine-full-length ExoU fusion proteins were produced and purified as described previously (14). Linear yeast pentaubiquitin was cloned into pET15b from the chromosome of Saccharomyces cerevisiae BY4741. The protein was expressed and purified as described for ExoU. Labeling with the methanethiosulfonate nitrooxide spin label MTSL3 was performed as described (31) with the exception that proteins were purified by FPLC gel filtration using Superose 6 resin and 10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 20% glycerol buffer. All ubiquitin proteins were expressed and purified as described for ExoU with the exception that tag removal was accomplished by exposure of purified tagged proteins to 10 units/mg thrombin in an overnight room temperature cleavage reaction. Recombinant clones expressing [15N]ubiquitin were induced in modified M9 medium under similar conditions to rich medium induction and purified as described. Residues 480–683 of ExoU used in NMR titration experiments were constructed as SUMO1 fusions in a modified pET28a vector. Recombinant strains were grown in terrific broth with 50 μg/ml kanamycin and 30 μg/ml chloramphenicol. The induction step was initiated when the culture reached an A600 of 1.0 by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside and carried out for 3 h at 30 °C. After purification steps similar to those used for full-length ExoU, the SUMO tag was cleaved overnight at 4 °C with recombinant His-tagged Ulp1 SUMO protease. Protease and uncleaved proteins were removed by passage through affinity resin, and the flow-through was concentrated, purified by size exclusion chromatography (Sephacryl-200 HiPrep 26/60), and concentrated to 1.6 mM. Concentrations for full-length ExoU, ubiquitin, and ExoU 480–683 proteins were determined from A280 absorbances in 20 mM sodium phosphate, pH 6.0, 6 M guanidine hydrochloride using extinction coefficients of 29,160, 1,280, and 13,940 M−1 cm−1, respectively. Site-directed mutagenesis was accomplished using Change-IT site-directed mutagenesis kits (USB) according to the manufacturer’s protocol.

*Circular Dichroism—Monoubiquitin proteins were diluted to 0.1 mg/ml in 20 mM sodium phosphate, pH 7.0 and analyzed from 250- to 190-nm wavelengths in a Jasco J-710 spectrometer. Samples were measured at a scan speed of 50 nm/min for an average of five scans in 1-mm quartz cells. Recombinant ExoU was assayed at 0.1 mg/ml by using the same conditions as those used for ubiquitin.

*In Vitro Phospholipase Assay—ExoU enzymatic assays were performed in 96-well black plates as described previously (14). Briefly, fluorescence (415-nm excitation/580-nm emission) due to the cleavage of a quencher group in the phospholipid mimic PED6 (Invitrogen) was measured on a Spectramax M5 plate reader (Molecular Devices). Assays included 33 nM ExoU and a saturating amount of PED6 substrate (100 μM). Concentrations of monoubiquitin and diubiquitin ranged from 0 to 500 and 0 to 80 μM, respectively. The assay buffer included 50 mM MES, pH 6.3 and 750 mM monosodium glutamate. Results were obtained from a minimum of two independent titrations. Data were fit to a theoretical curve using non-linear regression analysis with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). The error values represent S.E. Catalytic

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3 The abbreviations used are: MTSL, S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate; EPR, electron paramagnetic resonance; DEER, double electron-electron resonance; PED6, N-[(6-(2,4-di-nitrophenyl)amino)hexanoyl]-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diace-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt; SUMO, small ubiquitin-like modifier.
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constants are reported as nmol of PED6 cleaved s\(^{-1}\) M\(^{-1}\) enzyme.

**Solid-phase Binding Assay**—96-well polystyrene plates were coated with 50 mM sodium bicarbonate buffer, pH 9.6 or buffer containing 250 ng of recombinant linear yeast pentaubiquitin, monoubiquitin, or monoubiquitin site-specific derivatives at room temperature for 2 h. Wells were washed with Buffer A (50 mM sodium phosphate, 150 mM NaCl) and then blocked overnight at 4 °C with Buffer A with 3% BSA (Buffer B). The next day the GST fusion proteins in Buffer B were applied to coated wells for 2 h at room temperature and washed with Buffer A. A 1:16,000 dilution of anti-GST monoclonal antibody (GE Healthcare) in Buffer B was applied to each well. After another 2-h room temperature incubation, the wells were washed with Buffer A and a 1:10,000 dilution of an anti-mouse IgG horse-radish peroxidase (HRP)-conjugated secondary antibody was applied. Following another set of washes, a substrate-peroxidase solution (1:10 ratio; QuantaBlu fluorogenic peroxidase substrate kit, Thermo Scientific) was added to the wells for 30 min at room temperature. Stop solution was added to conclude the assay, and fluorescence was measured at 340-nm excitation/415-nm emission on a Spectramax M5 plate reader.

