UNIVERSITY OF CALIFORNIA, SAN DIEGO

Determining the Mechanism of Action of ExoU, a Cytotoxin from the Bacterial Pathogen *Pseudomonas aeruginosa*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Chemistry

by

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This dissertation of Rebecca M. Phillips is approved, and it is acceptable in quality and form for publication on microfilm:

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The text of chapter 2, in full, is a reprint of the material entitled "*In Vivo* Phospholipase Activity of the *Pseudomonas aeruginosa* Cytotoxin ExoU and Protection of Mammalian Cells with Phospholipase A_2 Inhibitors" as it appears in the Journal of Biological Chemistry. The dissertation author was the primary researcher and/or author and the co-authors (D. Six, E. Dennis, and P. Ghosh) listed in this publication contributed to or supervised the research which forms the basis for this chapter.

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ABSTRACT OF THE DISSERTATION

Determining the Mechanism of Action of ExoU,

a Cytotoxin from the Bacterial Pathogen *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic bacterial pathogen that causes severe infections in individuals with compromised immune systems. In cystic fibrosis patients, infection with *P. aeruginosa* is the leading cause of death. A number of highly virulent strains of *P. aeruginosa* have been found to express the cytotoxic protein ExoU. Correspondingly, ExoU has been shown to increase virulence in animal models of pneumonia. ExoU is transported by the *P. aeruginosa* type III secretion system directly into the cytosol of host cells, where it exerts cytotoxicity. At the start of the studies described in this dissertation, the mechanism of action of ExoU was unknown.

Presented here are studies uncovering the mechanism of action of ExoU from *P. aeruginosa*. Evidence points to ExoU functioning as a host-cell activated phospholipase A_2 enzyme. Mutagenesis in the putative catalytic site or the presence of phospholipase A_2 inhibitors is found to block ExoU-induced cytotoxicity. Furthermore, cytotoxicity of an ExoU-expressing strain of *P. aeruginosa* is blocked by phospholipase A_2 inhibitors, suggesting a novel mode of treatment for *P. aeruginosa* infections. ExoU has no *in vitro* phospholipase A_2 activity without the presence of a cellular extract, suggesting that ExoU requires one or more host cell factors for activation.

Genetic and biochemical experiments were carried out to determine the host cell factors involved in ExoU activation. Genetic experiments with yeast, which are found to be susceptible to ExoU-induced cytotoxicity, suggest that the gene required for ExoU activation may be essential for viability or have a redundant function. Biochemical purification of the ExoU activator from a yeast extract suggests that it is a heat-stable, anionic and glucose-containing saccharide. However, this molecule is unable to induce rapid activation of ExoU, as is seen *in vivo*, suggesting the involvement of another factor, termed the ExoU accelerator. The ExoU accelerator is found to be heat-labile and part of a very large complex containing RNA. Surprisingly, these studies show that the saccharide activator does not co-purify with this factor. This suggests that there may be two completely different ExoU activators, or that the saccharide activator is an unusual glycosylation modification on the ExoU accelerator.

I.

Introduction

Introduction

Pseudomonas aeruginosa is an opportunistic, Gram-negative bacterial pathogen that causes severe infections in immunocompromised individuals such as cystic fibrosis, AIDS, burn, and neutropenic chemotherapy patients (Afessa and Green, 2000; Brennan and Geddes, 2002; Lyczak et al., 2000; Lyczak et al., 2002). *P. aeruginosa* is a major cause of hospital-acquired pneumonia, perhaps second only to *Staphylococcus aureus* in its frequency (Schulert et al., 2003). However, in contrast to infections caused by *S. aureus*, those caused by *P. aeruginosa* tend to be especially severe and often lead to mortality (Hauser et al., 2002). Treatment of infection by this ubiquitous bacterium is often problematic due to antibiotic resistance.

A number of bacterial factors have been implicated in *P. aeruginosa* pathogenesis, and prominent among these is the type III secretion system (TTSS). Approximately three-quarters of clinical isolates of *P. aeruginosa* express one or more TTSS proteins when tested *in vitro* (Hauser et al., 2002; Roy-Burman et al., 2001), and the great majority of cystic fibrosis patients have antibodies reactive against the TTSS protein PopB (Moss et al., 2001). *P. aeruginosa* strains that express TTSS proteins are associated with greater mortality rates in patients and increased severity of disease (Hauser et al., 2002; Roy-Burman et al., 2001). Additionally, vaccination with a TTSS component (PcrV) has been shown to decrease lung inflammation and injury in challenged mice (Sawa et al., 1999).

P. aeruginosa uses the TTSS to export effector proteins directly into the cytosol of host mammalian cells (Fig. 1.1) (Frank, 1997). The delivery of certain effectors is dependent on their association in the bacterial cytosol with specific chaperone proteins, such as OrfA or SpcU, which remain in the bacterial cytosol after translocation.

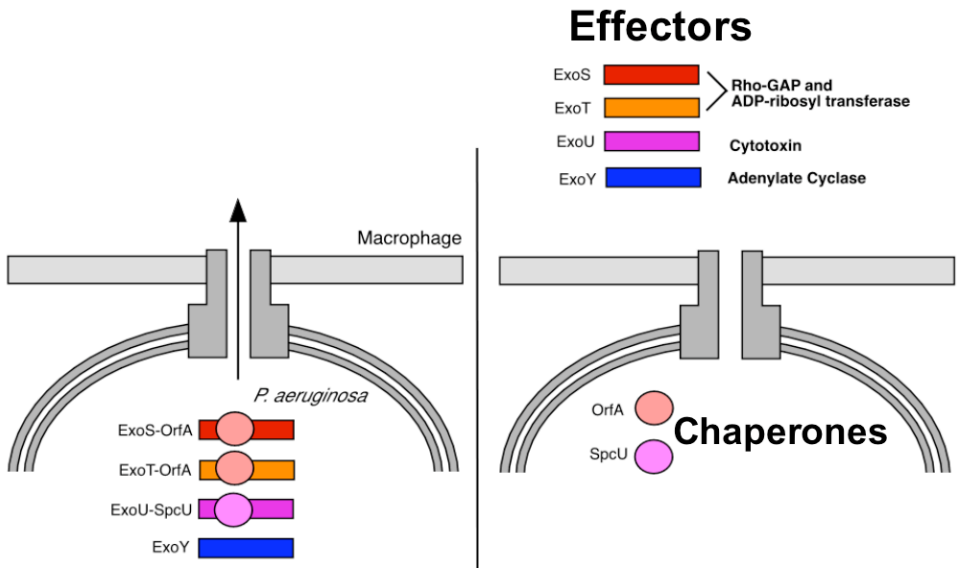

Figure 1.1. Schematic of *Pseudomonas aeruginosa* type III secretion of effectors. Four know effectors ExoS, ExoT, ExoU, and ExoY are translocated directly from the bacterium into the host cell cytosol. The bacterial chaperone OrfA is required for the translocation of the effectors ExoS and ExoT, and the chaperone SpcU for the translocation of the effector ExoU.

Four effectors, ExoS, ExoT, ExoU, and ExoY, have been identified for *P. aeruginosa* (Fig. 1.1), although most strains generally do not express all four at the same time (Feltman et al., 2001; Hauser et al., 2002; Roy-Burman et al., 2001). ExoS and ExoT are highly related and have dual functions, acting as both a GTPase-activating protein (Garrity-Ryan et al., 2000; Goehring et al., 1999; Kazmierczak and Engel, 2002; Krall et al., 2000) and ADP-ribosyl transferase (Knight and Barbieri, 1997), and ExoY has been identified as an adenylate cyclase (Yahr et al., 1998). 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 (Fu et al., 1993), and unidentified factors for ExoY (Yahr et al., 1998). Of these four effectors, ExoU (74 kDa) is the most cytotoxic, but its biochemical function was not known before the studies presented in this dissertation (Finck-Barbancon et al., 1997; Hauser et al., 1998).

ExoU is found in about one-third of clinical isolates, and these ExoU-expressing strains are associated in 90% of cases with severe disease (Feltman et al., 2001; Hauser et al., 2002). ExoU has been shown to cause fatality in a mouse model of lung infection (Finck-Barbancon et al., 1997), and expression of ExoU in *P. aeruginosa* strains lacking it increases virulence in a mouse model of acute pneumonia (Allewelt et al., 2000). The exact role of ExoU in virulence is uncertain, but it is implicated along with ExoT in the onset of septic shock (Kurahashi et al., 1999). 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) (Garrity-Ryan et al., 2000). 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 (Garrity-Ryan et al., 2000; Geiser et al., 2001).

The goal of the research presented in this dissertation is to determine the mechanism of cytotoxic action by ExoU, where no mechanism existed before. We provide evidence that ExoU functions as a phospholipase A_2 enzyme (Fig. 1.2) and show that cytotoxicity caused by ExoU-expressing *P. aeruginosa* is blocked by phospholipase A_2 inhibitors. These data provide the basis for development of novel antimicrobial therapeutics directed at the highly cytotoxic ExoU. Furthermore, like other *P. aeruginosa* effectors, we find that ExoU is dependent on a host cell factor for activity. Genetic and biochemical experiments were carried out to elucidate the host cell factors involved in ExoU activation.

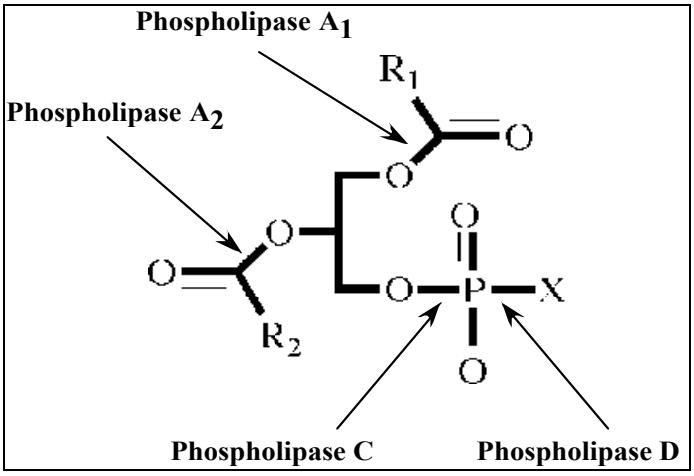

Figure 1.2. Schematic of phospholipase action. Phospholipase A_1 hydrolyzes the fatty acyl chain (R_1) at the *sn*-1position, and phospholipase A_2 hydrolyzes the fatty acyl chain (R_2) at the *sn*-2 position. Phospholipase B (not shown) hydrolyzes either *sn*-1 or *sn*-2. Phospholipases C and D hydrolyze the head group (X), removing the phosphate from or leaving it on the lipid, respectively. Lysophospholipases cleave the fatty acid from a lysophospholipid, which contains one rather than two fatty acyl chains.

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II.

Determining the mechanism

of cytotoxicity by ExoU

Abstract

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.

Introduction

Pseudomonas aeruginosa is an opportunistic, Gram-negative bacterial pathogen that causes severe infections in cystic fibrosis, AIDS, burn, and neutropenic chemotherapy patients (Afessa and Green, 2000; Brennan and Geddes, 2002; Lyczak et al., 2000; Lyczak et al., 2002). The *P. aeruginosa* type III secretion system (TTSS) exports four proteins (ExoS, ExoT, ExoU, and ExoY) directly into the cytosol of host cells (Frank, 1997) to subvert host defenses. Of these four effectors, ExoU (74 kDa) is the most cytotoxic, but its biochemical function is not known (Finck-Barbancon et al., 1997; Hauser et al., 1998).

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 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 (Clarke and McNeil, 1992). Cells were centrifuged (700 x *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 x 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 x *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 (Hauser and Engel, 1999) 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 radio-assays were quenched and analyzed using a modified Dole protocol (Kokotos et al., 2002). 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 10x 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 10x 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 10x 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 10x 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 10x concentrated solution.

Thin Layer Chromatography Assay—Approximately 2.1 g 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 x 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 x *g* for 1 min. The supernatant was used for LDH quantification and extracted using the modified Dole protocol (Kokotos et al., 2002). 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 (Clarke and McNeil, 1992). A swift and marked cytotoxicity, as measured by exclusion of trypan blue or release of intracellular lactate dehydrogenase, is observed (Fig. 2.1*A*). ExoU-mediated cytotoxicity is reported to occur through necrosis rather than apoptosis (Hauser and Engel, 1999), 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 (Finck-Barbancon et al., 1997). The cytotoxic effect is specific to ExoU, as demonstrated by papain-digested ExoU, bovine serum albumin, or buffer alone lacking cytotoxicity (Fig. 2.1*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. 2.1*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).

