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In Vivo Phospholipase Activity of the *Pseudomonas aeruginosa* Cytotoxin ExoU and Protection of Mammalian Cells with Phospholipase A_2 Inhibitors*

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A number of clinical isolates of *Pseudomonas aeruginosa* are cytotoxic to mammalian cells due to the action of the 74-kDa protein ExoU, which is secreted into host cells by the type III secretion system and whose function is unknown. Here we report that the swift and profound cytotoxicity induced by purified ExoU or by an ExoU-expressing strain of *P. aeruginosa* is blocked by various inhibitors of cytosolic ($cPLA_2$) and Ca^{2+}-independent ($iPLA_2$) phospholipase A_2 enzymes. In contrast, no cytoprotection is offered by inhibitors of secreted phospholipase A_2 enzymes or by a number of inhibitors of signal transduction pathways. This suggests that phospholipase A_2 inhibitors may represent a novel mode of treatment for acute *P. aeruginosa* infections. We find that 300–600 molecules of ExoU/cell are required to achieve half-maximal cell killing and that ExoU localizes to the host cell plasma membrane in punctate fashion. We also show that ExoU interacts *in vitro* with an inhibitor of $cPLA_2$ and $iPLA_2$ enzymes and contains a putative serine-aspartate catalytic dyad homologous to those found in $cPLA_2$ and $iPLA_2$ enzymes. Mutation of either the serine or the aspartate renders ExoU non-cytotoxic. Although no phospholipase or esterase activity is detected *in vitro*, significant phospholipase activity is detected *in vivo*, suggesting that ExoU requires one or more host cell factors for activation as a membrane-lytic and cytotoxic phospholipase.

Pseudomonas aeruginosa is an opportunistic, Gram-negative bacterial pathogen that causes severe infections in cystic fibrosis, AIDS, burn, and neutropenic chemotherapy patients (1–4). Treatment is often problematic due to antibiotic resistance of this pathogen. A number of bacterial factors have been implicated in *P. aeruginosa* pathogenesis, and prominent among these is the type III secretion system (TTSS).[1] Approximately three-quarters of clinical isolates of *P. aeruginosa* express one or more TTSS proteins when tested *in vitro* (5, 6), and the great majority of cystic fibrosis patients have antibodies reactive against the TTSS protein PopB (7). *P. aeruginosa* strains that express TTSS proteins are associated with greater mortality rates in patients and increased severity of disease (5, 6). Additionally, vaccination with a TTSS component (PcrV) has been shown to decrease lung inflammation and injury in challenged mice (8).

The proteins ExoS, ExoT, ExoU, and ExoY are exported directly into the cytosol of host mammalian cells through the TTSS of *P. aeruginosa* (9), although most strains generally do not express all four at the same time (5, 6, 10). ExoS and ExoT are highly related and have dual functions, acting as both a GTPase-activating protein (11–14) and ADP-ribosyl transferase (15), and ExoY has been identified as an adenylate cyclase (16). Interestingly, the ADP-ribosyl transferase activities of ExoS and ExoT and the adenylate cyclase activity of ExoY are dependent on host cell factors, the 14-3-3 protein FAS for ExoS and ExoT (17), and unidentified factors for ExoY (16). Of these four effectors, ExoU (74 kDa) is the most cytotoxic, but its biochemical function is not known (18, 19).

ExoU is found in about one-third of clinical isolates, and these ExoU-expressing strains are associated in 90% of cases with severe disease (6, 10). ExoU has been shown to cause fatality in a mouse model of lung infection (18), and expression of ExoU in *P. aeruginosa* strains lacking it increases virulence in a mouse model of acute pneumonia (20). The exact role of ExoU in virulence is uncertain, but it is implicated along with ExoT in the onset of septic shock (21). Septic shock, alveolar epithelial injury, and bacteremia are caused in a rabbit model of pneumonia by the wild-type *P. aeruginosa* clinical isolate PA103, which expresses ExoU and ExoT (but not ExoS and ExoY) (14). By comparison, deletion of ExoU and ExoT in PA103 abrogates these effects. Sepsis appears to arise from epithelial cell damage and subsequent leakage of proinflammatory cytokines into the bloodstream, giving rise to a systemic inflammatory response. The combined actions of ExoU as a cytotoxin and ExoT in its ability to inhibit bacterial internalization and wound repair are also seen to be important in a mouse model of acute pneumonia (14, 22).

We sought to determine whether inhibitors that block the cytotoxic action of ExoU could be identified. We report that cytotoxicity caused by purified ExoU internalized into mammalian cells or by *P. aeruginosa* PA103 is effectively blocked by

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[1] The abbreviations used are: TTSS, type III secretion system; MAFP, methyl arachidonyl fluorophosphonate; GFP, green fluorescent protein; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; $sPLA_2$, secreted PLA_2; PLC, phospholipase C; PLD, phospholipase D; CHO, Chinese hamster ovary; LDH, lactate dehydrogenase; DTT, dithiothreitol; BSA, bovine serum albumin; TX-100, Triton X-100; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; Lyso-PC, lysophosphatidylcholine; PPPC, 1-palmitoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; ATK, arachidonyl trifluoromethyl ketone; PTK, palmitoyl trifluoromethyl ketone; PIP_2, phosphatidylinositol 4,5-bisphosphate.

inhibitors of cytosolic and Ca^{2+}-independent phospholipase A_2 ($cPLA_2$ and $iPLA_2$) enzymes. Importantly, this suggests that inhibitors of phospholipase A_2 enzymes may serve as potential modes of treatment for acute pneumonias caused by ExoU-expressing cytotoxic strains of *P. aeruginosa*. In addition, we provide mutagenesis evidence consistent with ExoU functioning as a phospholipase and biochemical evidence indicating ExoU to be the direct target of inhibition. Although we find that ExoU lacks detectable phospholipase or esterase activity *in vitro*, experiments *in vivo* demonstrate significant phospholipase activity, indicating that one or more host cell factors are required for activation of ExoU as a membrane-lytic phospholipase.