**GST Pulldown**—Cells carrying GST fusion plasmids were induced, harvested, and lysed as described. The soluble supernatant fraction from bacterial lysates was collected after a 1-h 100,000 × g centrifugation and flash frozen in PBS, protease inhibitors, DNase I, RNase I, 1 mM EDTA. Glutathione magnetic bead slurry (80 μl; Pierce) was washed three times in 500 μl of Buffer A before incubation with saturating amounts of GST fusion soluble fractions (1 h at 4 °C with end-over-end mixing). The beads were washed five times with 500 μl of buffer before the addition of 12.5 μg of linear yeast pentaubiquitin. The incubation with pentaubiquitin was for 2 h at 4 °C after which an aliquot was removed for the input fraction. The beads were washed three times with 500 μl of buffer, and aliquots for the bound fraction were removed for Western blot analysis.

**NMR Spectroscopy**—\(^{1}\)H-\(^{15}\)N heteronuclear single quantum coherence NMR experiments were performed at 25 °C on a Bruker Avance III 500-MHz spectrometer equipped with a three-pulse resonance Bruker Avance III 500-MHz spectrometer equipped with a triple-resonance \(z\) axis gradient CryoProbe. All NMR samples contained 0.16 – 0.2 mM \[^{15}\text{N}\] ubiquitin and were prepared in 90% H\(_2\)O, 10% D\(_2\)O-containing Buffer A and 0.02% sodium azide. Unlabeled ExoU fragments (residues 480 – 683) ranged from 0- to 8-fold molar excess over ubiquitin over the titration. Chemical shift perturbations were tabulated according to \((\Delta \text{H} \times 5)^2 + (\Delta \text{D} \times 5)^2)^1/2\).

**EPR Spectroscopy**—Continuous wave EPR spectroscopy was carried out at room temperature on an Elexys E500 spectrometer (Bruker Biospin, Billerica, MA) equipped with a high-Q cavity operating at X-band. Samples (10 mM Tris, 150 mM NaCl, 20% glycerol, pH 7.0 buffer) were prepared by mixing unlabeled ExoU and standard MTSIL-labeled ubiquitin A28C (designated A28R1) monoubiquitin to give a final concentration of 50 μM ubiquitin and the desired final concentrations of ExoU or C-terminal domain fragment. Spectrometer conditions were as follows: time constant, 10.28 ms; conversion time, 20.48 s; scan time, 20.97 s; a 100-kHz field modulation amplitude of 1.0 G, 10-milliwatt microwave power, and a sweep width of 100 G. Four-pulse double electron-electron resonance (DEER) spectroscopy was carried out at Q-band (34 GHz) on an E580 spectrometer (Bruker Biospin) equipped with an EN5107D2 dielectric resonator and 10-watt microwave amplifier. Samples containing ~0.1 mM singly labeled ExoU and 0.1 mM singly labeled ubiquitin were brought to a final concentration of 25% (v/v) perdeuterated glycerol (Sigma-Aldrich) as cryoprotectant before placement in 1.1 × 1.6-mm glass capillaries andflash frozen by immersion into liquid N\(_2\). Sample temperature during the experiment was maintained at 80 K using an Oxford cryostat. Observer pulses were positioned at the center field maximum with \(\pi\) and \(\pi/2\) pulse lengths of 56 and 28 ns, respectively. Pump pulses were positioned at the low field maximum. Data were corrected for background decay assuming a homogeneous three-dimensional protein distribution and analyzed by model-free Tikhonov regularization using DeerAnalysis2011 software (32).

**Generation of the ExoU-Monoubiquitin Complex Model**—The sequence for full-length ExoU was submitted to the Phyre2 web server for generation of a model based on published crystal structure coordinates for ExoU. This new structure was identical to the reported crystal structures with the addition of elements including the Asp-344 catalytic region and a large loop connecting the two most C-terminal helices. A MacPyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.3, Schrödinger, LLC) file of this structure was generated and combined with a Protein Data Bank file (code 2G4S) containing ubiquitin. Ubiquitin and ExoU amino acids that were changed to cysteine residues were then introduced, and an MTSL label was modeled onto each substitution using the PyMOL fusion command. MTSL-labeled ubiquitin was then manually docked onto the ExoU structure using DEER distance measurements relative to S137R1 ExoU. Distances measured between MTSL labels in the model differ from distances derived from the DEER analyses by a range of 1-4 Å. Additionally, ExoU residues 580 – 683 were submitted to the ZDOCK server (version 3.0.2) along with ubiquitin for generation of docking models. Monoubiquitin residues perturbed by the ExoU C-terminal fragment by NMR analysis were specified for the contact site. The model shown in Fig. 6B represents a model from the top five predictions that most closely resembles the compilation of the data displayed in this work.