Internalized ExoU(S142A-GFP) was visualized by fluorescence microscopy, revealing localization to the plasma membrane (Fig. 2.1*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. 2.1*C*, *Focal Plane 2*), as observed by varying the focal plane, and is indicative of possible ExoU interaction with host cell components.

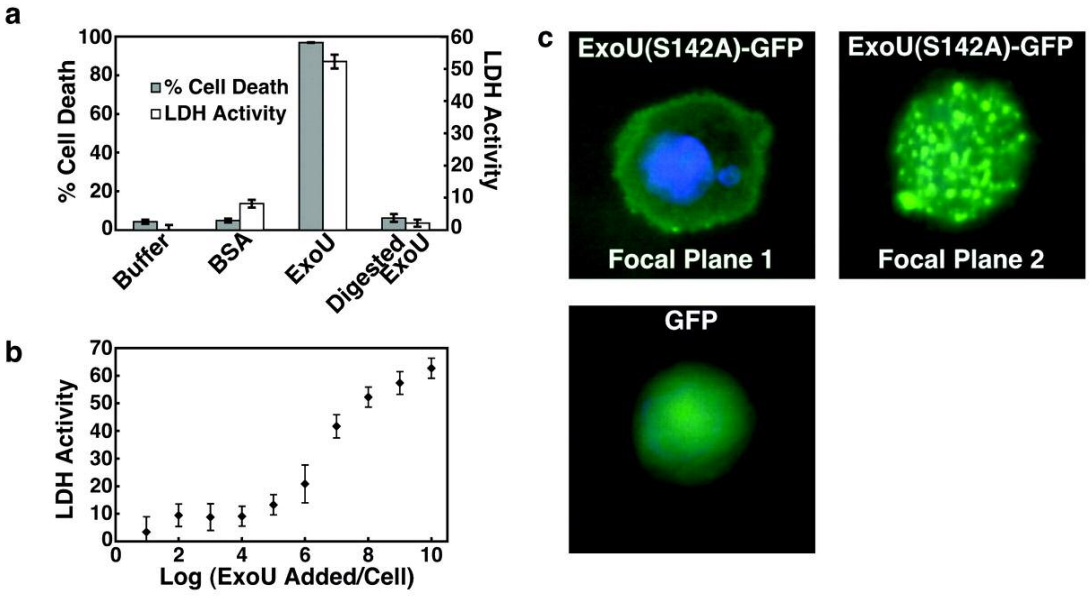

Figure 2.1. Cytotoxicity of purified ExoU. A, Buffer alone, BSA (50 µg), ExoU (50 µg), or papain-digested ExoU (50 µg) was syringe-loaded into CHO cells, and cytotoxicity was measured by trypan blue exclusion (percent of cell death, *gray*) or LDH activity (*white*, 100 x A_{490}), with LDH activity 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*).

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 (Balsinde et al., 1999; Six and Dennis, 2000). MAFP, an irreversible inhibitor of cytosolic PLA_2s ($cPLA_2$, also called Group IVA PLA_2) and Ca^{2+}-independent PLA_2s ($iPLA_2$, also called Group VIA PLA_2) (Balsinde and Dennis, 1996; Conde-Frieboes et al., 1996), blocks 82% of cell killing (Fig. 2.2*A*). In contrast, a specific inhibitor of $sPLA_2$, LY311727, fails to protect against cytotoxicity. Notably, $sPLA_2$s differ mechanistically from $cPLA_2$s and $iPLA_2$s 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 $cPLA_2$s and $iPLA_2$s (but not $sPLA_2$s) (Conde-Frieboes et al., 1996), are also found to be cytoprotective (Fig. 2.2*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 (Ono et al., 2002), and a specific inhibitor of endogenous $iPLA_2$, bromoenol lactone (Balsinde and Dennis, 1996), were examined. Pyrrophenone is not protective, whereas bromoenol lactone

shows only partial protection (Fig. 2.2*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 $iPLA_2$.

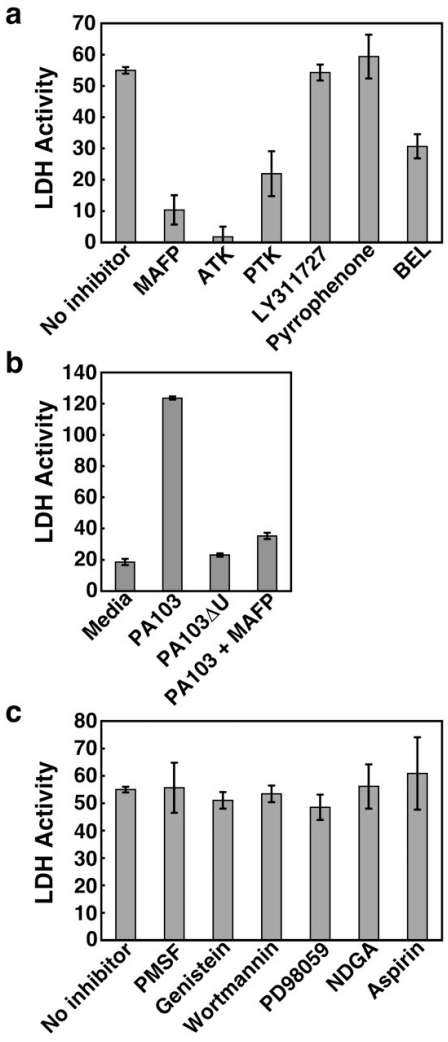

Figure 2.2. Effects of PLA_2 inhibitors (A and B) or non-PLA_2 inhibitors (C) in syringe-loading experiments or infection assays. As shown in **A** and **C**, inhibitors were syringe-loaded with ExoU (0.675 μM) into CHO cells and included in culture medium afterward. Inhibitors were used at the following 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.

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 (Garrity-Ryan et al., 2000) is non-cytotoxic (Fig. 2.2*B*). This is consistent with previous work showing loss of cytotoxicity through deletion of ExoU (Finck-Barbancon et al., 1997). We find that treatment of target cells with MAFP also renders wild-type *P. aeruginosa* PA103 non-cytotoxic (Fig. 2.2*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. 2.2*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 (Evans et al., 1998), we find that the protein tyrosine kinase inhibitor genistein offers no protection against ExoU (Fig. 2.2*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. 2.2*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 (Finck-Barbancon et al., 1997), we have detected homology to the $cPLA_2$ and $iPLA_2$ families using pattern-specific iterative BLAST (Altschul et al., 1997) (Fig. 2.3*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 (Dessen et al., 1999). 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 $cPLA_2$s and $iPLA_2$s (Dessen et al., 1999; Hirschberg et al., 2001; Pickard et al., 1996; Pickard et al., 1999; Tang et al., 1997; Tevzadze et al., 2000; van Tienhoven et al., 2002).

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. 2.3*B*). In comparison, deletion of the N-terminal 45 amino acids of ExoU has no effect on cytotoxicity (Fig. 2.3*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 (Finck-Barbancon et al., 1998) and the C terminus to be required for cytotoxicity (Finck-Barbancon and Frank, 2001). We have further 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. 2.3*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).

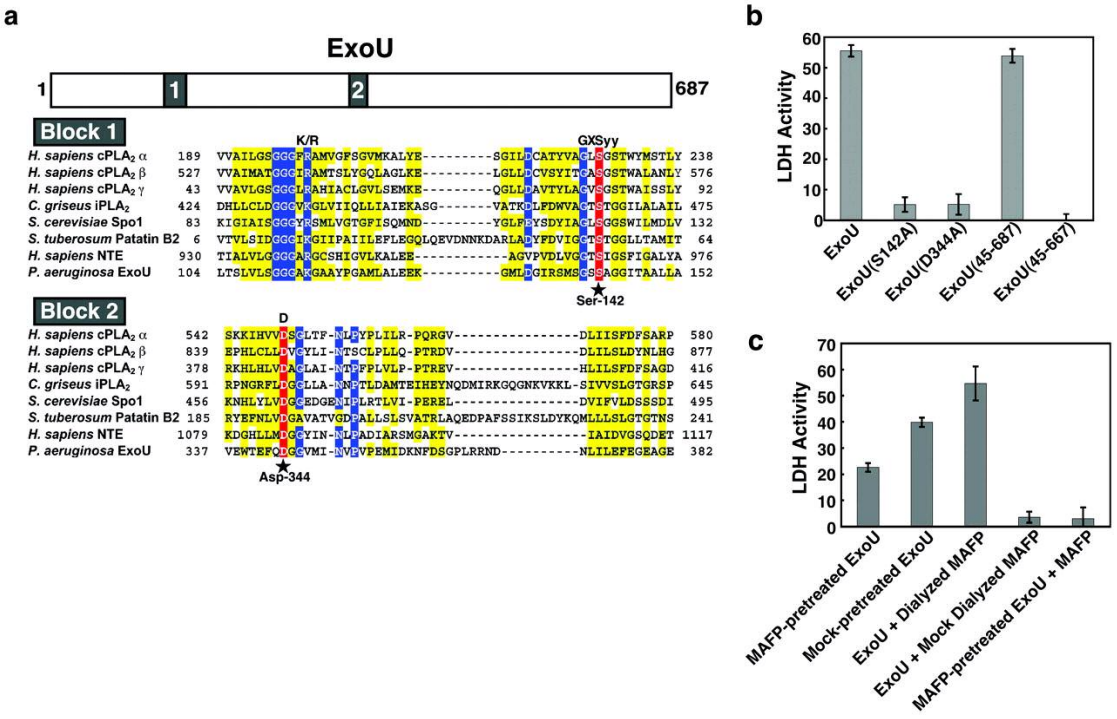

Figure 2.3. Homology of ExoU to phospholipase A_2. As shown in **A,** two blocks of homology are found between ExoU and cPLA$_2$s (human cPLA$_2$α, β, and γ, Spo1) and iPLA$_2$s(*Cricetulus griseus* iPLA$_2$, patatin B2, neuropathy target esterase (*NTE*)). The catalytic Ser (*Block 1*, Ser-142 in ExoU, *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*).

***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 2.1). 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 $cPLA_2\alpha$ 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. 2.3*A*) (van Tienhoven et al., 2002).

Table 2.1. ***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 (Yang et al., 1999).

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. 2.3*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. 2.3*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. 2.3*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. 2.3*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 (Balsinde and Dennis, 1996; Lin et al., 1992; Wang et al., 1992). 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 (Kokotos et al., 2002). 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. 2.4). 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.

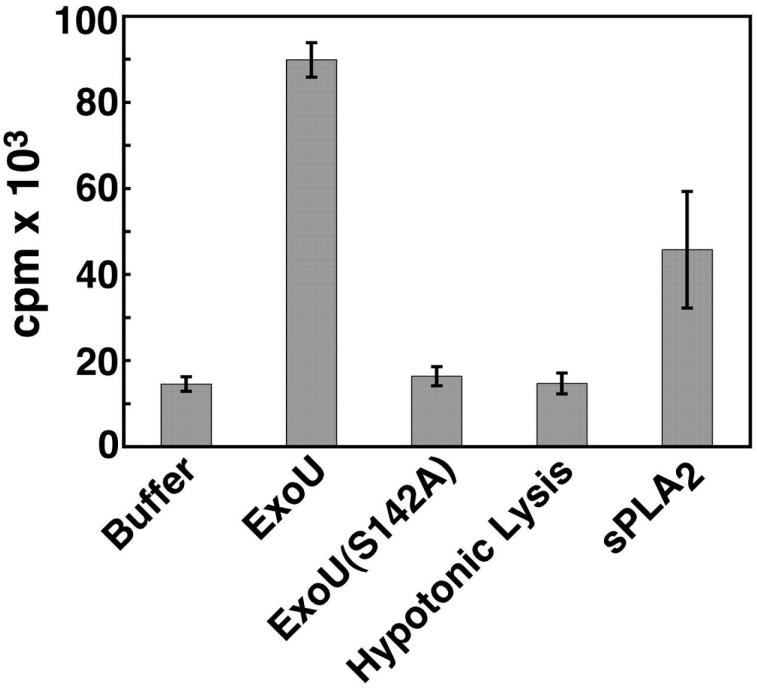

Figure 2.4. ExoU exhibits *in vivo* phospholipase activity. Buffer alone, ExoU (50 μg), ExoU(S142A) (50 μg), or sPLA$_2$ (10 μg) was syringe-loaded into CHO cells metabolically labeled with [^{3}H]arachidonic acid. Medium from cells was collected, extracted by a modified Dole protocol, and quantified by scintillation counting. Hypotonic lysis was carried out by resuspending labeled CHO cells in water. *Error bars* indicate the standard deviation of duplicate experiments.

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. 2.1*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 (Fu et al., 1993; Yahr et al., 1998).