EXPERIMENTAL PROCEDURES

Expression and Purification

ExoU, cloned from *P. aeruginosa* PA103 chromosomal DNA by PCR, was expressed in pET28b (Novagen) with a thrombin-cleavable N-terminal histidine tag (MGSSHHHHHHSSGLVPRGSHMAS). DNA sequencing verified the integrity of this and other constructs. ExoU expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (25 °C) in *Escherichia coli* BL21 (DE3), and bacteria were lysed by sonication in 150 mM NaCl, 50 mM phosphate buffer, pH 8.0, 10 mM β-mercaptoethanol, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml DNase. ExoU, present in the supernatant from the centrifuged lysate, was purified by metal chelation chromatography (Poros MC/M), and the histidine purification tag was removed by thrombin digestion. ExoU was further purified by size exclusion chromatography (Superdex 200) and concentrated to ~21 mg/ml (in 10 mM Tris, pH 8.0) and stored at −80 °C.

ExoU variants S142A, D344A, ExoU-(45–687), ExoU-(45–657), and ExoU-(45–667) were generated by PCR using standard means. ExoU-GFP, ExoU(S142A)-GFP, and ExoU-(45–667)-GFP fusion proteins contain a 6-residue linker sequence (GSGTSG) separating N-terminally located histidine-tagged ExoU and C-terminally located green fluorescent protein (GFP) (GFP_{UV}, Clontech). Unfused GFP contains an N-terminal histidine tag (MGSSHHHHHHSSGLVPRGSHMASLEAGSGTSG). These proteins were expressed in pET28b and purified as above, except that no thrombin cleavage was carried out. The cytotoxic activity of ExoU is not altered by the histidine tag.

Syringe Loading

Approximately 10^6 Chinese hamster ovary (CHO) cells in 900 μl of culture medium (Ham's F-12 medium, 10% fetal bovine serum), 100 μl of 20% Pluronic F-68, and 50 μl of 1.0 mg/ml ExoU (in 10 mM Tris-Cl, pH 8.0) were drawn up and expelled slowly (~0.2 ml/s) through a 30-gauge syringe needle six times (23). Cells were centrifuged (700 × *g*, 5 min), and the syringe-loading supernatant was quantified for lactate dehydrogenase (LDH) activity (Cytotox Kit, Promega). Culture medium (1 ml) was added to the cells, which were then incubated for 20 min at 37 °C. The cells were again centrifuged, and the incubation supernatant was also quantified for LDH. Reported LDH activity ($100 \times A_{490}$) is a sum of syringe loading and incubation supernatants and is corrected for background LDH activity from buffer alone. ExoU has no LDH activity. Cells were then plated and incubated for 16 h at 37 °C. Percentage of cell death was determined by counting trypan blue-excluding cells prior to syringe loading and after the 16-h incubation following syringe loading. Papain-digested ExoU (digested at 50:1 ExoU:papain, 25 °C, 20 h) contains no polypeptides >14 kDa, as visualized by SDS-PAGE. In all cases, inhibitors syringe-loaded in the absence of ExoU showed no cytotoxicity.

Quantification and Visualization of Syringe-loaded ExoU

Approximately 10^6 CHO cells were syringe-loaded with 50 μl containing 5, 50, or 250 μg of ExoU(S142A)-GFP and were lysed by a 1-h incubation in 200 μl of lysis buffer (0.9% (v/v) Triton X-100 in phosphate-buffered saline). Fluorescence (excitation, 395 nm; emission, 509 nm) was quantified from the clarified lysate (centrifuged 15,800 × *g*, 5 min) using a JY Fluoromax-3 fluorimeter, corrected for background fluorescence, and evaluated using a linear standard constructed using ExoU(S142A)-GFP. The detection limit is ~5 ng of ExoU(S142A)-GFP. Incubation of ExoU(S142A)-GFP with cells in the absence of syringe loading results in no detectable fluorescence, and the presence of the cell lysate does not alter GFP fluorescence. For visualization by fluorescence microscopy, ~10^6 cells were syringe-loaded with 15 μM ExoU(S142A)-GFP or 15 μM GFP, plated on coverslips, and incubated for 16 h and then fixed for visualization with 3% paraformaldehyde in phosphate-buffered saline for 15 min.

Infection Assays

CHO cells were infected as described previously (24) with PA103 or PA103ΔU at a multiplicity of infection of ~35:1, and an LDH assay was carried out 3 h after infection. Methyl arachidonyl fluorophosphonate (MAFP) was used at 67.5 μM, and cells were supplemented with 67.5 μM MAFP every hour.

MAFP Pretreatment

ExoU (13.5 μM) was incubated with 1.35 mM MAFP (MAFP-pretreated ExoU) or without MAFP (mock-pretreated ExoU) for ~16 h at 4 °C and dialyzed extensively over 2 days (in 10 mM Tris, pH 8.0, using a 6-kDa molecular mass cut-off membrane). Samples were syringe-loaded without further MAFP treatment. A sample of MAFP (1.35 mM) was incubated and dialyzed in the absence of ExoU and then used in a syringe-loading experiment with ExoU (ExoU + dialyzed MAFP). A sample of MAFP (1.35 mM) was incubated for 2 days without dialysis and used at 67.5 μM in a syringe-loading experiment with ExoU (ExoU + mock dialyzed MAFP). MAFP-pretreated ExoU was also syringe-loaded with a fresh aliquot of MAFP (67.5 μM) (MAFP-pretreated ExoU + MAFP).

In Vitro Activity Assays

PAPC:TX-100—ExoU (10 μg) or $cPLA_2$ α (1 μg) in 50 μl of buffer (20 mM HEPES, pH 7.7, 100 mM KCl, 200 μM $CaCl_2$, 1 mg/ml bovine serum albumin (BSA), 1 mM DTT) was added to 250 μl of buffer and 200 μl of mixed micelles, which were composed of 1 mM [^{14}C]PAPC (200,000 cpm at *sn*-2) and 3 mM Triton X-100 (TX-100). Samples were vortexed and incubated at 40 °C for 60 min. Products of this and following radioassays were quenched and analyzed using a modified Dole protocol (25). Mixed micelles were made in the following way: 190 μl of 100 mM KCl, 20 mM HEPES, pH 7.7, were added to phospholipids and vortexed followed by addition of 10 μl of 150 mM TX-100 and vortexing.