**RESULTS**

**Localization of the Dominant Ubiquitin-binding Region of ExoU**—Initial approaches to identify the ExoU-ubiquitin interaction domain(s) utilized a GST fusion strategy to screen for cofactor interactions. GST-ExoU was determined to have ~60% wild-type activity when stimulated with linear yeast pentaubiquitin and ~70% wild-type activity when stimulated with monoubiquitin under saturating conditions (data not shown). We concluded that recombinant GST-ExoU retains its ability to bind both substrate and cofactors as a functionally active molecule. A solid-phase binding assay was subsequently developed as a tool to map GST-ExoU fragments that demonstrate ubiquitin binding activity (Fig. 1A). In previous studies, we showed that ExoU has a higher affinity for polyubiquitin chains compared with monoubiquitin; thus, we chose to immobilize
recombinant yeast pentaubiquitin to increase the dynamic range of the assay (14). GST-ExoU fusions were constructed by using published crystal structures of ExoU in complex with its cognate chaperone, SpcU (Protein Data Bank codes 4AKX and 3TU3; see Refs. 33 and 34), as a guide for domain boundaries.

Our results indicate that compared with the full-length protein the ExoU N terminus including the catalytic domain (residues 1–477) has a relatively low affinity for immobilized pentaubiquitin. Slightly higher affinity was achieved with C-terminal domain residues 480–599 and 480–659. In contrast, a construct including residues 480–683 bound to pentaubiquitin with an apparent affinity similar to that of full-length ExoU. The four C-terminal residues, KEFT, appear to be dispensable for cofactor binding. Previous studies have shown that ExoU is equally active in vitro and in vivo regardless of the presence or absence of this sequence (35). A GST fusion of PcrV, a protein at the tip of the type III secretion needle, was used as a negative control.

GST pulldowns were utilized to capture soluble pentaubiquitin-ExoU interactions as a complement to the ELISA (Fig. 1B). Results from the pulldown analyses were in good agreement with the solid-phase assay in that full-length ExoU and amino acids 480–683 bound to pentaubiquitin with roughly similar affinity, whereas most of the other fragments failed to co-precipitate the ligand. Unexpectedly, residues 480–659 appeared to display a higher affinity for pentaubiquitin in soluble form than when this protein was immobilized.

We postulate that the discrepancy between solid- and solution-phase binding may be related to steric hindrance. Residues 660–683 include a flexible region of ExoU (33, 34). This flexible structure, which is missing from residues 480–659, may account for the ability of full-length ExoU and ExoU 480–683 to bind both immobilized and soluble ubiquitin. Attempts to pull down monoubiquitin with any of the fragments were unsuccessful (data not shown), suggesting that the ExoU C-terminal domain may contain multiple ubiquitin binding sites working in concert to increase the avidity of the interaction. This hypothesis is supported by the fact that GST-ExoU 600–687 was unable to efficiently bind ubiquitin by itself; however, portions of this sequence are necessary for restoring binding affinity to parental or wild-type levels. A general schematic of the functional domain structure of ExoU is shown in Fig. 1C.

**Functional Analyses of the C-terminal Domain of ExoU**—Structures of full-length ExoU in complex with its chaperone reveal that residues 480–683 can fold into two, mostly helical domains (33, 34). Both solid- and solution-phase binding assays suggested that the highest affinity for ubiquitin was attainable.

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**FIGURE 1. Identification of the ExoU ubiquitin-binding domain.** A, solid-phase plate binding assay detecting the binding of ExoU-GST fusion proteins to immobilized linear yeast pentaubiquitin. Reported fluorescence values include the subtraction of nonspecific binding and fit to a theoretical binding curve by non-linear regression analysis using GraphPad Prism 5.0 software ($n = 3–5$, mean $\pm$ S.E. (error bars)). B, Western blot analysis of GST pulldown assays utilizing non-tagged linear yeast pentaubiquitin and different GST-ExoU fusions. C, schematic diagram of the functional domains of ExoU. The catalytic dyad is composed of Ser-142 and Asp-344. MLD, membrane localization domain; RFU, relative fluorescence units; Ub, ubiquitin.
when both of these domains were present in the fusion protein. Sequence alignments comparing ExoU 480–683 with known ubiquitin-binding motifs did not reveal convincing similarity. Thus, a structural homology approach was used to search for proteins with an overall three-dimensional similarity. This analysis led us to postulate that the large helix (residues 587–619; see Ref. 34) and the large flexible loop (residues 658–673) may be involved in binding. The large helix resembles a structure similar to the S5a proteasome helix (residues 216–245) containing a ubiquitin interaction motif (37). ExoU shares many traits with ubiquitin interaction motifs such as S5a in that both have a low affinity for monoubiquitin versus higher order structures, and both proteins bind to multiple ubiquitin linkage types (14, 37). These motifs sometimes promote ubiquitylation of the protein encoding them (ExoU is modified by a Lys-63-linked diubiquitin molecule at Lys-178), and they are often associated with trafficking proteins that interact with ubiquitylated cargo (28, 35). The loop in this case partially mimics a zinc finger domain from isopeptidase T (Fig. 2, A and B). Arg-221, Tyr-261, and Asp-264 make contact with ubiquitin and are important for isopeptidase T activation (38). These residues, along with Phe-224, seem to be structurally analogous to Tyr-619, Asp-625, Arg-661, and Phe-663 of ExoU. As an initial test, we incorporated single point mutations at sites as shown in Fig. 2, B and C, to determine how these modifications modulate ExoU phospholipase activity. We then assayed the effect these mutations had on the ubiquitin binding properties of the ExoU C-terminal region.