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$_2$s 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 (Girod et al., 2002; Leidich et al., 1998; Zadori et al., 2001). Interestingly, phospholipase A$_2$ activity is implicated in phagosome lysis by *Rickettsia prowazekii* (Ojcius et al., 1995), 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. 2.3*A*, *Block 1*) are also present in the genomes of the bacterial pathogens *Treponema pallidum, Bacillus anthracis,* and *Mycobacterium tuberculosis*, suggesting 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*.

The text of chapter 2, in full, is a reprint of the material entitled "*In Vivo* Phospholipase Activity of the *Pseudomonas aeruginosa* Cytotoxin ExoU and Protection of Mammalian Cells with Phospholipase A_2 Inhibitors" as it appears in the Journal of Biological Chemistry. The dissertation author was the primary researcher and/or author and the co-authors (D. Six, E. Dennis, and P. Ghosh) listed in this publication contributed to or supervised the research which forms the basis for this chapter.

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III.

Genetic approach to identify genes required in ExoU activation

Abstract

The majority of ExoU-expressing clinical strains of *Pseudomonas aeruginosa* are found to be responsible for severe and acute infections in patients, often leading to mortality. Reports from our lab and others demonstrate that ExoU acts as a membrane-lytic phospholipase A_2 and requires one or more host cell factors for activation. It has also been reported that ExoU is cytotoxic to yeast. Here, we sought to identify the cellular processes required for ExoU activation using a forward genetics approach to identify *S. cerevisiae* genes whose loss, attenuation, or over-expression confers resistance to ExoU-mediated cytotoxicity. Analysis of ~5000 viable yeast deletion mutants, ~50,000 germicidal ultraviolet light yeast mutants, and ~17,500 suppressor screen mutants, yielded no yeast mutants able to survive ExoU-induced cytotoxicity. These experiments reveal that the mechanism of ExoU activation is likely conserved from yeast to mammals and may involve a gene essential for viability or having a redundant function.

Introduction

The highly cytotoxic protein ExoU (74 kDa) is a major virulence factor of the opportunistic bacterial pathogen *Pseudomonas aeruginosa*. Reports from our lab and others demonstrate that ExoU acts as a membrane-lytic phospholipase A_2 and requires one or more host cell factors for activation (Phillips et al., 2003; Sato et al., 2003). Two reports also demonstrate that ExoU is cytotoxic when expressed in *Saccharomyces cerevisiae* (Rabin and Hauser, 2003; Sato et al., 2003). In this study, we sought to identify genes involved in ExoU activation and cytotoxicity using a forward genetic approach in *S. cerevisiae*. A variety of screens were carried out to test the effect of loss, attenuation, or over-expression of genes on ExoU-mediated cytotoxicity. A library of deletions in all non-essential *S. cerevisiae* genes was screened for those that confer resistance to ExoU-induced cytotoxicity, and we report that no ExoU-resistant mutants were identified. Furthermore, random mutagenesis and multicopy suppressor screens yielded similar results of no ExoU-resistant yeast strains isolated. Based on our studies, we can conclude that the host-cell activation mechanism of ExoU is probably conserved from yeast to mammals and surmise that a gene essential for viability or having a redundant function may encode the ExoU activator.

Experimental Procedures

Yeast toxicity assays

Saccharomyces cerevisiae strain LPY6491 (Research Genetics Knock-out Consortium Project wild-type strain (Winzeler et al., 1999)), *MAT*α *ADE2 his3Δ1 leu2Δ0 LYS2 met15Δ0 TRP1 ura3Δ0*) or LPY1248 (*MAT*a, *can1-100 his3-11 leu2-3,112 lys*Δ *trp1-1 ura3-1*) was grown in minimal medium (Difco) with 2% glucose, raffinose, or galactose (Fisher) as the carbon source. Standard methods were used to transform yeast with recombinant plasmids (Gietz and Woods, 2002). Yeast were incubated at 30°C for 48 hours. Yeast strains and DNA plasmids pYES2 and pRS316 were generous gifts from the Lorraine Pillus lab (UCSD).

Yeast pathogenic genetic array

In collaboration with Dr. Charles Boone (U. Toronto), ExoU-pRS316 plasmid was transformed into the synthetic genetic array (SGA) yeast strain (S288c, *MAT*α*, can1delta::MFA1pr-HIS3 lyp1*Δ) and used for pathogenic genetic array (PGA) analysis, modeled after the synthetic genetic array (SGA) analysis (Tong et al., 2001). The transformed strain was mated with the *MATa* deletion array yeast, and after 1 day on YEPD media, the mated cells were pinned onto SD –ura +G418 to select for G418 resistant diploids carrying the plasmid. The cells were then sporulated for 5 days on low nutrient sporulation media and then pinned onto SD –his –arg –lys –ura +Canavanine +Thialysine to select for *MATa* deletion yeast containing the ExoU-

pRS316 plasmid. Yeast were pinned onto selection media containing 2% galactose and assessed for survival.

Yeast UV-mutagenesis screen

A *UV*-crosslinker (Hoefer) was used to irradiate yeast with germicidal ultraviolet light (254 nm UV) (Lawrence, 1991; Lawrence, 2002). A mutagen dose giving 50% yeast survival was experimentally determined to be 2000 μJ/cm^2. Approximately 102,000 LPY6491 yeast colony forming units containing the low-copy number plasmid ExoU-pGFP-pRS316 were plated onto uracil-deficient minimal media agar containing 2% glucose. Plates were exposed to UV light with lids off, and then grown in the dark for 2 weeks at room temperature to allow for survival of light- and temperature-sensitive mutants. Surviving mutants were replica plated by means of velvet onto uracil-deficient minimal media agar containing 2% galactose and grown for two more weeks as before. Survivors of ExoU-pGFP expression were streaked onto uracil-deficient minimal media agar containing 2% glucose and 0.1% 5-fluoroorotic acid (5-FOA) to eliminate plasmid. Mutant yeast were transformed with fresh plasmid and the toxicity assay was performed again. Fluorescence microscopy was used to assess ExoU-pGFP expression in yeast mutants.

Yeast multicopy suppressor screen

Yeast (strain LPY6491) containing the low-copy number plasmid ExoU-pGFP-pRS316 were transformed with the *Saccharomyces cerevisiae* AB320 genomic library cloned into the high-copy number 2-μm plasmid YEp13 (containing a *LEU2*

marker) (ATCC) (Ganster et al., 1993). Yeast were plated onto uracil and leucine-deficient minimal media agar containing 2% galactose and incubated 2 weeks at 30°C. Survivors of ExoU-pGFP expression were streaked onto leucine-deficient minimal media agar containing 2% glucose and 0.1% 5-FOA to eliminate ExoU plasmid. Yeast were transformed with fresh plasmid and toxicity assay was performed again.

Yeast dilution assays

Yeast strain LPY6491 was transformed with pYES2 plasmid alone or the *Rickettsia prowazekii* gene *rp534* in pYES2. Yeast cultures were grown for 2 days at 30°C in uracil-deficient minimal medium. In series dilutions (1:5 in water) were made of each culture and replica plated onto uracil-deficient minimal media containing 2% glucose or galactose. Plates were incubated at 30°C for 3 days.

Results

ExoU expression is cytotoxic in *S. cerevisiae*

To determine if ExoU expression is cytotoxic in yeast, ExoU or the non-cytotoxic ExoU(S142A) mutant was expressed in *S. cerevisiae* from the *GAL1* galactose-inducible promoter using the high-copy number (2μ origin) plasmid pYES2. As seen in Figure 3.1, repression of expression with glucose medium allowed growth of yeast. However, yeast failed to grow during ExoU expression with galactose medium. Even basal expression of ExoU with raffinose medium rendered the yeast inviable. In contrast, yeast growth comparable to plasmid control was observed upon mutation in the putative catalytic site of ExoU (S142A) with galactose medium. Similar effects were seen in both *MAT*a (LPY1248, derivative of W303) and *MATα* (LPY6491, derivative of S218c) backgrounds. These results are consistent with reported results (2, 47). These data imply that, in addition to mammalian cells, yeast are also able to produce the activator of ExoU and, as a result, are susceptible to ExoU toxicity.

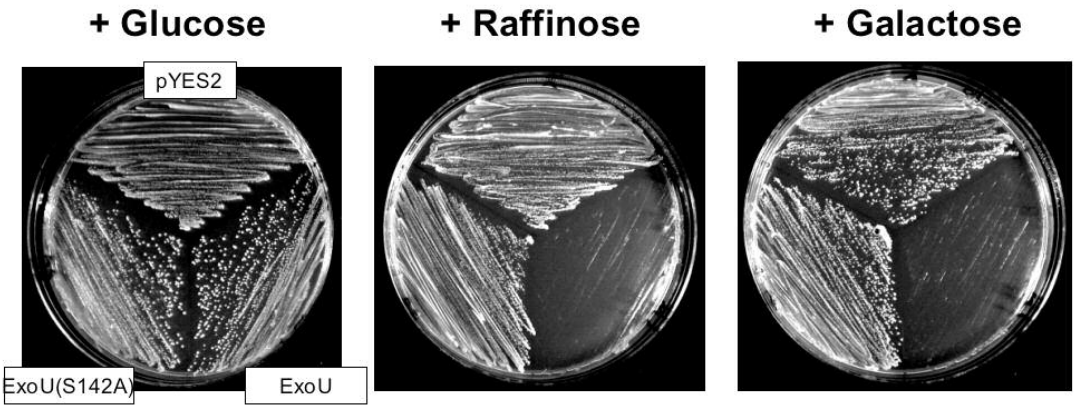

Figure 3.1. ExoU expression is cytotoxic to yeast. Expression of ExoU from the *GAL1* promoter (using pYES2) was derepressed with raffinose or induced with galactose, and resulted in cytotoxicity in both cases. In contrast, expression of the non-cytotoxic ExoU S142A point mutant did not produce cytotoxicity, and neither did the vector (pYES2) alone.

A screen of non-essential genes does not identify ones conferring resistance to ExoU-induced cytotoxicity

To determine which genes in yeast contribute to ExoU-induced cytotoxicity, a library of deletions in all non-essential *S. cerevisiae* genes was screened for those that confer resistance to ExoU-induced cytotoxicity using a synthetic genetic array (SGA) (Tong et al., 2001). Of the ~6200 predicted *S. cerevisiae* genes, greater than 80% (~5000) are non-essential. These ~5000 non-essential genes have been deleted individually and replaced with a marker gene and a specific identifier sequence ('barcode'), and this collection of strains has been placed in an ordered array. The SGA is manipulated by robotic means in the Boone laboratory.

The high-copy number ExoU-expressing plasmid (ExoU-pYES2) was transformed into a query yeast strain (S288c *MAT*α), which was then crossed against the ~5000 strains in the SGA. The resulting diploid strains were sporulated and selected for the presence of both the ExoU-expressing plasmid and the marked deletion; this was carried out without inducing ExoU expression. Unfortunately, no yeast survival was observed following the sporulation step with repression of ExoU expression on glucose-medium. This result was reproduced a second time, suggesting a problem with the high-copy number plasmid used in these experiments. To address this problem, ExoU was instead cloned into the low-copy number (CEN6) plasmid pRS316 (ATCC), and ExoU-pRS316 was confirmed to cause cytotoxicity (data not shown). Colonies were then pinned from non-inducing to inducing plates (i.e., galactose), and growth of these colonies was scored. The screen was carried out in duplicate, and four mutants (IZH3, PRC1, APP2, and ACE2) in which both duplicates supported growth were found (Fig. 3.2). For other mutants, only one of the duplicates supported growth, indicating that these mutants were likely to be false positives.