PIP_2:PAPC:TX-100—ExoU (10 μg) or $cPLA_2$ α (5 ng) in 50 μl of assay buffer (100 mM HEPES, pH 7.5, 80 μM $CaCl_2$, 0.1 mg/ml BSA, and 2 mM DTT) was added to a solution containing 400 μl of assay buffer and 50 μl of a 10× concentrated stock of mixed micelles, whose final concentration contained 97 μM [^{14}C]PAPC (100,000 cpm at *sn*-2), 3 μM PIP_2, and 400 μM TX-100. The reaction was carried out as above, except that the incubation time was 30 min. The micelles were prepared as above, except that phospholipids were resuspended in assay buffer (48.7 μl) as a 10× concentrated stock, and TX-100 (1.3 μl of 150 mM) was then added.

PPPC:TX-100—ExoU (10 μg) or $cPLA_2$ α (1 μg) in 50 μl of assay buffer (100 mM HEPES, pH 7.7, 0.1 mg/ml BSA, and 1 mM DTT) was added to a solution containing 400 μl of assay buffer (containing 5 mM Ca^{2+} or 5 mM EGTA) and 50 μl of a 10× concentrated stock of mixed micelles, whose final concentration contained 100 μM [^{14}C]PPPC (100,000 cpm at *sn*-2) and 400 μM TX-100. The reaction was carried out for 60 min at 40 °C. Mixed micelles were made as for PIP_2:PAPC:TX-100.

LysoPC—ExoU (10 μg) or $cPLA_2$ α (1 μg) in 50 μl of assay buffer (100 mM KCl, 20 mM HEPES, pH 7.7, 0.1 mg/ml BSA, and 1 mM DTT) was added to a solution of 400 μl of assay buffer (containing 5 mM Ca^{2+} or 5 mM EGTA) and 50 μl of a 10× concentrated stock of pure micelles, whose final concentration contains 1 mM [^{14}C]LysoPC (200,000 cpm on palmitic acid at *sn*-1 or *sn*-2). The reaction was carried out for 60 min at 40 °C. The LysoPC micelles were formed by thorough vortexing after addition of buffer (100 mM KCl and 20 mM HEPES, pH 7.7) to LysoPC to make a 10× concentrated solution.

Thin Layer Chromatography Assay—Approximately 2.1 g of of liver polar lipid extract (Avanti) per sample was dried down and resuspended in 4.4 ml of reaction buffer (150 mM NaCl and 50 mM Tris, pH 8.0) by alternating between vortexing and warming in a 37 °C water bath for 5 min. Lipids were sonicated on ice until no residual lipids could be seen on the glass and the solution became clear. The lipid sample (400 μl) was mixed with 100 μl of enzyme and incubated ~18 h at 37 °C with shaking. The following enzyme amounts were used: 0.25 μg of secreted PLA_2 ($sPLA_2$) (*Naja naja* venom, Sigma), 172 μg of PLC (*Clostridium perfringens*, Sigma), 2 μg of PLD (*Streptomyces chromofuscus*, Sigma), 890 μg of ExoU. Replicate samples were supplemented with 3 mM $CaCl_2$. Lipids were extracted using the Bligh-Dyer procedure. Briefly, 1.5 ml of 1:2 $CHCl_3$:MeOH was added to samples followed by 0.5 ml of $CHCl_3$ and by 0.5 ml of 60 mM HCl; samples were vortexed vigorously

at each addition. The lower organic layer was transferred into glass tubing and dried down to one-fifth of the original volume under an N_2 stream.

Approximately 25 μl of each sample was spotted onto a TLC plate (20 × 20 cm), as were standards (oleic acid, LysoPC, POPC, and dioleoylglycerol). The chromatographic separation was run for 2.5 h in 65:25:4 $CHCl_3$:MeOH:H_2O. Development of the spots was achieved by incubation in an iodine chamber for 10 min, and Sigma spray reagent molybdenum blue was used for detection of phosphorus-containing compounds.

Phenyl Valerate Esterase Assay—ExoU (1.65 mg) or cPLA$_2$ α (45 μg) in 1 ml of assay buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA) was added to 1 ml of phenyl valerate (0.5 mg/ml) (Oryza Laboratories, Chelmsford, MA) in 0.03% TX-100. Phenyl valerate was initially solubilized at 15 mg/ml in dimethylformamide and diluted into 0.03% TX-100. Reactions were incubated for 20 min or 3 h at 37 °C and stopped through addition of 1 ml of 3.4% (w/v) sodium dodecyl sulfate in assay buffer containing 0.25 mg/ml 4-aminoantipyrine (Sigma). The reactions were mixed with 500 μl of 0.4% $K_3Fe(CN)_6$ (Sigma) and incubated for 10 min, and absorbance at 510 nm was measured.

In Vivo Phospholipase Activity Assays

Tritiated arachidonic acid or palmitic acid (60–100 Ci/mmol; PerkinElmer Life Sciences) was added (~0.5 μCi/10^6 cells) directly to CHO cells (in Ham's F-12 medium), and cells were incubated for 24 h. Medium was removed, and cells were washed three times with phosphate-buffered saline containing 1 mg/ml BSA. Approximately 10^6 cells were syringe-loaded as described above, except that cells were incubated in the syringe-loading solution for 20 min and then centrifuged at 720 × *g* for 1 min. The supernatant was used for LDH quantification and extracted using the modified Dole protocol (25). Radioactivity in the sample extracted by the Dole protocol was quantified by scintillation counting. Dole extraction was seen to be successful in separating free fatty acids from phospholipids. This was done by addition of exogenous [^{3}H]arachidonic acid or [^{14}C]PAPC to medium from buffer-loaded or hypotonically lysed cells, and it was found that >89% of [^{3}H]arachidonic acid is extracted as compared with only <2% of [^{14}C]PAPC.