**Modulation of the Putative Ubiquitin-binding Domain and the Effects on Activation and Ubiquitin Binding**—Mutations were constructed in full-length ExoU to determine the effect of amino acid substitutions on in vitro catalytic constants. Non-conservative mutations were chosen to disrupt ExoU function due to the apparent tolerance of the enzyme to various mutations (30, 36). Placement of each mutation was aimed at altering the surface of each hypothesized binding site without affecting interhelical contacts. Mono- or diubiquitin was titrated into the assay to measure initial rates of ExoU activity (Table 1). With monoubiquitin as a cofactor, wild-type ExoU displayed a catalytic constant of 55 nmol of substrate cleaved s^{-1} m^{-1} enzyme.
and an activation constant ($K_{\text{act}}$) of 111 μM. Substitutions that included R600E, V604R, and F663E result in enzymes that exhibit substrate cleavage rates equal to or slightly faster than the parent molecule. F663E ExoU possesses a decreased activation constant compared with wild type and thus appears to be a more efficient enzyme. It is possible that replacement of a bulky residue with a hydrophobic residue in this flexible region facilitates ubiquitin interaction in solution. ExoU R600E requires a 2-fold increase in ubiquitin concentration to reach its activation constant compared with wild type, suggesting that either this residue participates in an interaction with cofactor or that Arg-600 stabilizes an active conformation. However, the retention of significant catalysis suggests that it is not a primary site of contact with either substrate or cofactor.

Substitutions that include K611F, Y619E, Q623G, and R661E greatly diminish ExoU enzymatic activity with monoubiquitin added as the cofactor. Monoubiquitin was not saturable in these assays, and the activation constants reported represent the highest concentrations tested for comparison purposes (Table 1). Enzyme activity was most severely affected by Y619E and R661E ExoU, whereas K611F and Q623G ExoU activities were moderately decreased. A D625R substitution does not affect maximal substrate cleavage rates; however, it does appear to be inhibitory to cofactor interactions. These data support a model in which the tip of the four-helix bundle is involved in cofactor binding and activation.

To test this hypothesis, a diubiquitin titration was performed. The greater activation efficiency of diubiquitin over monoubiquitin (~100-fold) allowed kinetic saturation to be achieved with several of the ExoU derivatives. All ExoU mutants defective for monoubiquitin activation except Y619E and R661E possessed catalytic rates equal to or slightly better than wild-type enzymes given sufficient diubiquitin concentrations; however, their activation constants were roughly 3- (R660E) to 9-fold (K611F) higher over the native toxin. ExoU Y619E is unable to be significantly activated by diubiquitin. In contrast to Y619E, non-linear regression analysis of the R661E data resulted in an activation constant not significantly altered from wild-type levels. We interpret these data to suggest that Tyr-619 is a significant residue in the ExoU ubiquitin-binding domain that is likely supported by additional contacts from Arg-600, Lys-611, Gln-623, and Asp-625. Furthermore, it is possible that the R661E substitution may negatively affect substrate binding (39).

ExoU derivatives possessing a decreased rate of substrate cleavage were reconstructed as a series of GST-C-terminal domain clones, and the proteins were purified and assayed for their relative binding constants on immobilized pentabiquitin by an ELISA (Fig. 2D). Glutamic acid substitutions at arginine 600 (R600E) or 619 (Y619E) resulted in an approximate 5-fold decrease in apparent affinity for pentabiquitin. Fragments including R661E were more severely affected, possessing 12-fold weaker affinity compared with the native sequence. Circular dichroism spectra of full-length ExoU point mutants indicate that these proteins are folded and contain similar amounts of secondary structure (Fig. 2E). Interestingly, K611F, Q623G, and D625R proteins displayed no defect in ubiquitin binding by this method. We postulate that Arg-661 and Tyr-619 are important residues in the principle ubiquitin-binding interface of the C-terminal domain. The decrease in affinity of R600E ExoU and its location on α22 suggest the possibility that proper binding to a polyubiquitin chain may require that one ubiquitin molecule be positioned in the vicinity. The preservation of binding affinity with K611F and D625R ExoU was surprising, and additional experiments will be required to discern how these specific substitutions hinder catalysis. ExoU Q623G was designed to subtly destabilize the four-helix bundle by removing the glutamine side chain; however, as binding defects were not observed, we postulate that other residues may contribute to stability of the surrounding structure to minimize the impact of the glycine substitution in this type of assay. Overall, combining the binding and kinetic data, it appears that a large surface within the C-terminal domain of ExoU modulates both the interaction with ubiquitin and enzymatic activity by a mechanism that likely involves conformational changes and/or stabilization of a catalytically competent structure.