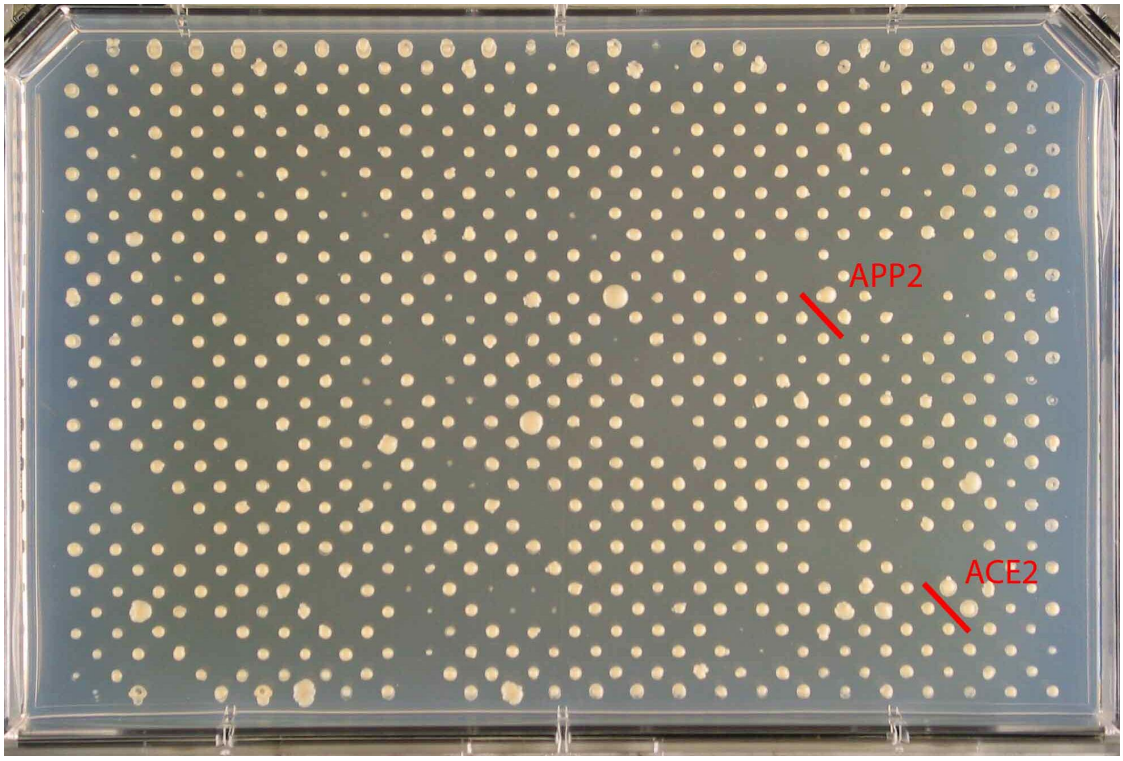

Figure 3.2. Plate from SGA screen for ExoU-resistance. Large colonies (e.g., duplicates labeled APP2 and ACE2) indicate mutants that support growth under ExoU-inducing conditions (i.e., galactose). Small colonies are indicative of residual cells from colonies picked robotically from non-inducing plates and pinned to the inducing plate. Also visible are large colonies that occur as singlets rather than duplicates, and are likely to be false positives.

The experiment was repeated a second time, and seven mutants (SPO16, SRY1, CPR6, EDC1, YER119C-A, YGR107W, and YLR311C) were identified in the second round by the ability of both duplicates to support growth under ExoU-inducing conditions. Unfortunately, none of the four strains identified from the first screen matched the seven identified in the second screen. Nevertheless, these total of eleven deletion-mutant strains, along with two others that appeared as singlets in both screens (i.e., only one of the duplicates were found to grow: ACB1 and HIR2), were tested for ExoU-resistance by transforming with the low-copy number ExoU plasmid (ExoU-pRS316). However, none were found to be resistant, indicating that these identified deletion mutants are false positives for ExoU resistance.

No ExoU-resistant yeast was isolated though random UV mutagenesis

Due to negative results from the SGA screen, we turned to a random mutagenesis approach. The wild-type yeast strain LPY6491 (*MATα*, derivative of S218c) transformed with ExoU-pGFP-pRS316 (green fluorescent protein fusion for expression detection, confirmed to cause cytotoxicity, data not shown) were exposed to UV radiation sufficient to result in 50% cell death (as determined experimentally) (Lawrence, 1991; Lawrence, 2002). The results are summarized in Table 3.1. Mutagenized yeast (approximately 50,000 colonies) were then induced for expression of ExoU-pGFP (using galactose medium), and ~1% of yeast colonies (456 strains) were found to survive.

Of course, many of these survivors represent strains in which the ExoU-pGFP-pRS316 plasmid is damaged and therefore lack ExoU expression. To sort this out, the ExoU-pGFP-pRS316 plasmid was cured from these strains by counterselection using 5-fluoroorotic acid (5-FOA) (since pRS316 carries the *URA3* marker). The ExoU-pGFP-pRS316 plasmid was then retransformed into these 456 strains and assessed for survival during ExoU expression on galactose medium. While 32 strains appeared to survive ExoU induction, the plasmid cured yeast were retransformed again and tested for survival. This time, none of the yeast were able to survive, suggesting that mutation had occurred only in the plasmid.

Table 3.1. Summary of results from random yeast *UV*-mutagenesis screen.

Total number of cells irradiated	~100,000
Cells surviving UV irradiation	~50,000
Cells surviving ExoU induction	456
Cells surviving ExoU induction with re-transformed pYES2-ExoU	32
Cells surviving ExoU induction with re-transformed pYES2-ExoU: Second round (in duplicate)	0

Over-expression suppressor screen

To test the possibility that over-expression of a gene product might overcome ExoU-mediated cytotoxicity in yeast, a multi-copy suppressor screen was carried out.

We used a suppressor library that consists of 5-20 kb genomic fragments from *S. cerevisiae* AB320 cloned into the high copy number (2 μm) vector YEp13 (Ganster et al., 1993). Approximately 10^4 clones are needed for full coverage of the genome. Yeast containing the low-copy number plasmid ExoU-pGFP-pRS316 were transformed with the suppressor library and were plated on galactose medium to induce over-expression of both ExoU and the library fragment products. Of ~17,500 colonies screened, 180 colonies survived and were observed to grow poorly. To eliminate false positives, colonies were grown on leucine-deficient media containing 5-FOA, to retain the library YEp13 plasmid with the LEU2 marker while inducing loss of the ExoU plasmid with the URA3 marker, respectively. Of the 180 initial survivors, none were able to survive when ExoU-pGFP expression was induced from re-introduced plasmid, suggesting that they were false positives.

Effect of ExoU homologues on yeast survival

To test whether the closest homologue of ExoU, the *rp534* gene product from *Rickettsia prowazekii*, is also toxic to yeast, the gene was cloned into the high-copy number plasmid pYES2 and transformed into yeast. Although yeast were able to grow during expression of the *rp534* gene product with galactose medium, the colonies appeared to have a sick or slow-growth phenotype. To confirm this, dilution assays were performed to compare growth of yeast with plasmid only and plasmid containing the *rp534* gene. As seen in Figure 3.3, yeast expressing the *rp534* gene product have a sick or slow-growth phenotype compared to the plasmid control. These data suggest

that the *rp534* gene product from *Rickettsia prowazekii* may have a similar effect as ExoU, albeit to a lesser extent.

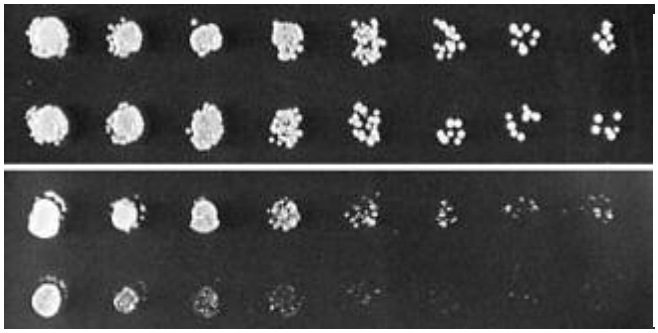

Figure 3.3. ExoU homolog causes a sick or slow-growth phenotype in yeast. In series dilutions of yeast transformed with vector alone (pYES2) or *Rickettsia prowazekii* rp534 gene product in pYES2. Yeast growth was compared on glucose medium (noninducing) and galactose medium (inducing).

Discussion

Our research demonstrates that ExoU expression is cytotoxic to yeast, consistent with reports published at the time of these experiments (Rabin and Hauser, 2003; Sato et al., 2003). This result not only implies that yeast produce the molecule that activates ExoU phospholipase A_2 activity, but also opens the door to genetic means for studying ExoU activation and cytotoxicity. Here, we used a forward genetics approach to identify *S. cerevisiae* genes whose loss, attenuation, or over-expression confers resistance to ExoU-mediated cytotoxicity. From this, we hoped to gain insight into the cellular processes required for ExoU activation.

To begin, an SGA screen was used to test a library of deletions in all non-essential *S. cerevisiae* genes for those that confer resistance to ExoU-induced cytotoxicity. Of the ~5000 screened *S. cerevisiae* mutants, none were able to survive ExoU expression. The results of this screen suggest one of two possibilities. First, these results suggest that the activator of ExoU may involve an essential gene product that, if deleted, would make the yeast inviable. The other possibility is that the activator of ExoU could be a non-essential gene product with a homologue or similar gene product with a redundant function that could compensate for the deletion and still activate ExoU.

Due to negative results from the SGA screen, we turned to a random mutagenesis approach. Because the ExoU activator may be an essential gene product,

we sought to isolate viable yeast with mutations in essential genes as well as non-essential genes. In this manner, we hoped it might be possible to identify ExoU-resistant conditional mutant yeast with a mutation in an essential gene that would not render the yeast inviable, yet would also not be able to activate ExoU.

To carry out a random mutagenesis screen, we chose germicidal ultraviolet light (254 nm UV), an efficient mutagen that has the advantage of producing a large range of substitutions and frameshift mutations (Kohalmi and Kunz, 1988; Kunz et al., 1987). We decided on a mutagen dose that yielded 50% yeast cell survival, which generally yields the highest proportion of mutants per treated cell (Lawrence, 1991; Lawrence, 2002). Growth of yeast was performed at room temperature in a light-free environment, so as to allow for growth of light- and temperature-sensitive yeast mutants. Finally, for the best chance at recovering an ExoU-resistant yeast mutant, a low-copy plasmid was used to express ExoU-pGFP (green fluorescent protein, for detection of expression). Nevertheless, of the 50,000 yeast mutants screened, none were ultimately able to survive ExoU expression.

Next, we addressed the possibility that over-expression of a gene product might be able to overcome ExoU-mediated cytotoxicity in yeast. For example, this gene product may sequester or degrade the ExoU activating molecule. To explore these and other possibilities, a multicopy suppressor screen was carried out. Over ten-fold the number of yeast colonies required to cover the library was screened for survival of ExoU-pGFP expression. Despite 180 initial survivors, all were false

positives, as seen in preceding genetic studies.

Finally, we have investigated the effect of yeast expression of an ExoU-related protein RP534 from *Rickettsia prowazekii*, an obligate intracellular bacterial pathogen and the cause of epidemic typhus. RP534 (68 kDa) is identified by a BLAST search to be related to ExoU (expectation value 4 x 10^{-7}, ~20% identity) (Andersson et al., 1998) and contains blocks of phospholipase A_2 sequence homology, including the putative catalytic site Ser and Asp. While the functional role of RP534 is unclear, it is intriguing to note that a phospholipase A_2 activity is implicated in escape of *R. prowazekii* from phagosomes (Ojcius et al., 1995).

We asked whether RP534 has the same effect as ExoU when expressed in yeast. Although not cytotoxic, RP534 expression in yeast was found to confer a sick or slow-growth phenotype. If RP534 is the phospholipase A_2 enzyme implicated in phagosomal escape, then this non-cytotoxic phenotype is understandable, as a cytotoxic phospholipase would defeat the bacterium's goal of intracellular survival by destroying the cell in which it is trying to survive. Nevertheless, our observation of a clear sick or slow-growth phenotype in yeast suggests that RP534 may be mechanistically related to ExoU.

Overall, while our genetic screens did not reveal specific cellular processes that might be involved in ExoU activation, they reveal that the mechanism of ExoU

activation is likely conserved from yeast to mammals and may involve a gene essential for viability or having a redundant function. In Chapters 4 and 5, these implications were kept in mind during the screening of ExoU activator candidates identified by biochemical means.

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IV.

Biochemical approach to characterize the ExoU activator

Abstract

The *Pseudomonas aeruginosa* cytotoxin ExoU acts a membrane-lytic and host-cell activated phospholipase A_2. *Saccharomyces cerevisiae* susceptibility to ExoU cytotoxicity suggests that yeast contain one or more factors involved in ExoU activation. Here, we sought to isolate and characterize the activator of ExoU by using biochemical means to fractionate it from a yeast cell lysate. Our results and those of others show that ExoU has *in vitro* phospholipase activity only when supplemented with a yeast cell lysate. We report biochemical fractionation of a yeast lysate and find surprisingly that the ExoU activator is likely an anionic and glucose-containing saccharide. This molecule appears to be conserved from yeast to mammals, but importantly, to be absent in bacteria. Our results also suggest that another molecule might play a role in accelerating ExoU activation. Furthermore, we describe a similar mechanism of activation for the ExoU-related protein RP534 from *Rickettsia prowazekii*, suggesting that phospholipases in other bacterial pathogens may sharea similar mechanism of activity.

