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A Sensitive Fluorescence-Based Assay for the Detection of ExoU-Mediated PLA₂ Activity

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Abstract

Background—*Pseudomonas aeruginosa* is an opportunistic pathogen that causes disease in immunocompromised individuals, burn victims, and cystic fibrosis patients. Strains that secrete ExoU induce host cell lysis and damage epithelial tissue, which can lead to severe outcomes including sepsis and mortality. ExoU is classified as an A2 phospholipase (PLA₂) and activity is dependent on the eukaryotic protein, superoxide dismutase 1 (SOD1).

Methods—A sensitive and low-background *in vitro* fluorescence-based assay was developed to detect ExoU activity using the fluorogenic substrate, PED6.

Results—The optimized assay enabled us to perform the first kinetic evaluation of the activation of ExoU (apparent K_m of 13.2 ± 1.5 $\mu\text{mol/l}$ PED6 and an apparent V_{max} of 42 nmoles/min/mg). An inhibitor study using the inhibitor, methyl arachidonyl fluorophosphonate (MAFP), yielded an IC_{50} of 13.8 ± 1.1 nmol/l and validated the use of high-throughput inhibitor screens using the assay. Most notably, the *in vitro* fluorescence-based activity assay was sensitivity enough to detect catalytically active ExoU injected into eukaryotic cells.

Discussion—The use of the fluorescence-based activity assay to study the mechanism of ExoU activation may lead to the development of potential therapeutics to reduce *P. aeruginosa*-associated mortality.

Keywords

Pseudomonas aeruginosa; ExoU; PED6; phospholipase A2

1. Introduction

P. aeruginosa is a Gram-negative bacterium that causes opportunistic infections in immunocompromised individuals. These infections can be life-threatening depending on the underlying condition of the patient, susceptibility of the strain to antibiotic treatment, and whether the infection leads to sepsis and bacteremia [1–4]. Severe infections occur in individuals with cystic fibrosis [5], AIDS [6], burn patients [7], organ transplant recipients, and individuals undergoing cancer chemotherapy or who are subjected to mechanical ventilation for several weeks [8–10].

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Bacterial pathogens utilize a wide range of virulence factors to survive within the host and to subvert the host's immune system. One particular pathogenic strategy is the secretion of lipases into the external environment or directly into the host cell cytoplasm to alter host membrane organization or lipid signaling cascades [11,12]. All 4 types of phospholipase proteins contribute to bacterial virulence in various pathogens [11,13]. This report specifically focuses on bacterial phospholipase A2 effector activity, which has been reported to enable *Yersinia enterocolitica* to colonize host tissue, *Helicobacter pylori* to degrade host-protective gastric mucus, and leads to poor clinical outcomes in patients infected with certain strains of *Pseudomonas aeruginosa* [11,14,15].

ExoU is a 74 kDa (687 amino acids) hydrophilic protein comprised of 2 functional domains. The N-terminal catalytic domain (amino acids 1–350) shares homology with the patatin and mammalian cPLA₂ phospholipase domains, specifically at 3 highly conserved amino acid motifs; a glycine-rich oxyanion hole and the catalytic dyad [16,17]. This domain shares 27% identity with the *Legionella pneumophila* VipD and *Rickettsia prowazekii* RP534 PLA₂ proteins [18]. The C-terminal domain (351–687) shares homology to no known protein and is hypothesized to play a role in enzymatic activation. Fluorescence microscopy data indicates that portions of ExoU's C-terminus may also be involved in interactions with the plasma membrane [19]. ExoU requires the presence of eukaryotic cofactor proteins for enzymatic activity, one of which has been identified as superoxide dismutase 1 [20].

Although progress has been made regarding ExoU's contribution to infection, the mechanism of activation (interfacial or activator-mediated) and the functional significance of the C-terminal domain are poorly understood. This is, in part, due to the lack of a sensitive *in vitro* assay. Previously published assays that measured ExoU activity did so directly using ¹⁴C-labeled liposomes or indirectly using the Ellman's reagent [21,22]. Although both assays report PLA₂ activity, the necessity of a eukaryotic protein activator, often supplied as a crude cellular lysate, yielded high background activity; likely from mammalian PLA₂ enzymes contaminating the lysates. As a result, quantitative analysis of ExoU activity has been limited. Fortunately, the identification of SOD1 as an activator provided us the opportunity to utilize a fluorescence-based assay in the presence of purified activator [20].