RESULTS

ExoU Cytotoxicity—To investigate the mechanism of ExoU-induced cytotoxicity, we purified recombinantly produced ExoU and introduced it into the cytoplasm of CHO cells by syringe loading (23). A swift and marked cytotoxicity, as measured by exclusion of trypan blue or release of intracellular lactate dehydrogenase, is observed (Fig. 1*A*). ExoU-mediated cytotoxicity is reported to occur through necrosis rather than apoptosis (24), consistent with our observations. Cytotoxicity occurs almost immediately after syringe loading (~10 min), and after 16 h, less than 5% of cells are viable. ExoU exhibits no cytotoxicity when added to the surface of cells, even at concentrations 10-fold higher than those used for syringe loading (data not shown), confirming that intracellular localization is required (18). The cytotoxic effect is specific to ExoU, as demonstrated by papain-digested ExoU, bovine serum albumin, or buffer alone lacking cytotoxicity (Fig. 1*A*). Similar results are observed in the human hepatocyte cell line HepG2 (data not shown).

Specificity of ExoU action is also confirmed by its dose-dependence (Fig. 1*B*). Half-maximal killing is observed with ~3 million ExoU molecules added per cell during syringe loading. However, the number of molecules internalized by CHO cells is much lower, estimated at ~0.01–0.02%. Therefore, half-maximal cytotoxicity appears to require only ~300–600 ExoU molecules internalized per cell, setting a value on the magnitude of export required of the TTSS *in vivo* to achieve cell killing. To determine the efficiency of internalization, a construct containing a non-cytotoxic variant of ExoU (S142A, see below) fused to GFP was produced, purified, and syringe-loaded into CHO cells. Fluorescence from internalized ExoU(S142A)-GFP was then quantified (data not shown). ExoU-GFP and ExoU are internalized in quantitatively similar ways, as shown by ExoU-GFP (containing wild-type ExoU) having the same half-maximal killing dose as ExoU (data not shown).

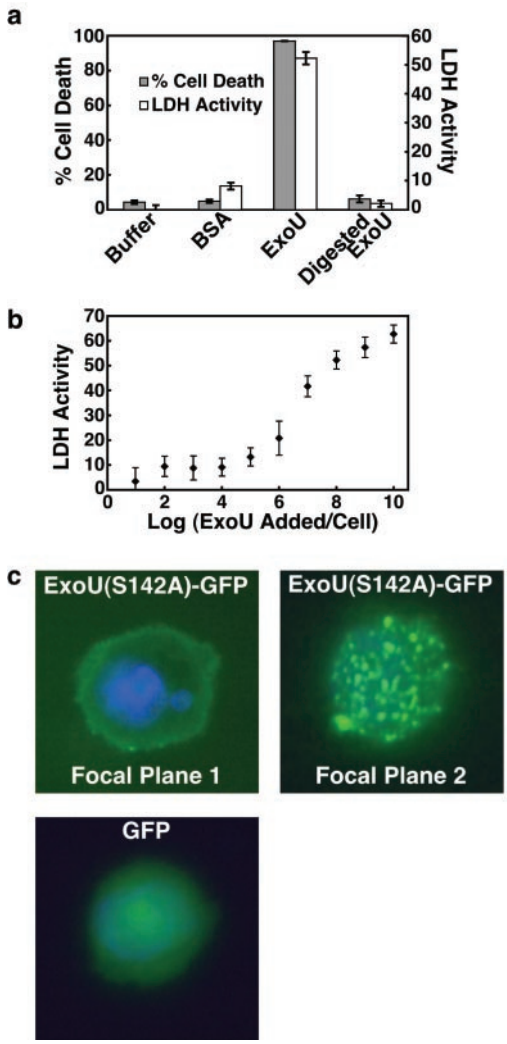

FIG. 1. **Cytotoxicity of purified ExoU.** *a*, buffer alone, BSA (50 μg), ExoU (50 μg), or papain-digested ExoU (50 μg) was syringe-loaded into CHO cells, and cytotoxicity was measured by trypan blue exclusion (percent of cell death, *gray*) or LDH activity (*white*, 100 × A_{490}), with LDH activity from buffer alone subtracted. *Error bars* indicate the standard deviation of triplicate experiments for this and the following figures. *b*, cytotoxic dose-response in CHO cells, as measured by LDH activity. ExoU was quantified using an experimentally determined absorption extinction coefficient (ϵ_{280} = 31,200 M^{-1} cm^{-1}) and CHO cells by counting trypan blue-excluding cells. As shown in *c*, ExoU(S142A)-GFP localizes to the plasma membrane in punctate fashion. A fluorescence microscopy image of CHO cells syringe-loaded with ExoU(S142A)-GFP or GFP is shown. Cell nuclei are visualized by Hoechst staining (*blue*), and GFP fluorescence is seen in *green*. For ExoU(S142A)-GFP, representative focal planes are shown to demonstrate circumferential plasma membrane (*Focal Plane 1*) and punctate localization (*Focal Plane 2*).

Internalized ExoU(S142A-GFP) was visualized by fluorescence microscopy, revealing localization to the plasma membrane (Fig. 1*C*). This contrasts with the diffuse localization pattern seen for syringe-loaded GFP. Furthermore, the localization of ExoU(S142A-GFP) occurs in punctate fashion (Fig. 1*C*, *Focal Plane 2*), as observed by varying the focal plane, and is indicative of possible ExoU interaction with host cell components.