**Identification of Ubiquitin Residues That Interface with the ExoU Ubiquitin-binding Domain**—Initial efforts to monitor ubiquitin-ExoU interactions by two-dimensional NMR were hampered by large peak broadening in the heteronuclear single quantum coherence spectrum of ubiquitin upon the addition of full-length ExoU protein. A truncated form of ExoU (residues 480–683) shown in this work to bind ubiquitin with similar affinity as the native toxin was subsequently used for these experiments. $^{15}$N-$^{1}$H heteronuclear single quantum coherence titrations between labeled ubiquitin and unlabeled ExoU fragment revealed chemical shift perturbations mostly surrounding residues corresponding to the hydrophobic patch (Leu-8, Ile-
44, and Val-70) region of monoubiquitin in solution (Fig. 3A). The most dramatic shifts occurred within a small loop C-terminal to Ile-44 in residues 46–48 (AGK) as well as C-terminal residues Val-70, Leu-71, and Arg-72. Leu-8 and Ile-44 did not display chemical shifting to the degree of Val-70. Ile-13 and Thr-14 shifts may be due to low affinity contact or are possibly an artifact of intramolecular chemical environmental disturbances of ubiquitin upon contact with ExoU. Interestingly, unlike isopeptidase T, the ExoU ubiquitin-binding domain does not seem to make extensive contacts to the ubiquitin C-terminal diglycine motif (Fig. 3B). Our working hypothesis from these data is that the ExoU C-terminal domain undergoes a binding interaction with monoubiquitin predominantly contacting the small loop distal to Ile-44 and resides Arg-42, Val-70, Leu-71, and Arg-72 (Fig. 3C).

Verification of Ubiquitin Residues Important for Binding and Activation of ExoU—To confirm the role of the hydrophobic patch in binding and activation, we constructed site-specific mutations of selected residues as well as Ile-36 and Glu-24 (Fig. 3C). Isopeptidase T contacts Ile-36, and our structural homology hypothesis would suggest that ExoU might also mimic this binding mode despite the NMR titration results suggesting minimal involvement compared with other surfaces (38). Glutamate 24 was chosen as a control and was expected to be an innocuous mutation. All mutations were made in a non-conservative manner to arginine as our alanine scan results showed that ubiquitin can accommodate many different point mutations without a loss in ExoU activation capacity (supplemental Fig. S1). Each modified recombinant protein was purified to homogeneity and examined by circular dichroism to evaluate overall folding (Fig. 4A). This analysis together with equivalent levels of expression, solubility, and Western blot detection (data not shown) suggested that the overall conformation of each protein was generally similar to that of parental ubiquitin. Activation of ExoU under steady-state conditions was unde-tectable with an L8R derivative under the conditions tested (Table 2). I36R and I44R substitutions were roughly 10-fold less efficient activators of ExoU. Ubiquitin derivative V70R displayed a 1.6-fold decrease in activation rate compared with parental ubiquitin. The substitution of an arginine for glutamic acid (E24R), a residue implicated by NMR analysis to be outside of an essential binding interface, did not have a significant effect and possesses an almost identical activation constant compared with parental ubiquitin (Table 2).

Interestingly, when ubiquitin derivatives were immobilized for solid-phase binding analysis, L8R displayed minimal defects in binding to the C-terminal domain despite being unable to activate ExoU. The most dramatic changes in binding affinity were observed for I44R followed by V70R. Although Ile-44 does not appear to make direct contact with ExoU by NMR, it is centrally located between two sites of contact, and placement of a long side chain at this position results in a 4-fold decrease in
apparent affinity using this method. V70R displayed a 3-fold decrease in affinity compared with parental ubiquitin. Molecular modeling of the I36R mutation into ubiquitin indicates that the new residue is likely to be disruptive to Leu-71 and Arg-72, which may impact the mechanism by which these mutants are defective for ExoU binding and subsequently activation. Ubiquitin E24R displays a functional binding phenotype similar to Glu-24 and shows minimal chemical shift perturbation (Fig. 4B). In summary, Leu-8 appears to be in a critical location for modulating enzyme activation but has a limited role in binding affinity, whereas Val-70 is critical for activation and binding. Isoleucine 44 is positioned between two major sites of contact, and insertion of a large side chain into this location is postulated to cause steric interference between the ExoU and monoubiquitin interface, resulting in the largest decrease in affinity.