This report outlines the optimization and validation of a sensitive and low background *in vitro* fluorescence-based assay and begins to characterize ExoU activation. The ability to conduct high-throughput inhibitor studies using a model PLA₂ inhibitor, methyl arachidonyl fluorophosphonate, is also addressed. Importantly, the assay is sensitive enough to detect intracellular ExoU activity in a HeLa cell model during *P. aeruginosa* infection. Utilization of the optimized fluorescence-based assay to ascertain the mechanism of ExoU activation may lead to the development of potential therapeutics that will inhibit ExoU activation and reduce *P. aeruginosa* associated mortality.

2. Materials and methods

2.1. Reagents

N-((6-(2,4-dinitrophenyl)amino) hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-2A,3A-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (PED6) was from Molecular Probes and suspended to a final concentration of 5 mmol/l in 100% dimethyl sulfoxide or 1 mmol/l in 100% ethanol. The structure of PED6 is shown in Figure 1A. 4,4-difluoro-5,7-dimethyl-4-bora-2A,3A-diaza-s-indacene-3-pentanoic acid (BODIPY FLC₅) was from Molecular Probes (Carlsbad, CA) and was suspended in 100% dimethyl sulfoxide to a final concentration of 5 mm. L-glutamic acid monosodium salt (MSG), L-glutamic acid potassium salt monohydrate, sodium phosphate,

sodium acetate trihydrate, and sodium citrate tribasic dihydrate were from Sigma Aldrich (St. Louis, MO) and the stock solutions adjusted to pH 6.3.

2.2. Bacterial strains, plasmids, and growth conditions

P. aeruginosa, PA103 Δ exoUexoT::Tc, with plasmids, pUCP18exoU, pUCP18exoU S142A, or pUCP18gfp [16,23,24], or PA103exoT::Tc pUCP18gfp [25] were grown on Vogel-Bonner minimal agar [26] containing 400 μ g/ml carbenicillin. *Escherichia coli* (*E. coli*) BL21 pLysS, containing the pET15bexoU or pET16bexoU S142A plasmids, were grown in Luria-Bertani (LB) medium with 100 μ g/ml ampicillin and 30 μ g/ml chloramphenicol. The *E. coli* strain, DH5 α pET16bexoU, was cultivated in LB medium containing 100 μ g/ml ampicillin. To remove the histidine tag from rExoU, *exoU* was cloned into pET15b, which introduces an N-terminal thrombin cleavage site. Briefly, *exoU* was extracted from pET16bexoU by digesting the plasmid with BamHI and NdeI and ligating the *exoU* fragment into the pET15b plasmid. All constructs were confirmed by nucleotide sequence analysis.

2.3. Protein expression, SDS PAGE, and immunoblot analysis

Overnight cultures of the BL21 pLysS pET15bexoU or BL21 pLysS pET16bexoU S142A strains were subcultured (1/50) in LB medium containing the appropriate antibiotics and grown at 30°C to an optical density of approximately 0.5 at 600 nm. The expression of rExoU was induced by the addition of 0.5 mmol/l isopropyl β -D-1 thiogalactopyranoside. The culture was shifted to 16°C and shaken overnight. Bacterial cells were collected by centrifugation at 7700 $\times g$ for 10 min, suspended in 1x binding buffer (50 mmol/l Na₂HPO₄ pH 7.0, 300 mmol/l NaCl, 5 mmol/l imidazole) with protease inhibitors (130 μ mol/l benzamidine, 84 μ mol/l leupeptin, 2.9 μ mol/l pepstatin A, and 6.8 μ mol/l phosphoramidon) and passed through a French pressure cell (4x at 16,000 psi). The total bacterial lysate was subjected to centrifugation at 16,200 $\times g$ for 10 min to separate unbroken cells and membrane debris from the smaller cellular components. The supernatant was subjected to ultracentrifugation at 100,000 $\times g$ for 1 h to remove insoluble material and most of the lipopolysaccharide. The supernatant from the ultracentrifugation step was passed through a 0.22 μ m syringe filter (PALL Corp.) and loaded directly onto Talon Co⁺² affinity resin. Affinity chromatography was performed according to the manufacturer's protocol (Clontech). The N-terminal 6x histidine tag fused to rExoU was removed by thrombin digestion. The digested material was passed through Sephacryl S100HR (Sigma) and benzamidine Sepharose 6B (GE Healthcare) resins to remove the histidine tag and thrombin, respectively. The rExoU preparation was stored in 10 mmol/l Tris pH 8.0, 15 mmol/l NaCl and 20% glycerol. The N-terminal 10x histidine tag associated with the catalytic null, rExoU S142A, was retained. This protein was purified by Talon Co⁺² affinity chromatography and dialyzed against 50 mmol/l NaCl and 50 mmol/l MOPS pH 6.3. Both protein preparations were stored at -80°C.