Inhibition of ExoU-mediated Cytotoxicity—Strikingly, ExoU-induced cytotoxicity is entirely eliminated or greatly reduced by specific inhibitors of phospholipase A_2 (PLA_2) enzymes (26, 27). MAFP, an irreversible inhibitor of cytosolic PLA_2s (cPLA$_2$,

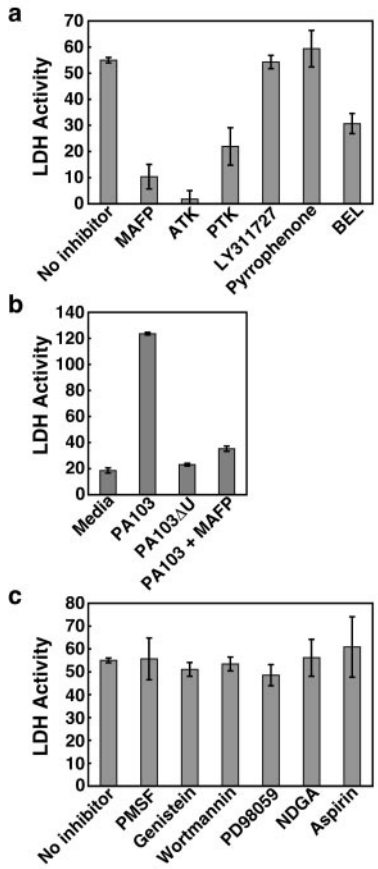

FIG. 2. **Effects of PLA_2 inhibitors (*a* and *b*) or non-PLA_2 inhibitors (c) in syringe-loading experiments or infection assays.** As shown in *a* and *c*, inhibitors were syringe-loaded with ExoU (0.675 μM) into CHO cells and included in culture medium afterward. Inhibitors were used at the following concentrations: MAFP, 67.5 μM; ATK, 67.5 μM; PTK, 67.5 μM; LY311727, 250 μM; pyrrophenone, 1 μM; bromoenol lactone (*BEL*), 67.5 μM; phenylmethylsulfonyl fluoride (*PMSF*), 1.0 mM; genistein, 200 μM; wortmannin, 100 nM; PD98059, 50 μM; nordihydroguaiaretic acid (*NDGA*), 100 μM; and aspirin, 100 μM. *b*, MAFP inhibition of cytotoxicity in CHO cells infected with *P. aeruginosa* PA103, as assessed by LDH activity. Background LDH activity from medium alone is shown and was not subtracted from sample values.

also called Group IVA PLA_2) and Ca^{2+}-independent PLA_2s (iPLA_2, also called Group VIA PLA_2) (28, 29), blocks 82% of cell killing (Fig. 2*A*). In contrast, a specific inhibitor of sPLA_2, LY311727, fails to protect against cytotoxicity. Notably, sPLA_2s differ mechanistically from cPLA_2s and iPLA_2s in that the former enzyme family uses a catalytic histidine, whereas the latter two families similarly use a catalytic serine. Consistent with the action of MAFP, arachidonyl trifluoromethyl ketone (ATK) and palmitoyl trifluoromethyl ketone (PTK), which are specific and reversible inhibitors of cPLA_2s and iPLA_2s (but not sPLA_2s) (29), are also found to be cytoprotective (Fig. 2*A*). Interestingly, although ATK blocks cell killing (96% protection) even more efficiently than MAFP, PTK is only partially protective (60% protection), implying a preference for arachidonate over palmitate in the target of these inhibitors.

To assess whether a cellular phospholipase is responsible for cytotoxicity, a specific inhibitor of endogenous cPLA_2 α, pyrrophenone (30), and a specific inhibitor of endogenous iPLA_2, bromoenol lactone (28), were examined. Pyrrophenone is not protective, whereas bromoenol lactone shows only partial protection (Fig. 2*A*). These results indicate that ExoU-mediated cytotoxicity is likely to involve an enzyme with similarity to cPLA_2 or iPLA_2 but unlikely to involve an endogenous cPLA_2 or iPLA_2.

Inhibition of P. aeruginosa Cytotoxicity—These data further suggest that MAFP might be an effective way to inhibit the cytotoxicity of ExoU-producing *P. aeruginosa* strains. To test this, we examined cell killing by the clinical *P. aeruginosa* isolate PA103. Wild-type *P. aeruginosa* PA103, which expresses ExoU and ExoT but not ExoS and ExoY, is seen to be cytotoxic, whereas the ExoU-deficient strain PA103ΔU (14) is non-cytotoxic (Fig. 2*B*). This is consistent with previous work showing loss of cytotoxicity through deletion of ExoU (18). We find that treatment of target cells with MAFP also renders wild-type *P. aeruginosa* PA103 non-cytotoxic (Fig. 2*B*). These results raise the possibility that MAFP and other phospholipase A_2 inhibitors may be successful in treating acute pneumonias.

Signal Transduction Pathways—Although these data offer insight into the process of cytotoxicity, they do not indicate whether phospholipase activity is itself cytotoxic or is mediated through signaling events. Since release of arachidonate by cPLA_2 leads to downstream signaling through cyclooxgenases and lipoxygenases, inhibitors of these enzymes were examined. Neither a cyclooxgenase inhibitor (aspirin) nor a lipoxygenase inhibitor (nordihydroguaiaretic acid) protects cells from ExoU (Fig. 2*C*). This rules out prostaglandins and leukotrienes as mediators of cell death. We also examined signaling pathways involving kinases. In contrast with an earlier report demonstrating dependence of *P. aeruginosa* cytotoxicity on tyrosine phosphorylation (31), we find that the protein tyrosine kinase inhibitor genistein offers no protection against ExoU (Fig. 2*C*). This indicates that phosphorylation-dependent but ExoU-independent mechanisms of cytotoxicity may operate in certain *P. aeruginosa* strains. Furthermore, inhibitors of mitogen-activated protein (MAP) kinase and phosphoinositide 3-kinase pathways of signal transduction (PD98059 and wortmannin, respectively) also fail to protect (Fig. 2*C*). Similarly, a covalent inhibitor of serine proteases, phenylmethylsulfonyl fluoride, fails to protect. These results are consistent with cytotoxicity arising from direct phospholipase action.

Lipase Motif in ExoU—Sequence analysis suggests that ExoU may itself be the phospholipase that causes cytotoxicity. Although previous work failed to detect homology in ExoU to proteins of known function (18), we have detected homology to the cPLA_2 and iPLA_2 families using pattern-specific iterative BLAST (32) (Fig. 3*A*). The overall identity between ExoU and these PLA_2s is minimal (<13%), but two small and functionally important blocks of homology are found. The first block contains the lipase motif G*X*Syy (*X* is any amino acid, and y is a small amino acid, such as Gly, Ser, or Thr), in which serine (Ser-142 in ExoU) is predicted to act as a nucleophile and form an unusual Ser-Asp catalytic dyad, as suggested by the structure of cPLA2 α (33). The first homology block also contains a conserved Arg or Lys, which is thought to interact with the phosphate group of phospholipid substrates. The second block contains the Asp of the catalytic dyad (Asp-344 in ExoU). Mutagenesis has confirmed the functional importance of the Ser and Asp in cPLA_2s and iPLA_2s (33–39).