Introduction

Reports from our lab and others demonstrate that the highly cytotoxic protein ExoU from *Pseudomonas aeruginosa* acts as a membrane-lytic phospholipase A_2 and requires one or more host cell factors for activation (Phillips et al., 2003; Sato et al., 2003). Because our genetic screens in *Saccharomyces cerevisiae* did not identify genes required for the activation of ExoU, we turned to a biochemical approach. In this study, we sought to isolate and characterize the molecule or molecules in a yeast lysate that are responsible for ExoU activation. Our results are consistent with those of others and show that ExoU has *in vitro* phospholipase activity only when supplemented with a yeast cell lysate (Sato et al., 2003). We used classical biochemical fractionation techniques based on size, polarity, ionic state, hydrophobicity, and heat-sensitivity to purify the ExoU activator, with *in vitro* phospholipase assays being used to follow the activator. Surprisingly, we find that the ExoU activator is likely a eukaryotic-specific, anionic, and glucose-containing saccharide. Furthermore, we provide evidence that another molecule may play a role in accelerating ExoU activation, although this saccharide activator is sufficient for ExoU activation. Last, we provide evidence that the ExoU-related protein RP534 from the bacterial pathogen *Rickettsia prowazekii* may use a similar mechanism of activation.

Experimental Procedures

In vitro phospholipase A_2 activity assays

Highly sensitive *in vitro* radioactive phospholipase assays were performed in reaction buffer composed of 20 mM MOPS, pH 6.5, 50 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Mixed micelles were composed of 1 mM radiolabeled POPC (L-α-1-palmitoyl-2-([1-^{14}C]oleoyl) phosphatidylcholine, 100,000 cpm) and 3 mM Triton X-100 in a final volume of 500 µl. The mixed micelle substrate was prepared as described previously (Phillips et al., 2003). Phospholipids were dried down under a liquid nitrogen stream and resuspended by vortexing in 392 µl reaction buffer to form a cloudy white solution of multilamellar vesicles. After resuspension of the phospholipids, Triton X-100 was added (8 µl of 150 mM per assay), resulting in a rapid clearing of the cloudy white vesicles as the clear mixed micelles form. After micelle formation, the mixed micelles (400 µl/assay) were added to siliconized glass test tubes. The reaction was initiated by the addition of one hundred micrograms of purified ExoU (~3 µM) and varying quantities of fractionated yeast extract in 0.1 mL total. The final reaction (500 µl) was incubated at 37°C for 16 hours, then quenched, and the fatty acids were extracted using a modified Dole protocol (Kokotos et al., 2002). Briefly, 1.5 mL Dole reagent (isopropyl alocohol:heptane:water:concentrated sulfuric acid in a ratio of 3000:750:146:4.2) was added to the reaction, followed by 1.5 mL each of water and heptane. The organic and aqueous layers were mixed by vortexing, and then allowed to separate. Free fatty acids (arising from phospholipase

activity) partition into the heptane layer at top. Half of the heptane layer (1 mL) was flowed over silica gel to separate any contaminating unreacted phospholipids and silica gel was washed with 1 mL ether. The heptane-ether phase was then added to scintillation fluid and scintillation counts were quantified. As a positive control, snake venom $sPLA_2$ (Sigma, 1 μg) was used. As negative controls, buffer only, ExoU only, or yeast lysate only were used. ExoU was purified as described previously (Phillips et al., 2003).

Yeast cell lysate and yeast extract preparation

Yeast cell lysate was prepared as described previously (Sato et al., 2003). Briefly, the yeast cell lysate is composed from the soluble fraction of *Saccharomyces cerevisiae* (strain LPY6491). For one phospholipase reaction, the washed yeast pellet from ~16 mL culture grown to density of A_{600} ~2 in YPD media was lysed with glass beads and vortexing in 150 μl phosphate buffer (50 mM sodium phosphate buffer, pH 8, 1 mM EDTA, 5% glycerol, and protease inhibitors [1 mM PMSF, 10 μg/ml E-64, 4 μg/ml pepstatin A, 40 μg/ml aprotinin, 40 μg/ml leupeptin, 40 μg/ml benzamidine hydrochloride, and 40 μg/ml phosphoramidon (all Sigma)]. Insoluble material and unlysed cells were removed by centrifugation (16,100 x g, 30 min, 4°C), and the soluble supernatant was retained.

Commercial powdered yeast extract (Fisher or VWR) was prepared by vortexing 125 mg powder into 1 mL water. For other chemicals (vitamins, minimal medias, salts, divalent metals, inositol, phosphatidylinositol, bile salts, gangliosides,

sulfatides, methylglucoside, glucose, maltose, isomaltose, heparin, glycogen, UDP-glucose, beta-cyclodextrin, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose), 10 mg was dissolved into 100 µl water and used for activity assays.

Boiling, 3 kDa-filtration, and chloroform extraction

Yeast cell lysate was boiled for 10 minutes at 100°C, insoluble material was removed by centrifugation (16,100 x g, 30 min, 4°C) and the supernatant was used for activity assays and further experiments. Boiled yeast lysate was passed through a 3 kDa-cutoff membrane (Microcon YM-3, Millipore), and both the retentate and filtrate were tested in activity assays. The 3 kDa-filtrate of boiled lysate was evaluated on a 500 dalton-cutoff filter (Amicon YC05 membrane, Millipore), and retentate and filtrate were tested in activity assays. Chloroform extraction was performed by adding an equal volume of chloroform to the 3 kDa-filtrate of boiled lysate, vortexing for 2 minutes, and allowing the phases to separate. The phases were separated into two tubes, and both were speed-vac evaporated to dryness. Pellets from each phase were resuspended in lysis buffer and tested in activity assays.

RNase, DNase, protease, and reducing agent treatment

Yeast extract in phospholipase reaction buffer was incubated with 100 µg/ml DNase (Sigma) supplemented with 1 mM $MgCl_2$, 100 µg/ml RNase (Sigma), or 2 mM reducing agent dithiothreitol, at room temperature for 1 hour with gentle agitation, and samples were tested in activity assays. For protease treatment, yeast extract was

incubated overnight at 37°C with a protease mixture containing chymotrypsin (Boehringer Mannheim), papain (Boehringer Mannheim), trypsin (Sigma), and proteinase K (Gibco) at 100 μg/ml, 80 μg/ml, 40 μg/ml, and 500 ug/ml, respectively. To quench protease activity, the sample was boiled 10 min at 100°C, and insoluble material was removed by centrifugation (16,100 x g, 30 min, 4°C). The supernatant was then passed through a 3 kDa-filter and the filtrate was incubated with protease inhibitors (see above) for 15 minutes. The sample was then tested in an activity assay. Controls included a protease-untreated lysate and a sample treated with proteases but supplemented with untreated lysate. SDS-PAGE was used to confirm efficiency of boiling and 3 kDa-filtration.

C-1 and C-18 reverse phase chromatography

A 3 kDa-filtered and chloroform-extracted yeast extract was applied to a C-18 (Vydac, 4.6 mm x 250 mm) or C-1 (Allsphere Alltech, 4.6mm x 250 mm) column. A gradient of 0-100% acetonitrile (starting from 0.1 % trifluoroacetic acid in water) over 20 column volumes at a flow rate of 1 ml/min was used to elute samples from the columns. Chromatography runs were monitored by measuring absorbance 205 nm and 250 nm. Fractions were collected, dried to completeness by speed-vac, and resuspended in water for activity assays.

Anion exchange and cation exchange chromatography

A 3 kDa-filtered and chloroform-extracted yeast extract was applied to a cation (Poros HS/M, 10 mm x 100 mm) or anion exchange (Poros HQ/M, 10 mm x 100 mm)

column in water at a flow rate of 15 ml/min. A 20-1000 mM NaCl gradient followed by a step to 3 M NaCl over 20 column volumes was used for sample elution. Fractions were concentrated on a 500 dalton-cutoff membrane (YC05, Millipore), and used in activity assays. Following anion exchange, activity assays were supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$.

Size-exclusion chromatography

A 3 kDa-filtered and chloroform-extracted yeast extract was applied to a size-exclusion column (Superdex Peptide, Pharmacia, 10 mm x 300 mm) and run in water at a flow rate of 0.5 ml/min. Fractions were speed-vac dried from 1 mL to 100 µl and used for activity assays. Size exclusion column standards were prepared by dissolving 100 µg myoglobin, 20 µg vitamin B12, 20 µg chloroquine, 20 µg riboflavin, and 20 µg tryptophan in 100 µl water. Standards were used to calibrate molecular mass with elution times.

Electrospray ionization (ESI) mass spectrometric analysis

Yeast extract (12.5 g) was chloroform-extracted, and purified by anion-exchange, C-1 reversed-phase and size-exclusion chromatographies. ESI mass spectrometric analysis (both positive and negative mode) was carried out on the purified sample by Dr. Su at the Department of Chemistry Mass Spectrometry Facility.

Time-course, pre-incubation, and dose-dependence activity assays

Time-course assays were carried out by incubating mixed micelles with 12.5 mg yeast extract and 100 μg ExoU over a 25 hour period at 37°C. At designated time points, the Dole assay was used to quench the reactions and determine phospholipase activity.

Pre-incubation assays were carried out by incubating only two of the three following components overnight at 37°C: 12.5 mg yeast extract in 100 μl water, 100 μg ExoU, or 400 μl mixed micelles. The third component was added to each of the samples and incubated for 30 minutes more at 37°C. The modified Dole assay was used to terminate the reactions and determine phospholipase activity.

Dose-dependence assays were carried out by pre-incubating varying amounts of yeast extract (from 0 to 5 mg yeast extract in 100 μl water) with 100 μg ExoU overnight at 37°C. Mixed micelles (400 μl) were added to the ExoU/ yeast extract samples and incubated for 30 minutes at 37°C, after which the modified Dole assay was used to terminate the reactions and determine phospholipase activity.

Pull-down of yeast extract with His-ExoU

His-tagged ExoU (100 μg) was incubated with 12.5 mg yeast extract in 100 μl binding buffer (50 mM phosphate buffer and 300 mM NaCl) overnight at 37°C. Ni-NTA beads (Sigma) pre-equilibrated with binding buffer were incubated with His-ExoU/ yeast lysate sample for 2 hours at room temperature with gentle agitation.

Beads were washed with binding buffer, and His-ExoU was eluted with elution buffer (50 mM phosphate buffer, 300 mM NaCl, and 300 mM imidazole) and used in an activity assay. An identical sample was mixed with fresh yeast extract and also tested in an activity assay.

Determination of specific activity

Specific activity was used to determine sample purity during each fractionation step. Specific activity was determined by relating the measured activity for the ExoU activator at a certain time-point to the quantity of fractionated yeast extract (i.e., reaction rate per milligram of fractionated sample). Activity assay measurements were determined by pre-incubating ExoU with activator sample overnight at 37°C, followed by incubation for 30 minutes with mixed micelles at 37°C. This 30 minute time-point was chosen because at this point, the reaction satisfies linear, initial rate conditions (see Fig. 4.5B). The quantity of fractionated yeast extract was determined by drying down the fraction and determining the resulting dry weight.

Nuclear magnetic resonance (NMR) of ExoU activator

Approximately 5 mg ExoU activator was purified from 25 g yeast extract. NMR (500 Mhz Varian Inova, in D_2O) was used to measure proton shifts of ExoU activator. Two-dimensional NMR was used to measure proton-proton shift coupling and carbon-proton shift coupling. The purified sample was analyzed by C-18 liquid chromatography-mass spectrometric (LC-MS) analysis and was found in the flow-through of the C-18 column.

Monosaccharide analysis

Consecutive purification steps of 3 kDa-filtration, tandem C-1 chromatography steps, and size-exclusion chromatography were used to purify approximately 1 mg ExoU activator from 25 g yeast extract. Monosaccharide composition analysis was performed at the UCSD Glycobiology Core by treating the sample with 2 M TFA at 100°C for 4 h to cleave all glycosidic linkages. After drying the hydrolyzate, sample was dissolved in water and analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA-1 column (Dionex) eluted with a low concentration of sodium hydroxide. Common monosaccharide standards found in vertebrate oligosaccharides (Man, Gal, Glc, GlcNAc, GalNAc, Fuc and Xyl) were treated in parallel and used for calibration of HPAEC-PAD response. The printout of the results shows the profile and individual monosaccharide content expressed in nanomoles present in the volume injected. The aforementioned monosaccharides were identified by elution position. Blanks (that represent background from the methodology used for sample preparation) were used for quantitative results.

Concavalin A-Sepharose 4B pull-down

Cancanavalin A (Con A) Sepharose 4B beads (Amersham Biosciences) were washed with binding buffer (20 mM Tris pH 7, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 300 mM NaCl) and incubated with 3 kDa-filtered glycogen in binding buffer for 1 hour at room temperature with gentle agitation. Beads were washed three times with binding buffer. Any bound sugars were eluted with binding buffer containing 1 M α-

D-methylglucoside (Sigma). All fractions were used in activity assays. Pre-column sample was used as a positive control, and binding and elution buffers were used as negative controls.