All proteins were analyzed by SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels (13.5%), which were stained with Coomassie brilliant blue or transferred to nitrocellulose membranes for immunoblot analysis. The fractions from the cellular infections were normalized to the HeLa cell number. To detect ExoU, nitrocellulose membranes were probed with the U29F8 monoclonal anti-ExoU antibody (1/20,000; 1/15,000 for injected ExoU). Green fluorescence protein (GFP) was detected with a monoclonal anti-GFP antibody (1/4,000; Covance). The bound monoclonal antibodies were detected with a horseradish peroxidase-conjugated monoclonal antibody directed against mouse IgG (1/10,000; Roche). Human SOD1 was detected with a polyclonal sheep anti-human SOD1 antibody (1/10,000; Calbiochem) and the bound antibody was detected with a horseradish peroxidase-conjugated donkey antibody directed against sheep IgG (1/10,000; Jackson ImmunoResearch). The membrane was incubated with Supersignal West Pico chemiluminescent substrate (Thermo Scientific) to visualize peroxidase activity by chemiluminescence.

2.4. Fluorescence-based ExoU activity assay

N-((6-(2,4-dinitrophenyl)amino) hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-2A,3A-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (PED6) was utilized as a substrate to detect and quantify ExoU phospholipase activity. Fluorescence intensity was measured by using a Spectramax M5 spectrophotometer (Molecular Devices) at an excitation wavelength of 488 nm and an emission wavelength of 511 nm (495 nm cutoff filter).

The initial assay conditions were modeled after Sato *et al* with minor modifications [20]. In a 50 μ l reaction volume, 50 mmol/l NaCl, 50 mmol/l MOPS pH 6.3, 6.25 μ mol/l bovine SOD1 (Sigma), 30 μ M PED6, and various amounts (30 – 500 ng) of rExoU were added to a 96-well microplate (Costar), vortexed, and the fluorescence intensity (relative fluorescence units, RFU) was measured for 4 h at room temperature.

The reaction conditions (50 μ l) for the optimized assay included 50 mmol/l MES pH 6.3, 750 mmol/l MSG, 100 μ mol/l PED6, 9.4 μ mol/l bovine SOD1 and upon addition of 125 ng rExoU, the fluorescence intensity was measured at room temperature. For both assay conditions, the normal background fluorescence intensity associated with PED6 was determined by performing the assay in the absence of wild type rExoU and in the presence of the catalytic null, rExoU S142A, and subtracting this value from the sample value. Kinetic constants are reported as apparent constants due to the unknown critical micelle concentration of PED6.

Various amounts (0.22 – 486 nmol/l, final concentration) of MAFP (methyl arachidonyl fluorophosphonate; BIOMOL International), an irreversible inhibitor of cytosolic and Ca^{+2} -independent PLA₂ enzymes, were added to the optimized fluorescence-based activity assay prior to the addition of rExoU. The half maximal inhibitory concentration (IC₅₀) was determined by fitting ExoU activity (RFU) at the various log concentrations of MAFP to a sigmoidal dose response regression model (Prism GraphPad).

2.5. BODIPY FLC₅ standard curve

Various amounts (0.0625 – 5.0 nmoles) of the purified fluorescent product of hydrolyzed PED6, BODIPY FLC₅ (4,4-difluoro-5,7-dimethyl-4-bora-2A,3A-diaza-s-indacene-3-pentanoic acid) were added to the reaction buffer (50 mmol/l MES pH 6.3, 750 mmol/l MSG) and the fluorescence intensity was measured.

2.6. Background PLA₂ activity from mammalian cells

Background PLA₂ activity associated with mammalian cellular lysates was evaluated in the fluorescence-based assay. A549 and HeLa cells were grown (37°C, 5% CO₂) in culture dishes (58 cm² growth area) in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum to a final cell density of 3.5×10^6 cells. Cells were washed with Hank's balanced salt solution (HBSS) with calcium and magnesium, scraped, and suspended in lysis buffer (100 mmol/l HEPES pH 7.4, 1 mmol/l EDTA, 255 mmol/l sucrose, 1 mmol/l phenylmethylsulphonyl fluoride, and 84 μ M leupeptin). After multiple passages through a 22-ga double needle, cellular lysates were subjected to centrifugation at $16,200 \times g$ for 10 min followed by ultracentrifugation at $100,000 \times g$ for 1 h to isolate soluble cytosolic material from insoluble material. The soluble cytosolic fraction was collected, protein concentration determined (BCA Protein Quantification Assay, Thermo Scientific) and various amounts (5 – 15 μ g) were directly added to the optimized fluorescence-based activity assay in the absence of rExoU.