Mutagenesis also provides evidence for the importance of these residues in ExoU. Alanine substitutions were created at either of the two positions, and although both ExoU(S142A) and ExoU(D344A) are monomeric and soluble *in vitro* like wild-type ExoU, neither is cytotoxic *in vivo* (Fig. 3*B*). In comparison, deletion of the N-terminal 45 amino acids of ExoU has no effect on cytotoxicity (Fig. 3*B*, ExoU-(45–687)), demonstrating the precise perturbation of function in ExoU(S142A) and ExoU(D344A). The N terminus of ExoU has been shown to interact with the specific bacterial chaperone SpcU (40) and the C terminus to be required for cytotoxicity (41). We have further

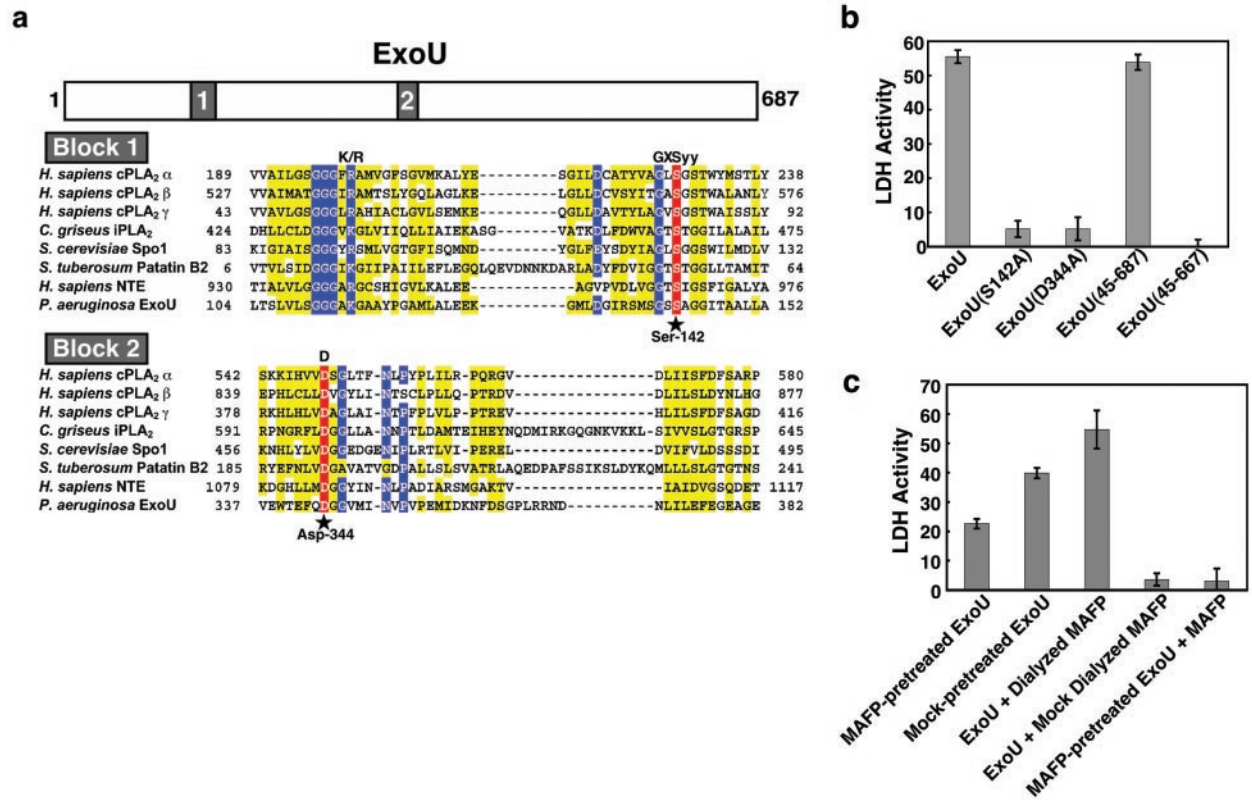

FIG. 3. **Homology of ExoU to phospholipase A_2.** As shown in *a*, two blocks of homology are found between ExoU and cPLA$_2$s (human cPLA$_2$ α, β, and γ, Spo1) and iPLA$_2$s (*Cricetulus griseus* iPLA$_2$, patatin B2, neuropathy target esterase (*NTE*)). The catalytic Ser (*Block 1*, Ser-142 in ExoU, *starred*) and Asp (*Block 2*, Asp-344 in ExoU, *starred*) dyad are in *red*, highly conserved residues in are in *blue*, and chemically similar residues are in *yellow*. GenBank™ accession numbers are as follows: cPLA$_2$ α (P47712), cPLA$_2$ β (AAD32135), cPLA$_2$ γ (AAC32823), iPLA$_2$ (I15470), Spo1 (NP_014386), patatin B2 (P15477), neuropathy target esterase (CAA06164), and ExoU (AAC16023). As shown in *b*, ExoU Ser-142 and Asp-344 are essential for cytotoxicity, as are the C-terminal 20 residues. Cytotoxicity of syringe-loaded wild-type ExoU and variants in CHO cells, as assessed by LDH activity, is shown. As shown in *c*, MAFP pretreatment of ExoU is cytoprotective. ExoU was either pretreated with MAFP (*MAFP-pretreated ExoU*) or mock pretreated with MAFP (*Mock-pretreated ExoU*). As controls, MAFP was incubated and dialyzed in the absence of ExoU (*ExoU + Dialyzed MAFP*) or incubated and not dialyzed (*ExoU + Mock Dialyzed MAFP*) and used in syringe-loading experiments. MAFP-pretreated ExoU was also syringe-loaded with a fresh aliquot of MAFP (*MAFP-pretreated ExoU + MAFP*).

limited the functional portion of the C terminus, finding that deletion of even the last 20 residues abrogates cell killing, as seen in the ExoU-(45–667) deletion mutant (Fig. 3*B*). This C-terminal region has no similarity to phospholipases and appears unique to ExoU. It is worthwhile noting that deletion of the C-terminal 20 residues does not change localization within CHO cells, as assessed with a GFP fusion to ExoU-(45–667) (data not shown).