Glycosidase digestions

A 3 kDa-filtered glycogen sample was prepared by dissolving 125 mg glycogen (Oyster, Type II, Sigma) into 1 mL water, passing through a 3 kDa membrane, and using the filtrate for further analysis. Glycosidases (β-glucosidase, α-glucosidase, α-amylase, chitinase, dextranase [all from Sigma except β-glucosidase, which was from VWR]) were diluted in water to 10 mg/ml. The 3 kDa-filtered glycogen sample was incubated with 500 μg glucosidase overnight at 37°C. To eliminate phospholipase contamination of glycosidases, digests were passed through 3 kDa-filters (Microcon YM-3, Millipore). The filtrate was then used in activity assays. Controls included glycogen alone or all glycosidases alone.

NMR and Saccharide linkage analysis of activator purified from glycogen

A 3 kDa-filtered glycogen sample was purified by C-1 and size-exclusion chromatographies. Approximately 1 mg purified activator was obtained from 500 mg glycogen. The purified sample was subjected to NMR analysis as above. For monosaccharide linkage analysis at the UCSD Glycobiology Core, purified ExoU activator was methylated according to the Ciucanu-Kerek method (Ciucanu and Kerek, 1984). Purification methods included extraction, Sep-Pak cartridge, dialysis and chromatography over LH-20. Permethylated products were cleaved by acid

hydrolysis. Partially methylated monosaccharides were reduced, and the alcohols were derivatized to obtain partially methylated alditol acetates. Analysis of these products was done by glycosyl-linkage composition electrospray ionization mass spectrometry (GLC EIMS), and identification achieved by using a combination of retention times (as compared to those of known standards analyzed under the same conditions) and EIMS patterns.

Normal-phase chromatography

One mL 3 kDa-filtered glycogen was speed-vac dried to 30 µl and applied to a Carbohydrate ES column (Alltech, 4.6 mm x 250 mm). A gradient of 10-100% water (starting from 90% acetonitrile) over 20 column volumes at a flow rate of 0.5 ml/min was used to elute sample from the column. Chromatography runs were monitored by measuring absorbance at 205 nm and 250 nm. Fractions were collected, dried to completeness by speed-vac, and resuspended in water for activity assays.

RP534 expression/purification/activity assays

RP534 from *Rickettsia prowazekii* cloned into an expression vector (pET15b, Novagen) was a gift of Dr. Herbert Winkler (University of South Alabama). DNA sequencing verified the integrity of the construct. His-RP534 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 (βME), 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml DNase. His-RP534, present in the

supernatant from the centrifuged lysate, was purified by metal chelation chromatography (Poros MC/M), followed by size exclusion chromatography (Superdex 200, Pharmacia). His-RP534 was concentrated on a 10 kDa-cutoff filter (Amicon YM-10, Millipore) to ~2 mg/ml, dialyzed into buffer (10 mM Tris·Cl, pH 8.0, 500 mM NaCl, 10 mM βME), and used immediately in activity assays. His-RP534 was found to precipitate after storage at -80°C and -20°C, and to degrade and precipitate over time when stored at 4°C.

Results

A yeast cell extract activates ExoU as a phospholipase

In vitro assays reveal that purified ExoU has no detectable phospholipase, lysophospholipase, or esterase activity under a variety of conditions and on a variety of substrates (Phillips et al., 2003; Sato et al., 2003; Tamura et al., 2004). However, addition of the soluble fraction of a yeast cell lysate to ExoU results in marked phospholipase A_2 activity (Fig. 4.1), as has been reported in the literature (Sato et al., 2003). The phospholipase activity is due entirely to ExoU, as the yeast cell lysate by itself has no phospholipase activity (Fig. 4.1). At this point, it remains formally possible that this activity is an indirect consequence of ExoU.

The data shown in Figure 4.1 is from using mixed micelles containing ^{14}C-labeled phospholipids and Triton X-100, although ExoU-dependent activity has also been shown using liposomes (specifically, small unilamellar vesicles, data not shown). While this experiment shows ExoU-dependent activity on 1-palmitoyl-2-oleoyl-3-phosphatidylcholine (POPC, with the label on the *sn-2* oleoyl chain), other phospholipids have been found to be substrates. These include 1-palmitoyl-2-arachidonoyl-3-phosphatidylcholine (PAPC) and 1-palmitoyl-2-palmitoyl-3-phosphatidylcholine (PPPC) (data not shown). Other substrates that have been shown in other studies include 1-palmitoyl-2-linoleoyl-3-phosphatidylethanolamine (PLPE) and lyso-palmitoyl-1-phosphatidylcholine (lysoPC) (Sato et al., 2005). These results demonstrate that ExoU is likely a host-cell activated, non-specific phospholipase and

does not have a unique mammalian substrate. Figure 4.1 also shows ExoU to be highly efficient in its activity, as complete hydrolysis of substrate is equivalent to release of 50,000 cpm.

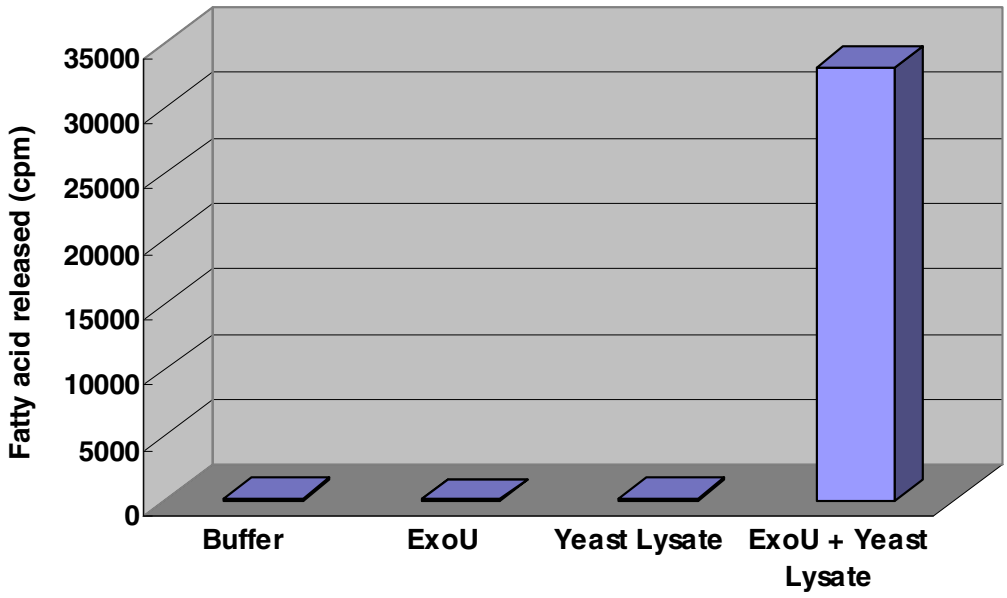


Figure 4.1. I*n vitro* phospholipase A_2 activity of ExoU. Buffer alone, ExoU (~3 μM), yeast cell lysate, or ExoU (~3 μM) supplemented with yeast cell lysate was incubated at 37°C for 16 hours with mixed micelle substrate, which was composed of Triton X-100 and L-α-1-palmitoyl-2-([1-^{14}C]oleoyl) phosphatidylcholine. Release of ^{14}C-oleic acid was extracted using a Dole assay and measured by scintillation counting.

Remarkably, commercial powdered yeast extract (Fisher, or other sources) was found to activate ExoU to the same extent as the yeast cell lysate (data not shown). In contrast, synthetic media (either for bacteria or yeast) was found not to activate ExoU, ruling out various monosaccharides, amino acids, inositols, vitamins, and salts as candidates for the ExoU activator. We also found another eukaryotic powdered extract, bovine brain-heart infusion, was found to activate ExoU to the same extent as yeast extract (data not shown), although we continued experiments with yeast extract, reasoning that it is probably a less complex starting mixture.

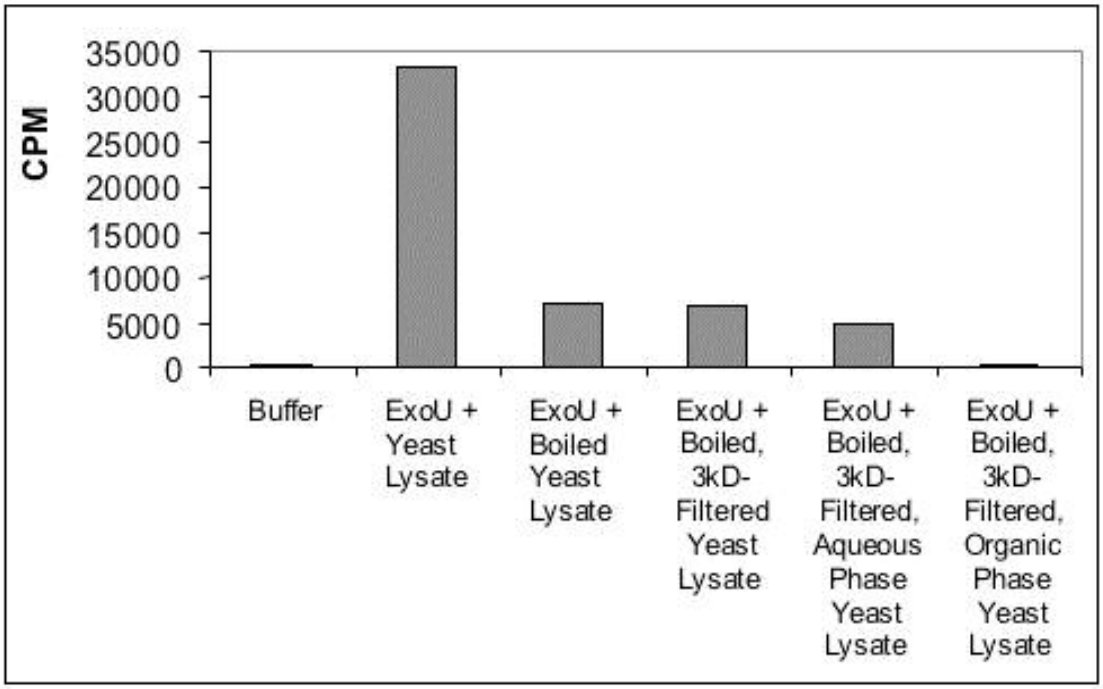
As another control for specificity, lysates from *E. coli* prepared similarly to those prepared from yeast were found not to activate ExoU (data not shown). Notably, lack of activity was only found when *E. coli* were grown in minimal media. However, when *E. coli* were grown LB (which contains powdered yeast extract), lysates were able to activate ExoU to the same extent as yeast, suggesting that the activator in powdered yeast extract may become bound to the *E. coli* cells.

The ExoU activator is heat-stable, low molecular weight, and hydrophilic.

We sought to provide initial characterization of the ExoU activator. To carry this out, a yeast cell lysate was boiled for 10 minutes at 100°C, and insoluble material was removed by centrifugation. Interestingly, the supernatant of the boiled extract was found to contain the ExoU activator (Fig. 4.2A). It must be noted that the boiled lysate activates ExoU to a lesser extent than does an unboiled lysate, but the level of

ExoU activation brought about by the boiled lysate is nevertheless substantial. At present, it is unknown why less activity is found in the boiled lysate, with possibilities being that some loss of activity occurs due to heating or that a separate heat-labile accessory factor is involved. The second possibility is consistent with the observation that boiling does not destroy any of the activity in powdered yeast extract.

After boiling the yeast lysate, the supernatant was passed through a 3 kDa-cutoff membrane, and the ExoU activator was found entirely in the filtrate (i.e., portion passing through the membrane) (Fig. 4.2A). This is consistent with the activator having a molecular mass less than 3 kDa. Furthermore, the ExoU activator was found in the retentate, not the filtrate, of a 500 dalton-cutoff filter, further setting a limit on its size and ruling out very small molecules such as amino acids and salts (data not shown). The 3 kDa-filtrate of boiled lysate was then subjected to chloroform extraction to assess the hydrophilicity of the ExoU activator. The ExoU activator was found to be retained entirely in the aqueous rather than the organic phase, indicating that the ExoU activator is hydrophilic.