2.7. Cellular infection and fractionation

ExoU activity associated with a HeLa cell infection model was evaluated. HeLa cells were cultured as described and infected with either PA103 Δ exoUexoT::Tc pUCP18exoU, PA103 Δ exoUexoT::Tc pUCP18exoU S142A, PA103exoT::Tc pUCP18gfp, or PA103 Δ exoUexoT::Tc pUCP18gfp at a multiplicity of infection of 5 in DMEM. Uninfected HeLa cells served as the negative control. Starting at 2 h post-infection and continuing through 5 h post-infection, the spent DMEM supernatant was removed from the culture dish and HeLa cells were scraped from the dish into lysis buffer (500 μ l DMEM, 1 mmol/l PMSF, and 84 μ mol/l leupeptin). The mammalian cells were lysed by multiple passages through a 22-gauge double needle and the cellular lysate was subjected to centrifugation at $16,200 \times g$ for 5 min to remove bacteria and unbroken mammalian cells. The supernatant was subjected to ultracentrifugation at $100,000 \times g$ for 1 h to obtain a soluble HeLa cell cytosolic fraction. The cytosolic fraction was passed through a 0.22 μ m syringe filter and a 25 μ l volume was added directly to the fluorescence-based activity assay (50 mmol/l MES pH 6.3, 750 mmol/l MSG, 100 μ M PED6). Activity was monitored for 7 h.

3. Results

3.1. Monosodium glutamate enhances the fluorescence-based ExoU activity assay

PED6, a glycerophosphoethanolamine lipid analog that possesses the BODIPY FL dye on its *sn*-2 acyl chain and a dinitrophenyl quencher group on its head group was utilized as the fluorogenic substrate (Fig. 1A and [20]). The close proximity of the fluorescent dye to the quencher group prevents fluorescence emission but upon cleavage by a PLA₂ enzyme, the dye is freed from the intramolecular quenching effect and fluorescence intensity directly relates to product formed. We initially observed that PA103 culture supernatants, induced for ExoU secretion, exhibited an increase in ExoU activity when directly added to the initial fluorescence-based activity assay (data not shown). To determine if the culture medium, TSBDU (trypticase soy broth dialysate for the purpose of ExoU secretion), contributed to an enhanced ExoU activity, rExoU was added to the initial fluorescence-based assay in the presence or absence of culture medium. The rate of ExoU activity was 40-fold higher in the presence of TSBDU than in its absence (Fig. 1B). The assay was performed in the absence of the ExoU activator, bovine SOD1, and exhibited no increase in fluorescence activity, indicating that the TSBDU components did not cleave PED6 nonspecifically (data not shown). These data suggested that a medium component was enhancing ExoU phospholipase activity, prompting us to analyze alternative reaction conditions to optimize the ExoU activity assay.

The TSBDU medium components have been well studied and widely utilized for the production of the secreted effectors [27–29]. Various components of the TSBDU medium, nitrilotriacetic acid (NTA), glycerol, and MSG, were examined for their ability to enhance ExoU activity. Neither NTA nor glycerol significantly affected ExoU activity (data not shown).

We next evaluated whether the salt concentration in the TSBDU medium enhanced ExoU activity. TSBDU is formulated with 85.5 mmol/l NaCl and 100 mmol/l MSG. As the amount of MSG was increased in the assay, the fluorescence intensity exhibited a dose dependent increase followed by a plateau (Fig. 1C). The titration of NaCl into the assay also elevated the fluorescence intensity but at a lower activity than MSG. The maximal rates of reaction, 227 RFU/min and 72 RFU/min, were observed at 500 mM of MSG and NaCl, respectively. The addition of 500 mM MSG enhanced ExoU activity 113-fold more than under assay conditions in which the salt concentration was not at optimal concentrations. No increase in fluorescence intensity was observed when bovine SOD1 was eliminated from the assay, indicating that neither salt served as an alternate activator of ExoU (data not shown).

3.2. The assay progression curve has a lag-phase followed by a steady-state phase

To evaluate the fluorescence-based activity assay in terms of meaningful kinetic data, relative fluorescence units were converted to absolute values (nmoles PED6 cleaved). A standard curve demonstrated that fluorescence intensity was linearly related to the concentration of the fluorescent PED6 cleavage product, BODIPY FLC₅ (Fig. 2A). Product inhibition or aberrant fluorescence quenching appeared to affect the rate of ExoU activity when > 10% of the product, BODIPY-FLC₅, was added to the reaction (data not shown) and we avoided analyzing data when more than 10% of the substrate was hydrolyzed. The ExoU activity was dependent on time and produced a progression curve that began with a 75 min lag-phase followed by a steady-state phase (Fig. 2B). Pre-steady-state rates (lag-phases) associated with PLA₂ enzymes have been well documented [30–32]. As a consequence of the observed pre-steady-state phase, ExoU activity was evaluated within the steady-state phase at the 90 min time point, after performing linear regression on the steady-state phase and using the value at the x-intercept as the zero time point (Fig. 2B, dotted line).