In Vitro Assays—Despite sequence and mutational evidence implicating ExoU as a phospholipase A_2 enzyme, we failed to detect phospholipase or esterase activity for ExoU *in vitro* (Table I). Sensitive radioactive assays for PLA$_1$, PLA$_2$, and lysophospholipase activities were carried out with ExoU. Substrates carrying ^{14}C-labeled arachidonic acid at the *sn*-2 position in PAPC:TX-100 mixed micelles or ^{14}C-labeled palmitic acid at *sn*-2 in PPPC:TX-100 mixed micelles were unaffected by ExoU. Addition of PIP$_2$, which increases the activity of the mammalian cPLA$_2$ α toward PAPC:TX-100 mixed micelles, had no effect, and neither did inclusion of Ca^{2+}. As a test for lysophospholipase activity, LysoPC micelles containing a mixture of labeled *sn*-1 and *sn*-2 palmitic acids were incubated with ExoU. Again, ExoU showed no activity. In addition, small unilamellar vesicles composed of a polar lipid extract from hepatocytes, which are shown in this study to be susceptible to ExoU killing, were incubated with ExoU, PLA$_2$, PLC, or PLD and analyzed by TLC. The lipid extract contains at least five substrates that could be resolved by TLC, with the most abundant ones being phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and cholesterol. Activity was observed for all enzymes except ExoU (data not shown). Lastly, ExoU with or without added cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+}) exhibited no activity in a phenyl valerate esterase assay (data not shown); in contrast, cPLA$_2$ and the serine protease chymotrypsin both exhibit activity in this assay. The phenyl valerate esterase assay has been used previously to demonstrate activity in the PLA$_2$-related enzyme neuropathy target esterase (Fig. 3*A*) (39).

TABLE I
In vitro radioactive assays

Assays were performed using L-α-1-palmitoyl-2-([1-^{14}C]arachidonoyl) phosphatidylcholine ([^{14}C]PAPC) in TX-100 mixed micelles ([^{14}C]PAPC:TX-100), [^{14}C]PAPC with PIP$_2$ in TX-100 mixed micelles (PIP$_2$:[^{14}C]PAPC:TX-100), L-α-1-palmitoyl-2-([1-^{14}C]palmitoyl) phosphatidylcholine in TX-100 mixed micelles ([^{14}C]PPPC:TX-100), or L-lyso-1-[1-^{14}C]palmitoyl-3-phosphatidylcholine in pure micelles ([^{14}C]LysoPC) as substrate.

Assay description	Control enzyme	Control specific activity	ExoU specific activity
		nmol/min/mg	*nmol/min/mg*[a]
[^{14}C]PAPC:TX-100	cPLA$_2$ α	137	<1
PIP$_2$:[^{14}C]PAPC:TX-100	cPLA$_2$ α	12,600	<1
[^{14}C]PPPC:TX-100	cPLA$_2$ α	8	<0.2
[^{14}C]PPPC:TX-100	iPLA$_2$	4,500[b]	<0.2
[^{14}C]LysoPC	cPLA$_2$ α	1,800	<5

[a] Values reported correspond to detection limits of the assays.
[b] Taken from Ref. 45.

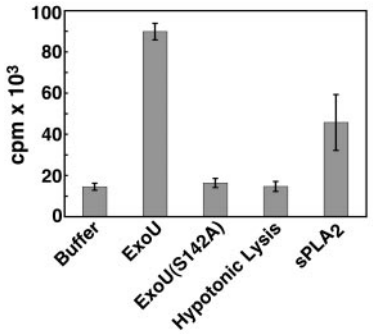

FIG. 4. **ExoU exhibits *in vivo* phospholipase activity.** Buffer alone, ExoU (50 μg), ExoU(S142A) (50 μg), or $sPLA_2$ (10 μg) was syringe-loaded into CHO cells metabolically labeled with [^{3}H]arachidonic acid. Medium from cells was collected, extracted by a modified Dole protocol, and quantified by scintillation counting. Hypotonic lysis was carried out by resuspending labeled CHO cells in water. *Error bars* indicate the standard deviation of duplicate experiments.

MAFP Pretreatment—As an alternative to an *in vitro* enzymatic assay, we asked whether ExoU interacts with MAFP *in vitro*. In studies above, inhibitors were added to CHO cells during syringe loading and also incubated with cells afterward. To examine whether ExoU is the direct target of MAFP inhibition, MAFP was preincubated *in vitro* with ExoU but not used on cells during or after syringe loading. Free MAFP was removed from pretreated ExoU by extensive dialysis. Significantly, pretreatment of ExoU with MAFP is found to be cytoprotective, whereas mock pretreatment of ExoU is not (Fig. 3*C*). This cytoprotection is not the result of free MAFP carried through dialysis, as a control shows that free MAFP is effectively removed by dialysis. To assess this, MAFP was dialyzed in the absence of ExoU and then tested for cytoprotective activity, and found to have none (Fig. 3*C*, *ExoU + Dialyzed MAFP*).

It must be noted that MAFP pretreatment is only partially cytoprotective (<50%) and requires incubation of ExoU and MAFP at concentrations 20-fold greater than those used during syringe loading. The lack of full inhibition is not due to time-dependent inactivation of MAFP, as shown by retention of cytoprotective activity in a sample of MAFP that was incubated over time but not dialyzed (Fig. 3*C*, *ExoU + Mock Dialyzed MAFP*). Furthermore, the >50% of ExoU not inhibited by MAFP pretreatment does not represent a resistant fraction of ExoU, as addition of further MAFP during syringe loading completely eliminates cell killing (Fig. 3*C*, *MAFP-pretreated ExoU + MAFP*). The lack of full cytoprotection and requirement for high concentrations are consistent with the *in vitro* enzymatic assays, which suggest that ExoU is largely inactive prior to internalization. This pretreatment experiment does, however, provide evidence for ExoU being a direct target of MAFP inhibition.