**EPR Analysis of the ExoU-Monoubiquitin Interaction** — Electron paramagnetic resonance of spin-labeled proteins is a powerful method to acquire structural and dynamic information about a given protein as it behaves naturally in solution. We began this analysis with a study of full-length ExoU binding to spin-labeled monoubiquitin to obtain an accurate dissociation constant. Numerous spin-labeled variants of ubiquitin were analyzed with the best data resulting from ExoU binding to A28R1. Alanine 28 is located in the middle of a prominent α-helix with its side chain exposed at the protein surface, making it an ideal site for placement of a nitroxide spin label in a location that will not perturb the folding or tertiary structure of ubiquitin. It is located on the opposite face from the hydrophobic patch so that it should not interfere with binding to ExoU (similar to Glu-24) and shows minimal chemical shift perturbation upon titration with the ExoU 480–683 construct (Fig. 3B).

A28R1 exhibits an identical affinity to native ubiquitin in our solid-phase binding assay (data not shown).

EPR spectra of A28R1 in the presence and absence of full-length ExoU are shown in Fig. 5A. In the absence of ExoU, the EPR spectrum of A28R1 is characteristic of fast rotational motion with a rotational correlation time on the order of 2 ns. A significant amount of the motional averaging can be attributed to the overall tumbling of the relatively small (8.5-kDa) monoubiquitin molecule itself. Upon binding to the much larger ExoU (74 kDa), the rotational motion of A28R1 is slowed considerably, and a second component appears in the EPR spectrum corresponding to a population of spin-labeled ubiquitin molecules with reduced rotational mobility (Fig. 5A, arrow). Titration of A28R1 with ExoU results in a concomitant increase in the motional restricted population until at high ExoU:ubiquitin molar ratios the EPR spectrum assumes a shape characteristic of a surface-exposed α-helical labeling site (40). Spectra were deconvoluted into bound and free components (Fig. 5B), and the fraction of bound A28R1 was used to generate a binding curve indicating a $K_d$ of 54 ± 9.8 μM.

An additional series of titrations was performed with the C-terminal domain of ExoU (residues 480–683) to quantify its binding affinity to A28R1 monoubiquitin in solution, and the resultant dissociation constant was 170 ± 23 μM (Fig. 5C). We postulate that although this domain appears to form the bulk of the ExoU-ubiquitin contact surface other parts of the catalytic domain may participate in stabilizing this structure in addition to providing lower affinity contacts with the cofactor to induce an active conformation.

When A28C was introduced into the L8R and V70R backgrounds and spin-labeled with MTSL (to generate L8R/A28R1 and V70R/A28R1, respectively), the EPR spectra of these constructs were indistinguishable from A28R1 in a wild-type background (supplemental Fig. S2). L8R/A28R1 bound to ExoU with an affinity similar to that of wild-type A28R1 with a $K_d$ of 78 ± 12 μM (supplemental Fig. S2). In contrast, V70R/A28R1 showed only weak binding to ExoU such that an accurate dissociation constant could not be obtained. Thus, in solution, monoubiquitin variants associate with ExoU with similar trends as in the ELISA format.

**Model for the ExoU-Monoubiquitin Complex** — We next performed a series of DEER experiments in which one spin label was placed on full-length ExoU and the other was placed on...
monoubiquitin. These data can be used to derive the location and orientation of ubiquitin in the context of a complete enzyme. ExoU proteins were singly labeled with MTSL at S137C, S643C, or E636C. S137R1 proteins retain 50% of wild-type catalytic activity, whereas S643C and E636C are fully active (Ref. 31 and data not shown). Ubiquitin was labeled at Q2C, T12C, A28C, or S57C to test several separate surfaces of the protein. None of these residues displayed dramatic chemical shifting in NMR studies, and each labeled protein retains at least 40% of wild-type activity. Representative Q-band DEER spectra are shown in supplemental Fig. S3, and a summary of the distances between each spin label pair is given in Table 3.