3.3 Optimization of the fluorescence-based ExoU activity assay

To generate kinetic constants related to ExoU activity we adjusted each aspect of the assay to obtain optimal activity. The initial assay, as developed by Sato et al., utilized a stock PED6 solution (1 mmol/l) dissolved in 100% ethanol [20], whereas the optimized assay utilizes a stock solution of PED6 (5 mmol/l) dissolved in 100% dimethyl sulfoxide. Both ethanol and dimethyl sulfoxide solvents abrogated ExoU activity when added at concentrations above 6% (v/v), but PED6 is most soluble in DMSO, allowing the preparation of highly concentrated stock solutions while keeping the percentage of organic solvent low in the assay. The optimal pH for the fluorescence-based activity assay was 6.0 – 6.5 (data not shown and [20]). To more closely approximate this pH range we replaced the original MOPS buffer with MES in the assay buffers.

The effect of MSG in the reaction was verified by establishing the optimal concentration for ExoU-mediated phospholipase activity. The rate of ExoU activity exhibited a dose-dependent increase as MSG (250 – 750 mmol/l) was added to the assay (Fig. 3A). At higher concentrations of MSG (1 mol/l), we noticed that the substrate appeared to form visible precipitates, which may account for the observed variation in activity at this concentration. To further understand how salt was affecting the fluorescence-based activity assay, ExoU activity was examined in the presence of various salts, all at a final concentration of 750 mmol/l (Fig. 3B). Under the optimized assay conditions, potassium glutamate and MSG enhanced ExoU activity similarly, whereas sodium citrate enhanced activity 1.5 fold more than MSG and potassium glutamate. Similar to the addition of 1 mol/l MSG to the assay, 750mmol/l sodium citrate began to precipitate the PED6 substrate, which accounted for the observed higher standard deviation. Little ExoU activity was detected in the presence of sodium phosphate or sodium acetate. These data indicate that higher molecular weight anions enhanced the activity of ExoU.

Under the initial assay conditions, ExoU activity, as a function of enzyme concentration, fit a nonlinear Hill sigmoidal model, peaking at 811 nmol/l [20]. Unexpectedly however, ExoU at concentrations above 811 nmol/l exhibited a dose-dependent decrease in activity. The combination of nonlinear activity followed by a decrease in activity as a function of ExoU concentration suggested that the kinetics of the reaction changed as ExoU concentrations changed. This observation prompted us to examine the reaction kinetics under optimal assay conditions that included the MES buffer at pH 6.3, 5 mmol/l dimethyl sulfoxide-dissolved PED6, and 750 mmol/l MSG. As higher concentrations of ExoU were added to the optimized assay, the rate of ExoU activity exhibited a linear, dose-dependent response (Fig. 3C). At concentrations above 270 nmol/l, ExoU activity began to plateau, as expected at saturating conditions. We used a final concentration of 33.8 nmol/l in the optimized assay.

An additional parameter in this assay is the necessity of the protein activator, SOD1. SOD1 was added to the assay in increasing concentrations (0.34 – 18.2 $\mu\text{mol/l}$) to determine the optimal amount for ExoU activation. Interestingly, SOD1 concentrations up to 18.2 $\mu\text{mol/l}$ activated ExoU linearly and a saturation condition was not observed (Fig. 3D). Although unable to saturate ExoU activity, SOD1 was kept constant at 9.4 μM throughout the optimization process.

Under initial assay conditions, the addition of substrate above 60 $\mu\text{mol/l}$ abrogated ExoU activity, most likely due to the limitation imposed by the ethanol solvent used to prepare the substrate stocks. The suspension of PED6 in DMSO and the stabilizing affects of MSG on the assay prompted us to perform Michaelis-Menten reaction kinetics. ExoU activity as a function of PED6 concentrations yielded a Michaelis plot (rectangular hyperbola) with an apparent K_m of 13.2 ± 1.5 $\mu\text{mol/l}$ PED6 and an apparent V_{max} of 42 nmoles/min/mg (Fig. 3E).