In Vivo Phospholipase Assay—We next asked whether ExoU exhibits phospholipase activity *in vivo*. CHO cells were metabolically labeled with [^{3}H]arachidonic acid, which is incorporated primarily into phospholipid pools (42–44). ExoU was syringe-loaded into these labeled cells, and after a 20-min incubation, medium from the cells was collected. The medium was extracted using a modified Dole protocol to isolate free fatty acids, and the radioactivity in the extract was quantified (25). Using this *in vivo* phospholipase assay, we find that syringe loading ExoU results in the production of large amounts of free fatty acids, consistent with high levels of phospholipase activity in ExoU (Fig. 4). As expected, *in vivo* phospholipase activity is also detected when the membrane-lytic and cytotoxic phospholipase A_2 from snake venom ($sPLA_2$) is syringe-loaded into CHO cells. Notably, although equimolar amounts of ExoU and $sPLA_2$ were used in these experiments, ExoU exhibits much greater phospholipase activity, in agreement with its rapid cytotoxic effect. In contrast, no phospholipase activity is detected when syringe loading buffer alone or the inactive ExoU(S142A) mutant or when cells are simply lysed by hypotonic treatment. Similar results were seen when [^{3}H]palmitic acid was incorporated into CHO cells (data not shown). Consistent with the effects of inhibitors on cytotoxicity, [^{3}H]arachidonic acid release induced by ExoU was inhibited by MAFP but not by LY311727 or pyrrophenone (data not shown). In summary, these results demonstrate that ExoU, in its intracellular form, generates significant amounts of free fatty acids. These results also suggest that ExoU functions as a highly active phospholipase *in vivo* and causes cell death directly through this activity.

DISCUSSION

Our results clearly identify certain inhibitors of $cPLA_2$ and $iPLA_2$ enzymes as effective antagonists of ExoU-induced cytotoxicity. MAFP and ATK are seen to offer the greatest protection against purified ExoU syringe-loaded into CHO cells, and MAFP is shown to protect CHO cells nearly completely from *P. aeruginosa* PA103-induced cytotoxicity. Importantly, this raises the possibility of a novel mode of treatment using phospholipase inhibitors for acute infections caused by cytotoxic, ExoU-expressing strains of *P. aeruginosa*.

Our work also provides evidence for identification of ExoU as a $cPLA_2$/$iPLA_2$-like enzyme. ExoU is shown to have two small but functionally critical blocks of homology to $cPLA_2$ and $iPLA_2$ families of phospholipases. The putatively catalytic serine and aspartate in these blocks are found to be crucial to the cytotoxic activity of ExoU, as assessed by syringe-loading wild-type and mutant ExoU into mammalian cells. Furthermore, the $cPLA_2$/$iPLA_2$ inhibitor MAFP is shown to interact with ExoU *in vitro*. Although no phospholipase, lysophospholipase, or esterase activity is detected for ExoU *in vitro*, significant phospholipase activity is detected *in vivo*. These results suggest that ExoU is inactive prior to entry into mammalian cells but becomes activated once within mammalian cells through the action of one or more host cell factors. This possibility is also consistent with experiments showing localization of ExoU to the plasma membrane in punctate fashion (Fig. 1*C*), suggestive of interaction and co-localization of ExoU with host cell factors. Interestingly, a requirement for host cell factors has been observed for the other *P. aeruginosa* TTSS effectors ExoS, ExoT, and ExoY (16, 17).

Toxicity caused by phospholipase action is not unprecedented, in that the snake venom toxins belonging to the $sPLA_2$ family have long been known to be membrane-lytic and cytotoxic. Direct cytotoxic action through ExoU agrees with the swift and profound cell death observed, the lack of cytoprotection offered by a number of signaling pathway inhibitors, and the high *in vivo* phospholipase activity. Phospholipases A_2s are known to be promiscuous in having lysophospholipase activity as well. It is possible that ExoU not only removes the *sn*-2 fatty acid from phospholipids but continues as a lysophospholipase and removes both fatty acids. Enzymes with phospholipase A_2 activity are known to be important to the pathogenesis of *Candida albicans*, parvovirus, and adeno-associated virus type 2 (46–48). Interestingly, phospholipase A_2 activity is implicated in phagosome lysis by *Rickettsia prowazekii* (49), and genome sequencing of this bacterial pathogen reveals a putative protein (RP534) related to ExoU. Potential proteins with the PLA_2-like lipase motif (Fig. 3*A*, *Block 1*) are also present in the genomes of the bacterial pathogens *Treponema pallidum*, *Bacillus anthracis,* and *Mycobacterium tuberculosis*, suggest-

ing that the PLA_2-like family of enzymes may be useful as antimicrobial targets.

In summary, our work provides direct evidence that cytotoxicity induced by purified ExoU or from ExoU-expressing *P. aeruginosa* is blocked by inhibitors of phospholipase A_2 enzymes and is consistent with ExoU functioning as a host-activated and membrane-lytic phospholipase. These results also suggest a novel mode of treatment for acute infections caused by ExoU-expressing strains of *P. aeruginosa*.

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Note Added in Proof—Similar conclusions regarding ExoU activation and function on heterologous expression of the protein in yeast have been reported recently (Sato, H., Frank, D. W., Hillard, C. J., Feix, J. B., Pankhaniya, R. R., Moriyama, K., Finck-Barbancon, V., Buchaklian, A., Lei, M., Long, R. M., Wiener-Kronish, J., and Sawa, T. (2003) *Embo J.* **22,** 2959–2969).

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In Vivo Phospholipase Activity of the *Pseudomonas aeruginosa* Cytotoxin ExoU and Protection of Mammalian Cells with Phospholipase A_2 Inhibitors

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