We attempted to use these DEER distances to construct an initial model for the relative localization of monoubiquitin upon complex formation with ExoU (Fig. 6 A). Compilation of all four distance constraints from ubiquitin to S137R1 of ExoU (located in the core of the catalytic domain) placed ubiquitin in an orientation in which loop residues 46–48 were in proximity to Tyr-619 on ExoU. Residues Val-70 to Arg-72 appear to be able to make contacts either with the following helix, H9251, or to H9231 on the opposite side of Tyr-619 if it becomes solvent-accessible. Distances derived from measurements based on ExoU-SpcU crystal structures (33, 34) to ubiquitin in this conformation were not in strict agreement with DEER data of ExoU labeled at S643C, E635C, or E636C. In general, the distances were either longer or shorter than empirical results depending on the label pair (Table 3). Distances to E63R1 were longer than to S643R1 in every comparable measurement to ubiquitin; thus, we postulate that the orientation of the four-helix bundle may be altered in solution upon ubiquitin binding. We note that our model based on ubiquitin-S137C distances also places Lys-63 of monoubiquitin in close proximity (within 10 Å) to Lys-178 of ExoU, suggesting a possible correlation between the post-translational modification of ExoU by the host ubiquitylation system and a mechanism of toxin activation (35).

To complement the model derived utilizing DEER distances between ExoU and monoubiquitin, we utilized the ZDOCK server to predict a complex between ubiquitin and the C-terminal domain of ExoU. The model shown in Fig. 6B is derived from one of the top five models generated. It is the only model of the five that places Tyr-619 in the center of a postulated binding interface with the AGK loop of ubiquitin between Tyr-619 and Lys-611 and C-terminal residues VLR to the other side.

**FIGURE 5. Solution-phase EPR binding analysis of full-length ExoU or ExoU 480–683 to MTSL-labeled monoubiquitin.** A, EPR spectra of monoubiquitin A28R1 upon titration with ExoU. The arrow denotes the motionaly restricted spectral component due to formation of the ExoU-ubiquitin complex. The final ubiquitin concentration was 50 μM. Scan width, 100 G. B, EPR spectra of bound and free A28R1. C, binding isotherm based on the fraction of bound A28R1 (fB) as a function of ExoU (open circles, full-length enzyme; closed squares, residues 480–683) concentration. The dashed lines represent the best fit to a single binding site model, and the inset represents the double reciprocal plot of the binding data. Ub, ubiquitin.

**TABLE 3**

| Ubiquitin label site | ExoU label site | Intermolecular distance (Å) |
|----------------------|----------------|-----------------------------|
| A28R1® | S137R1® | 36.2 ± 2.2 |
| Ex53R1 | Ex66R1 | 53.6 ± 4.1 |
| Ex66R1 | S643R1 | >56 |
| T12R1 | S137R1 | 42.4 ± 2.4 |
| Ex66R1 | S643R1 | 56.5 ± 3.6 |
| Ex66R1 | S643R1 | 50.9 ± 4.0 |
| S57R1 | S137R1 | 24.8 ± 1.8 |
| Ex53R1 | Ex66R1 | 45.0 ± 2.1 |
| Ex66R1 | S643R1 | 48.2 ± 5.8 |
| Q2R1 | S137R1 | 46.8 ± 3.1 |

® R5, brominated MTSL label.
® R1, standard MTSL label.
The other four models (not shown) do not place ubiquitin in contact with Tyr-619 but instead on the C-terminal residues 679–683. We postulated that these models are less likely representatives of the complex based on our observation that GST-fragments lacking this helix retain the ability to interact with ubiquitin in solution. Further structural analysis of this interaction will be necessary to confirm the proposed model.

**DISCUSSION**

Previous reports focusing on the C-terminal domain of ExoU identified this region as an essential component of the enzyme for toxicity (30, 35, 36). Truncation of as few as eight residues (amino acids 687–679) abrogates toxicity (35). As assessed by fluorescence microscopy, C-terminal sequences of ExoU were associated with localization of the protein to the cytoplasmic membrane of host cells (35, 36). Our data suggest that an additional role of the C-terminal domain of ExoU is to mediate an interaction with the cofactor ubiquitin. It is conceivable that these two processes are linked and that cofactor binding is actually a prerequisite to membrane localization in a host cell. Numerous trafficking signals are mediated by post-translation modification with ubiquitin, particularly of Lys-63 type linkages, and ExoU may have evolved to usurp this mechanism. It has been shown previously that ExoU becomes modified by two ubiquitin monomers conjugated by a Lys-63 linkage shortly after injection (35). This modification, however, was shown not to have a significant impact on ExoU localization or toxicity (35). These data leave open the question of whether *in vivo* ubiquitylation facilitates ExoU activation. Based on our biophysical analyses and modeling, ubiquitylation of ExoU *in vivo* and ExoU activation may indeed be linked but are not absolutely required for phospholipase activity. Importantly, retention of parental localization and toxicity of K178R molecules may be an outcome if the functional significance of ExoU ubiquitylation is merely to place an activator molecule into a binding site already accessible to solvent.