The irreversible inhibitor, MAFP, was previously shown to inhibit ExoU activity with an IC_{50} of 20nmol/l [33]. To evaluate the inhibitory effect of MAFP on rExoU in the optimized assay, ExoU activity was monitored in the presence of various amounts of MAFP (0.22–486 nmol/l, final concentration). MAFP inhibited rExoU with an IC_{50} of 13.8 ± 1.1 nmol/l (Fig. 3F).

3.4. Detectable levels of PLA₂ activity from translocated ExoU

The activity of mammalian PLA₂ enzymes and the apparent low levels of injected ExoU have made detecting translocated, catalytically-active ExoU challenging [16,19,34]. We reasoned that the increased sensitivity of the optimized assay may now allow measurement of ExoU activity in intoxicated cells. The background PLA₂ activity associated with a soluble HeLa cytosolic lysate was first examined by adding various amounts of the lysate to the optimized assay in the absence of rExoU. There was no significant increase in fluorescence intensity as a function of lysate concentration and a slight increase (50 RFU/h) as a function of time (Fig. 4A). The possibility that HeLa cytosolic lysates alter the integrity of the fluorescence-based activity assay was eliminated when the addition of rExoU (125 ng) produced a normal progression curve (Fig. 4A. inset). A549 soluble cytosolic lysates produced similar results (data not shown).

As endogenous HeLa PLA₂ activity was virtually undetectable in the optimized assay, the activity associated with HeLa cells after injection of ExoU by the *P. aeruginosa* T3SS was examined next. HeLa cell cytosolic lysates, harvested from the infection and containing translocated ExoU or ExoU S142A, were examined for ExoU activity. At 3 h post infection, the fraction harvested from ExoU-intoxicated cells exhibited a 3.7-fold increase in fluorescence intensity relative to the fraction from the ExoU S142A injected control (Fig. 4B).

To examine the fidelity of the fractionation, HeLa cells were infected with *P. aeruginosa* expressing GFP and ExoU or GFP alone as a control. All fractions were probed for ExoU, SOD1, and GFP by immunoblot analysis (Fig. 4C). GFP was detectable in the bacterial pellet/unlysed HeLa cell fraction starting at 3 h post infection, although the band was light and required longer exposures for the signal intensity to be visible (not shown). GFP was not detectable in HeLa cell cytosolic fractions. The absence of a signal during late time points of infection is significant since the numbers of bacteria have increased as shown by the strong GFP signals at late time points in the pellet fraction (4 and 5 h post infection). These data indicate that the bacteria were not lysing during the mechanical shearing procedure. To verify our fractionation procedure using a bacterial enzyme we examined the soluble and bacterial pellet/unlysed HeLa cell fractions for the activity of the periplasmic protein, β -lactamase. There was no positive correlation between ExoU activity and β -lactamase activity in the soluble

fractions, further indicating that ExoU activity is not a result of the spurious release of ExoU (data not shown).

SOD1 was detected primarily in the soluble lysate fractions (Fig. 4C). In control infections with a strain not expressing catalytically active ExoU (PA103 Δ exoUexoT::Tc) SOD1 was readily detectable by Western blot analysis at all time points. Some cellular lysis likely occurs at 5 h post infection due to insertion of type III pore proteins in eukaryotic membranes. In contrast, a strain expressing and injecting active ExoU showed fairly equivalent SOD1 signals only at the 2 h time point. At 3 h post infection, the SOD1 signal was diminished and at 4 and 5 h undetectable. These results indicate that cellular lysis was occurring due to the action of ExoU and likely accounts for lack of intracellular ExoU activity at 4 and 5 h post infection (Fig. 4B). ExoU protein was detected in the bacterial pellet/unlysed HeLa cell fraction at 4 and 5 h post infection. These results indicate that toxin associated with bacterial cells is detectable at later points in the infection. Overall, the infection experiment demonstrates the dynamic nature of an asynchronous infection and indicates that intracellular ExoU activity can be detected within a narrow time window. This window appears to be related to the time it takes for the bacteria to associate with cells, produce, inject and accumulate ExoU to detectable levels. Detection appears to be limited to times prior to cellular lysis.

4. Discussion

Our studies have focused on the A2 phospholipase, ExoU, whose activity is correlated with poor outcomes in patients suffering from acute *P. aeruginosa* infections. Prior to the identification of SOD1 as an activator, the initial studies of ExoU activity utilized eukaryotic lysates as a source of the activator [16,21,22]. Although useful, these assays had a low dynamic range and sensitivity in the presence of eukaryotic lysates. In this study we outline several changes in the fluorescence-based assay has increased its sensitivity and dynamic range. Importantly, these modifications had no affect on the requirement for SOD1 as a cofactor and allowed detection of translocated ExoU in the intoxication of cells *in vitro*.