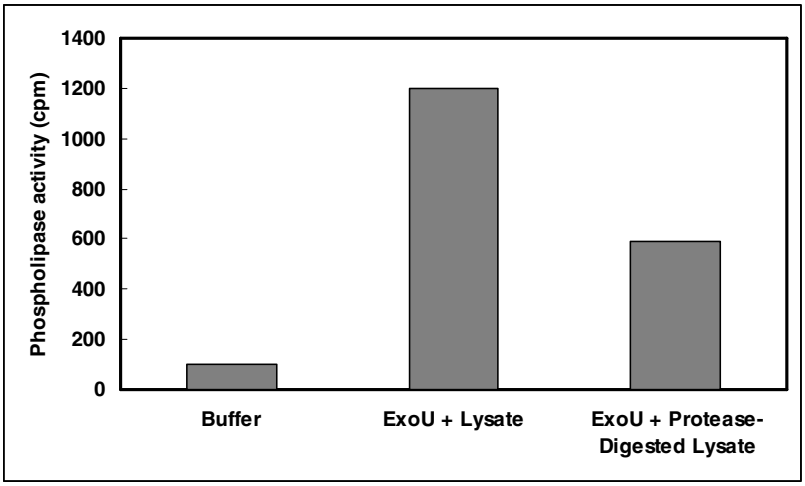


Figure 4.2. Temperature-resistance, size, and hydrophilicity of ExoU activator and effect of proteases on ExoU activator. A, Phospholipase assay with buffer alone or ExoU supplemented with yeast lysate that was untreated or treated as indicated. **B,** Phospholipase assay comparing boiled, 3 kDa-filtered yeast lysate alone or treated with a protease cocktail.

The ExoU activator is unlikely to be nucleic acid or protein

We tested to see if the ExoU activator is destroyed by treatment with DNase, RNase, or the reducing agent dithiothreitol (as might be expected for a peptide whose conformation is stabilized by disulfide bonds). None of these treatments had any effect on the ability of the ExoU activator to promote phospholipase activity (data not shown). We further show that treatment with relatively non-specific proteases (i.e., a protease mixture containing chymotrypsin, trypsin, papain, and proteinase K) causes only a partial decrease to ExoU activation, suggesting that the activator may not be a protein or peptide (Fig. 4.2B). We suspect that the partial decrease may be due to residual protease activity and the high susceptibility of ExoU to proteases (unpublished observations).

The ExoU activator flows through a C-18 but is retained by a C-1 reversed-phase column

As a means of purifying the ExoU activator, we carried out reversed-phase chromatography using a C-18 column, which binds highly hydrophobic molecules, and a C-1 column, which binds molecules of moderate to low hydrophobicity. Powdered yeast extract, which was solubilized in water, 3 kDa-filtered, and extracted with chloroform, was applied to the C-18 and C-1 columns, and a 0-100% acetonitrile gradient (starting from 0.1 % trifluoroacetic acid in water) was applied to elute samples from the columns. Fractions were then assayed for the presence of the ExoU activator using the mixed micelle phospholipase assay described above.

We find that the ExoU activator does not bind the C-18 column (Fig. 4.3A, boxed), but does bind the C-1 column and is eluted from this column at ~75% acetonitrile (Fig. 4.3B, boxed). We found that binding of the ExoU activator to the C-1 column required the presence of acid in the mobile phase, suggestive of anionic properties that are masked with acid. We also observed that the ExoU activator elutes as a discrete peak, suggestive of it being a specific molecule or molecular species. In summary, reversed-phase chromatography using the C-1 column provides a substantial step towards purification of the ExoU activator.

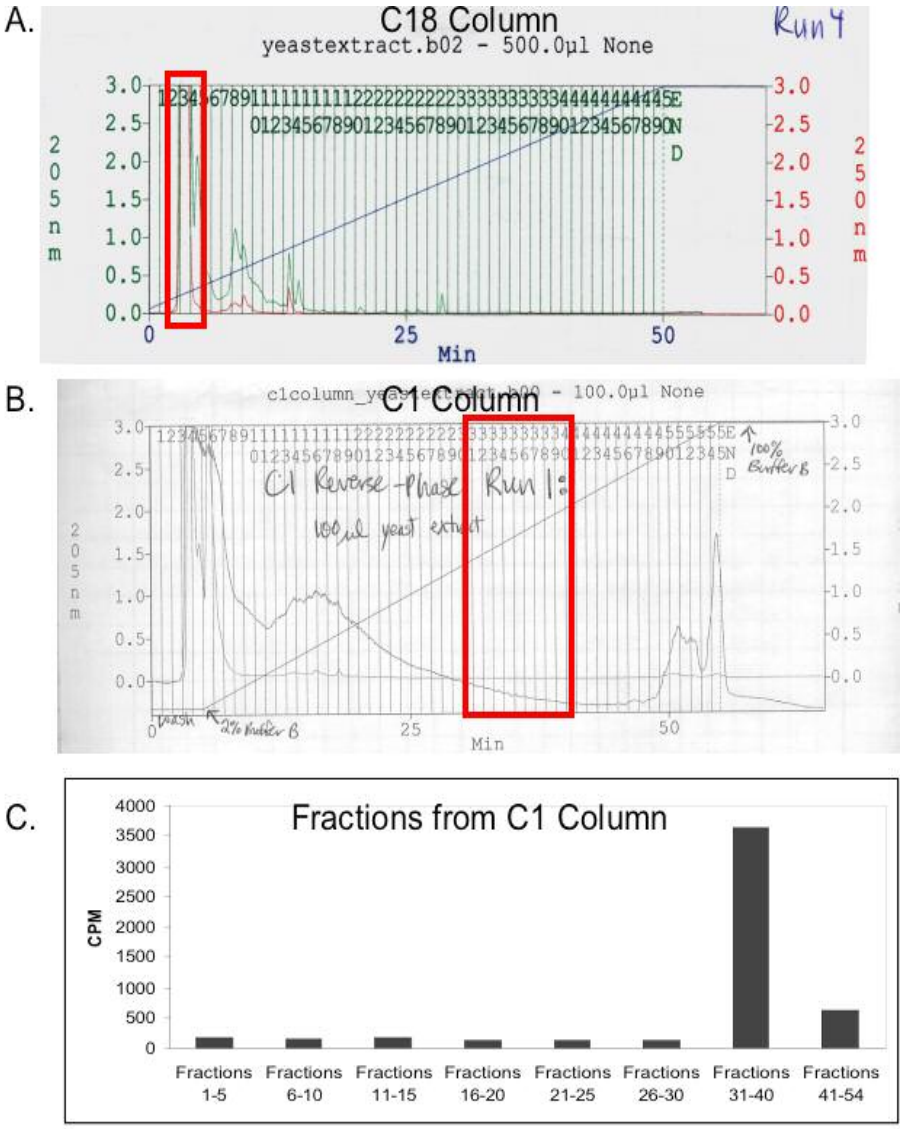


Figure 4.3. ExoU activator binds to a C-1 reverse-phase column. Filtered and chloroform-extracted yeast extract was applied to a **(A)** C-18 (Vydac, 4.6 mm x 250 mm) or **(B)** C-1 (Allsphere Alltech, 4.6mm x 250 mm) column. A gradient of 0-100% acetonitrile (sloped lines) was used to elute samples from the columns (starting from 0.1 % trifluoroacetic acid in water). Fractions were collected, dried to completeness, and resuspended in water for mixed micelle phospholipase assays. Fractions found to activate ExoU are boxed in panels A and B, and results from the phospholipase assay for fractions from the C-1 column are shown in **(C)**.

The ExoU activator is anionic and size-exclusion chromatography suggests a molecular mass of ~750 daltons

We used cation (Poros HS/M) and anion exchange (Poros HQ/M) chromatography to define the electrostatic characteristics of the ExoU activator. While the activator was not retained by the cation exchange column (data not shown), it was retained by the anion exchange column and was eluted in discrete fractions from this column at ~200-300 mM NaCl (Fig. 4.4A). These data indicate that the ExoU activator is likely to be negatively charged. The negative charge may be due to phosphates or sulfates. Our data also suggest that the small-molecule activator has tightly bound divalent cations (Mg^{2+} and/or Ca^{2+}) that are required for its function. These counterions are apparently stripped by ion exchange chromatography, resulting in decreased activity. This decrease in activity can be reversed by the addition of divalent cations in phospholipase activity assays.

We used size-exclusion chromatography (Superdex Peptide, optimum separation in 100-7000 dalton range) to provide an estimate of molecular mass. The ExoU activator was found to elute in discrete fractions, consistent with a molecular mass of ~750 daltons (Fig. 4.4B).

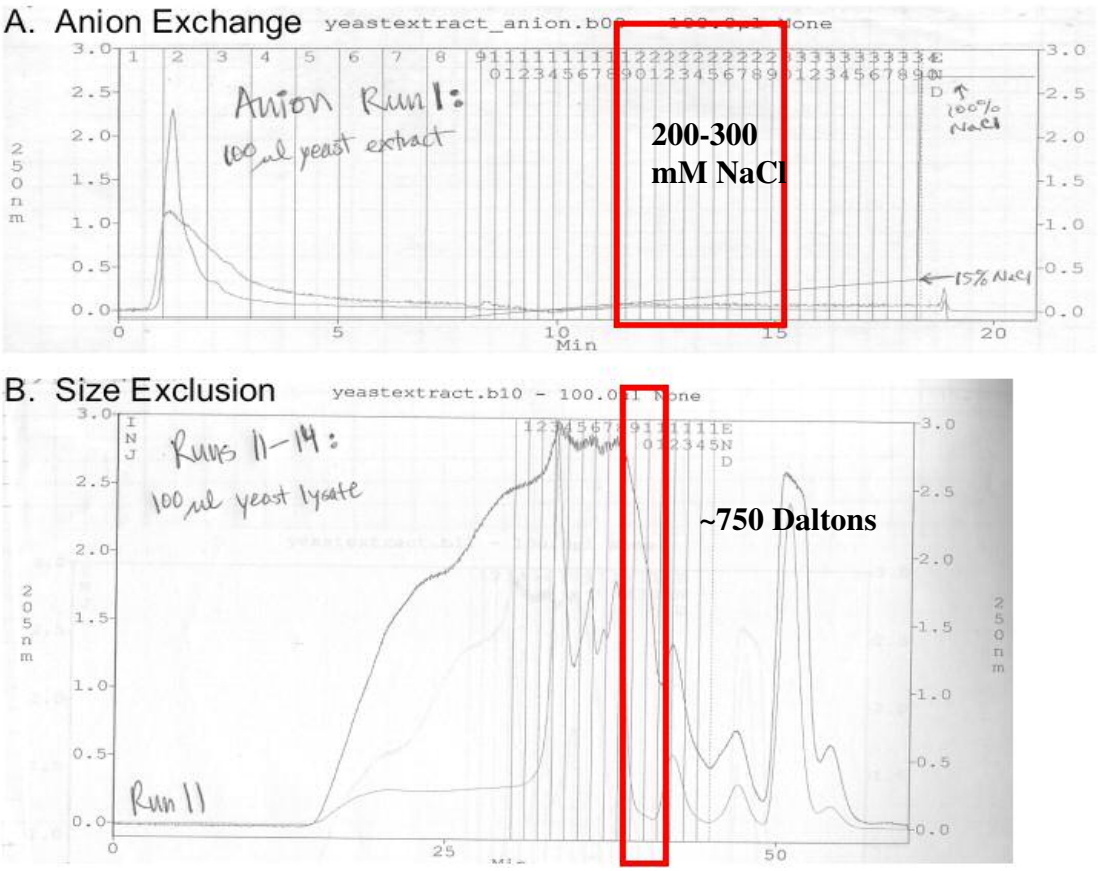


Figure 4.4. Chromatography traces of ExoU activator on anion and sizing columns. Filtered and chloroform-extracted yeast extract was applied to **(A)** an anion exchange column (HQ/M, Poros, 10 mm x 100 mm) or **(B)** size-exclusion column (Superdex Peptide, Pharmacia, 10 mm x 300 mm). Fractions found to activate ExoU are boxed. A 0-1000 mM NaCl gradient followed by a step to 3 M NaCl was used for the anion exchange column. For the size exclusion columns, standards (myoglobin, 17 kDa; vitamin B12, 1350 Da; chloroquine, 505 Da; riboflavin, 376 Da; and tryptophan, 204 Da) were applied to calibrate molecular mass with elution times.

Measuring specific activity — linearity of phospholipase assay

To monitor specific activity during large-scale purification of the ExoU activator, we sought to establish initial rate conditions for the mixed micelle phospholipase assay, and to verify that the assay is linearly sensitive to the amount of ExoU activator present. Surprisingly, no phospholipase activity was detected until 12 hours of incubation of ExoU, yeast extract and phospholipid substrate (Fig. 4.5A). These data contradict published data that demonstrates rapid activity (3 hours) in the presence of a yeast lysate (Sato et al., 2003; Tamura et al., 2004). This inconsistency suggests that another molecule might play a role in accelerating the activation of ExoU by the small molecule, and this hypothesis will be addressed further in Chapter 5. At this point, we continued with the small molecule activator purification, with the justification that while another molecule might be important for accelerating the activation of ExoU, the small molecule is a sufficient activator of ExoU.