Crystal structures of ExoU in complex with its bacterial chaperone, SpcU, detail the structure of the ExoU ubiquitin-binding region as a four-helix bundle (33, 34). Our bioinformatics analysis failed to identify potential ubiquitin-binding motifs in this structure; thus, we looked for structurally homologous domains (38). We postulated that the C-terminal domain of ExoU contained elements of structural homology to the S5a proteasome as well as the zinc finger ubiquitin-binding domain of isopeptidase T. Our experimental data implicate both elements in the protein-protein interaction with ubiquitin. This hypothesis was tested by introducing site-specific mutations of amino acid residues in both helix and loop structural components. Several site-specific substitutions were defective in phos-
Identification of the ExoU Ubiquitin-binding Domain

The ubiquitin-binding surface (derived from NMR chemical shifting) faces the distal end of ExoU helix 22 in our model. This site was shown by mutagenesis studies to be important for binding and activation, and thus the surface was used to computationally generate a docking model between the postulated ubiquitin-binding domain of ExoU and ubiquitin. One of the five top ZDOCK models closely resembled our DEER-based model and included the distal end of helix 22 as the predominant site of interaction. Further structural analysis will be required to confirm this hypothesis; however, several different approaches appear to agree in the implication of the residues in both enzyme and cofactor shown by these models. Additional information will be needed to refine the roles of some of these residues, and we suspect a few (e.g. Arg-600) may be involved in mediating binding events to polyubiquitin chains or dynamic conformational changes.

A limitation to our model is that it is based on the crystal structure of ExoU complexed with its cognate chaperone, SpcU (33, 34). This molecule is inactive by definition as it would be toxic to the bacterium producing it (14). ExoU is known to sample multiple conformations in solution that shift to a predominant state upon cofactor binding (31). Therefore, the model is likely an approximation of where ubiquitin first interacts with ExoU at its surface of highest affinity. In future analyses, we hope to determine whether or not the C-terminal four-helix bundle undergoes a specific conformational change upon cofactor binding.

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phospholipase activity and cofactor binding with the most dramatic impact stemming from the C-terminal end of helix 22 including Tyr-619. Additional defects with R661E and R600E mutants support the hypothesis that multiple surface contacts are involved in this protein-protein interaction. Other substitutions did not affect binding but did impact the efficiency of ubiquitin activation of the enzyme (K611F, Q623G, and D625R). In the proposed model (Fig. 6, A and B), we noted that these residues are located peripherally to Tyr-619, suggesting that they may facilitate conformational changes necessary for activation.

NMR analysis of isotopically labeled ubiquitin demonstrates a pattern of chemical shifts correlating to the area surrounding the hydrophobic patch, one of the most common sites of interaction between ubiquitin and other proteins. Minimal chemical shifts were observed involving Ile-36 or the C-terminal diglycine motif of ubiquitin, suggesting that ExoU may not mimic the binding mode of isopeptidase T and ubiquitin. Other substitutions at Ile-36 will be tested to determine whether arginine itself is disruptive to the interaction or whether the mechanism involves the presence or absence of a hydrophobic residue.

One interesting observation from this study was the phenotype of the ubiquitin L8R derivatives. These proteins retain their ability to bind ExoU yet did not produce activated enzymes, similar to the phenotype displayed by certain substitutions in the C-terminal domain of ExoU. These results separate cofactor binding into two functional classes: one class is likely most important for interaction and binding, and the other class may be key to conformational changes resulting in ExoU activation. Ubiquitin binding must promote some event leading to phospholipase activity, and it is possible that this happens in a specific sequence where an initial binding event is required to enable a subsequent conformational change in ExoU in which it can accept the activator molecule. Alternatively, upon ubiquitin binding, Leu-8 or a nearby surface may make contacts with ExoU that are necessary to induce a direct conformational change or stabilize a catalytically active conformation. In either case, the L8R mutation may provide a structural basis not only for a basic understanding of the enzymology of ExoU but also lead to the development of a targeted inhibitor molecule.

To construct a model describing the general localization of monoubiquitin relative to the complete ExoU toxin, we utilized DEER analyses and measured distances between singly labeled molecules. The only model that fit all four distance constraints to S137R1 is shown in Fig. 6A. Importantly, this model is supported by evidence obtained utilizing independent approaches. Biochemical analysis indicates that the high affinity site for cofactor binding is located in the C-terminal domain, principally between residues 600 and 683. Recombinant GST-ExoU 480–683 and GST-ExoU 480–659 were able to bind cofactor in solution with a similar affinity as GST-full-length ExoU, whereas GST-ExoU 480–599 could not. Mutational analysis of residues between 600 and 683 indicated that Tyr-619 and Arg-661, both located near one end of the C-terminal helical bundle, play a role in binding and activation. Kinetic data suggest that Arg-661 could function either in substrate interaction or cofactor binding.
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