In experiments that measured ExoU activity in *P. aeruginosa* cultures, we were surprised to observe that the spent TSBDU supernatants appeared to enhance ExoU activity. Tests of the inorganic medium components demonstrated that salt, specifically monosodium glutamate, augmented ExoU activity in the fluorescence-based activity assay. Monosodium glutamate was originally added to the TSBDU formulation as a replacement for the “expensive” α -alanine, glutamic acid, and aspartic acid nitrogen sources [27]. A literature search revealed that the addition of salt to reaction buffers is well characterized and is common practice in interfacial enzyme analyses. Specifically, salt lowers the critical micelle concentration of amphiphilic molecules [35–39] and anions induce a net negative charge on a zwitterionic interface [40]. A negative charge at the interface increases the affinity of PLA₂ enzymes to the interface [32, 41–44]. We postulate that the glutamate anion is responsible for inducing a negative charge on the PED6 interface as the PED6 head group is zwitterionic due to the quencher group. Data that support this hypothesis includes the enhancement of ExoU activity by potassium glutamate and sodium citrate. Interestingly, sodium chloride, sodium phosphate, and sodium acetate have a dramatically lower, if any, affect on ExoU activity, which suggests that the length of the anion plays a role in establishing the negative charge at the PED6 interface. Based on the literature cited above, MSG likely lowers the critical micelle concentration of the PED6 micelles and increases the affinity of ExoU to the PED6 interface by inducing a negative charge.

To evaluate the kinetic behavior of ExoU, we optimized the assay to best satisfy Michaelis-Menten assumptions; PED6 substrate is in excess and a steady-state is achieved, albeit after a lag-phase. Interfacial enzyme kinetics is complex and multiple kinetic pathways may exist. For this reason, our kinetic analyses are constrained to the steady-state phase and kinetic values

are reported as apparent values. Pre-steady-state rates have been linked to product accumulation at the interface [30,31]. To potentially eliminate the pre-steady-state phase we have attempted to titrate in higher levels of MSG to the assay, however PED6 visibly aggregates in MSG concentrations higher than 750 mmol/l.

We have shown that SOD1, at a concentration as high as 18 $\mu\text{mol/l}$, does not saturate ExoU activity. We speculate that the oligomeric structure or the post-translational modification state of the purified SOD1 preparations may be inflating the necessity for high concentrations of SOD1, although normal concentrations of SOD1 within cells range from 10–100 $\mu\text{mol/l}$ [45].

The amount of ExoU required for half-maximal killing of a CHO cell population has been estimated to be between 300 – 600 molecules/cell [17], which is below the level of immunoblot detection. Based on the low levels of background activity with cellular lysates observed with the optimized fluorescence-based activity assay, experiments were performed to determine if the assay was sensitive enough to detect the activity of ExoU injected in a population of cells during infection. Infections were performed over a time course and eukaryotic cells were lysed by a shearing procedure that leaves bacteria intact, as shown by the absence of GFP in the HeLa cell cytosolic fraction and confirmed, using an enzymatic bacterial reporter protein. The lysate fraction was cleared of membranes and filter sterilized to remove any bacterial contamination. From these cytoplasmic fractions, ExoU activity was detectable only at 3 h post-infection. Cytoplasmic fractions past this point did not have detectable activity. These later time points correspond with ExoU-mediated eukaryotic cell morphological changes over the time course of in a CHO cell model infection [23] and loss of the monolayer due to either release from the substrate or overt cell lysis. One indication of the loss of cells is the loss of SOD1 signal from cultures infected with strains expressing wild type ExoU versus strains expressing non-catalytically active ExoU (ExoU S142A; Fig. 4C). The difficulties in detecting catalytically active ExoU not only involve the sensitivity of the assay (injection and accumulation of enough ExoU) but the limitations of a dynamic infection system in which cells become permeable to large substrates and eventually lose their contents to the medium. We have, however, optimized the fluorescence-based activity assay so that we appear to be detecting injected ExoU based on our controls. We have provided data outlining a sensitive and specific fluorescence-based ExoU activity assay. The assay is sensitive enough to provide kinetic data and, for the first time, detects catalytically active ExoU in a HeLa cell infection model. We have also documented the utility of the fluorescence-based ExoU assay by verifying inhibitor study capabilities and showing a low, if any, background activity in the presence of a complex eukaryotic lysate. The optimization of the assay for the derivation of kinetic parameters is an important step towards understanding the mechanism of ExoU activation.

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List of abbreviations

PLA ₂	A2 phospholipase
SOD1	superoxide dismutase 1
PED6	N-((6-(2,4-dinitrophenyl)amino) hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-2A,3A-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt

MSG	monosodium glutamate
MAFP	methyl arachidonyl fluorophosphonate
<i>E. coli</i>	<i>Escherichia coli</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
BODPIY FLC ₅	4,4-difluoro-5,7-dimethyl-4-bora-2A,3A-diaza-s-indacene-3-pentanoic acid
RFU	relative fluorescence units
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MOPS	3-morpholinopropanesulfonic acid,3-(<i>N</i> -morpholino)propanesulfonic acid
DMEM	Dulbecco's modified Eagle medium
HBSS	Hank's balanced salt solution
PMSF	phenylmethylsulphonyl fluoride
TBSDU	trypticase soy broth dialysate for the purpose of ExoU secretion
NTA	nitrilotriacetic acid
GFP	green fluorescence protein
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt

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FIG. 1.

Identification of the TSBDU medium component responsible for the enhancement of ExoU activity. (A) The molecular structure of the PED6 substrate (Molecular Probes). The arrow denotes the site of ExoU-mediated hydrolysis. (B) Recombinant ExoU (135 nmol/l, final concentration) activity in the presence (●) or absence (■) of TSBDU medium. All values depict representative data of duplicate experiments. (C) Various concentrations of NaCl (■) and MSG (●) were added to the ExoU fluorescence assay under modified initial assay conditions (100 mmol/l MOPS pH 6.3, 30 μmol/l PED6, 6.25 μmol/l bovine SOD1, and 135 nmol/l rExoU). All values depict representative data of duplicate experiments.

FIG. 2.

(A) Standard curve exhibiting a linear relationship between fluorescence intensity (RFU) and fluorescent PED6 cleavage product (BODIPY FLC₅). (B) The reaction progression curve depicting a steady-state phase after a 75 min lag-phase (solid line), under optimized conditions (50 mmol/l MES pH 6.3, 750 mmol/l MSG, 33.8 ml/l rExoU, 9.4 μ mol/l bovine SOD1, and 100 μ mol/l PED6), Linear regression was performed with the steady-state time points (dotted line, $R^2 = 0.998$) and the value at the x-intercept was considered the 0 time point and used for rate calculations. Values represent the arithmetic mean and SD ($n=3$).

FIG. 3.

The fluorescence-based activity assay was optimized by evaluating the dependence of ExoU activity on (A) MSG, (B) various salts, (C) rExoU enzyme concentration, (D) activator concentration (bSOD1), and (E) the substrate (PED6) concentration. All analyses were performed under optimized conditions (50 mmol/l MES pH 6.3, 750 mmol/l MSG, 33.8 mol/l rExoU, 9.4 μ mol/l bovine SOD1, and 100 μ mol/l PED6), unless that particular condition was the parameter being evaluated. Linear regression was performed in the linear range of the rate of reaction as a function of ExoU concentration (B, dotted line, $R^2 = 0.984$). (F) MAFP (0.22–486 nmol/l) was added to the ExoU fluorescence assay under optimized conditions. The values reported represent the arithmetic mean and SD ($n=3$).

FIG. 4.

P. aeruginosa-injected ExoU is detectable in the fluorescence-based activity assay. (A) Background activity associated with native mammalian PLA₂ enzymes was examined by adding 5 μ g (●), 10 μ g (■), or 15 μ g (▲) of the soluble HeLa cell cytosolic fraction to the optimized fluorescence-based activity assay in the absence of rExoU. (A inset) Addition of rExoU to the reaction restored activity. (B) HeLa cell monolayers (3.5×10^6 cells/dish) were infected with either PA103 Δ exoUexoT::Tc pUCP18exoU or PA103 Δ exoUexoT::Tc pUCP18exoU S142A. An uninfected HeLa cell monolayer served as a negative control. At 2, 3, 4, and 5 h post-infection, HeLa cells were removed from the culture dish, lysed, and the soluble cellular cytosol isolated. The soluble HeLa cytosolic fractions were passed through a 0.22 μ m filter and added to the optimized activity assay lacking rExoU and bovine SOD1. The fluorescence intensity values represent the arithmetic mean and standard deviation of triplicate experiments. (C) HeLa cell monolayers (3.5×10^6 cells/dish) were infected with either PA103 Δ exoT::Tc pUCP18gfp or PA103 Δ exoUexoT::Tc pUCP18gfp. The cells were fractionated as discussed above and the bacteria and unbroken HeLa cell pellet (P) and soluble HeLa cell cytosolic fraction (S) were analyzed by immunoblot analysis with the anti-ExoU monoclonal antibody U29F8, anti-GFP, and anti-bovine SOD1 antibodies at all hours post infection. Lanes were normalized to HeLa cell number.