The data from this time-course experiment suggests a slow activation step followed by a quick phospholipase activity step. To test whether the slow activation step was due to ExoU-yeast extract, ExoU-phospholipid, or yeast extract-phospholipid interactions, we carried out pre-incubation experiments. Two of the three activity assay components (yeast extract, ExoU, or phospholipid mixed micelles) were incubated overnight at 37°C. The third component was added the next day, and the mixture was then tested for immediate phospholipase activity. ExoU pre-incubated with yeast extract had immediate phospholipase activity (Fig. 4.5B), although the other pre-incubated mixtures had no activity (data not shown).

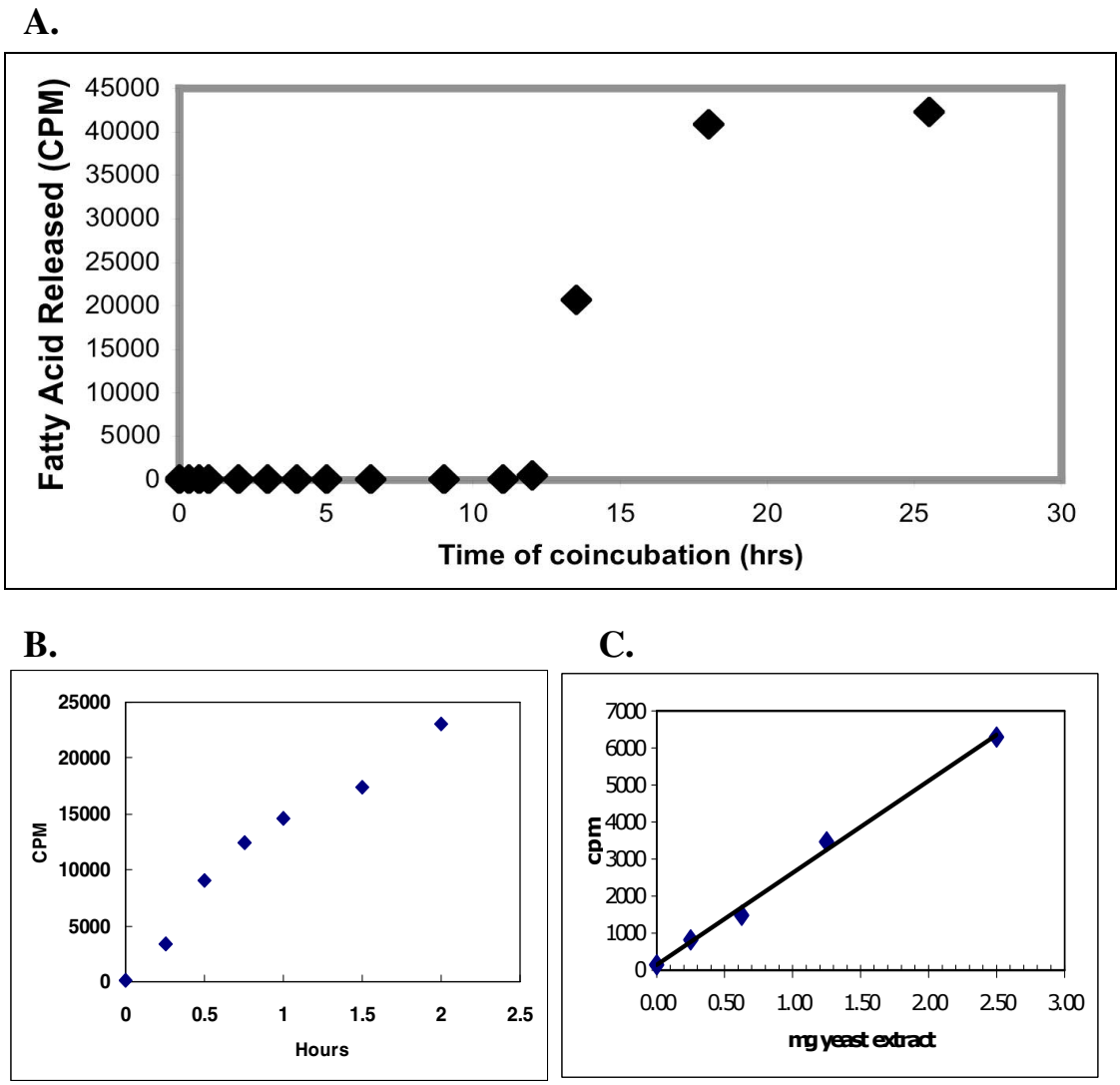


Figure 4.5. Time- and dose-dependence of ExoU phospholipase activity. **A,** Time course of mixed micelle phospholipase assay. Mixed micelles were incubated at 37°C with 1.25 mg yeast extract and ~3 μM ExoU over a 25 hour period and assayed for phospholipase activity at indicated time points. **B,** Phospholipase assay after overnight pre-incubation of the yeast extract with ExoU. **C,** Dose-dependence of mixed micelle phospholipase assay. Varying amounts of yeast extract were incubated for 30 minutes at 37°C with ~3 μM ExoU.

Figure 4.5B shows that linear, initial rate conditions are met in the first half-hour of the reaction, at which point ~10-15% of the substrate has been turned over (5000-7500 cpm out of a total of 50,000 cpm possible in the assay). Figure 4.5C shows that the assay responds linearly to changes in the amount of ExoU activator added. For this dose-response experiment, products were measured after 30 minutes of reaction (based on the data in Figure 4.5B).

Neither ExoU nor the small-molecule activator undergoes an irreversible change as result of co-incubation

To determine if an irreversible change, such as covalent modification, occurs to either ExoU or the small-molecule activator during co-incubation, His-ExoU was purified away from the activator with Ni-NTA beads. This sample of ExoU did not show 'rapid' (3 hour) phospholipase activity when incubated with a fresh aliquot of yeast extract, but instead required the standard 12-18 hr incubation time (data not shown). Likewise, yeast extract that had been incubated with ExoU for 18 hours, when purified away from ExoU and added to a fresh aliquot of ExoU, did not cause 'rapid' activation of ExoU (data not shown). These data demonstrate that neither ExoU nor small-molecule activator are permanently changed by overnight incubation. As a side note, it has been reported that ExoU is not permanently modified by yeast lysate either (Sato et al., 2003). These data also show that the interaction between ExoU and the activator is not such that the activator can be pulled-down by ExoU. Thus, the activator is not likely purifiable by an ExoU-affinity column.

ExoU activator may contain saccharide properties

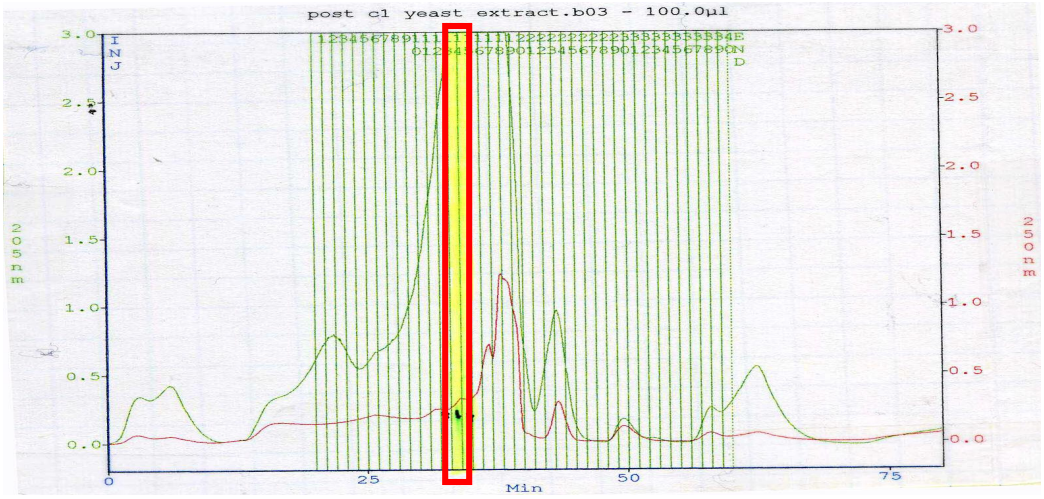
Consecutive purification steps of 3 kDa-filtration, C-1 and size-exclusion chromatography were used to purify ExoU activator from yeast extract. Only the purest fractions, as determined by specific activity, were pooled and used for each consecutive purification step. The small-molecule ExoU activator was found to elute in a single, discrete fraction from the size-exclusion column (Fig. 4.6A). Notably, the discrete peak observed on the size-exclusion column containing the ExoU activator is consistent with it being composed of a specific molecule or molecular species.

The small-molecule activator was analyzed by proton nuclear magnetic resonance (NMR) and mass spectrometry. Encouragingly, the NMR data shows relative simplicity of composition, indicating a high degree of separation efficiency (Fig. 4.6B). Interestingly, observed proton resonances are consistent with an oligosaccharide, having a β-linkage at the anomeric carbon, having a 6-deoxy group, and carrying acetylation. Mass spectrometry provides evidence that the compound is ~2000 daltons in molecular mass (data not shown). An oligosaccharide, due to its extended length, would not be expected to elute from a size-exclusion column at its true mass, explaining the difference between mass spectrometry and size-exclusion results. Of course, mass spectrometry is much more accurate for mass determination than is size-exclusion chromatography.

Further conclusions from the NMR data are that aromatic groups appear to be absent, indicating that the compound is not likely to have nucleic acid bases or other

aromatic groups. This is consistent with poor absorbance in 250-280 nm range. No peptide-like resonances are observed either, consistent with our observation that the small-molecule ExoU activator is not destroyed by various proteases (data not shown).

A.



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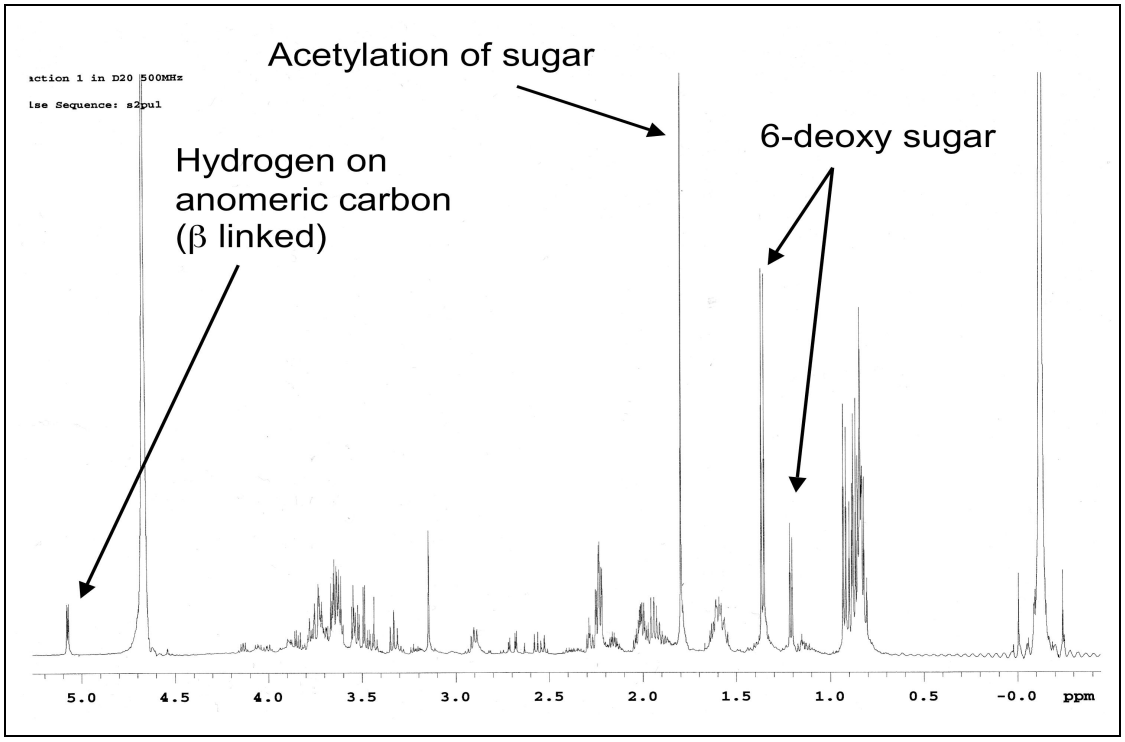


Figure 4.6. ExoU activator size-exclusion trace and proton NMR. A, Size-exclusion trace of post-C-1 column activator. Fractions used for NMR are boxed. **B,** Proton NMR (500 Mhz Varian Inova, in D_2O) of small-molecule ExoU activator, after C-1 reversed phase and size-exclusion chromatography of 3 kDa-filtered yeast extract. The sample was verified to activate ExoU in a phospholipase assay. Arrows point to characteristic resonances.

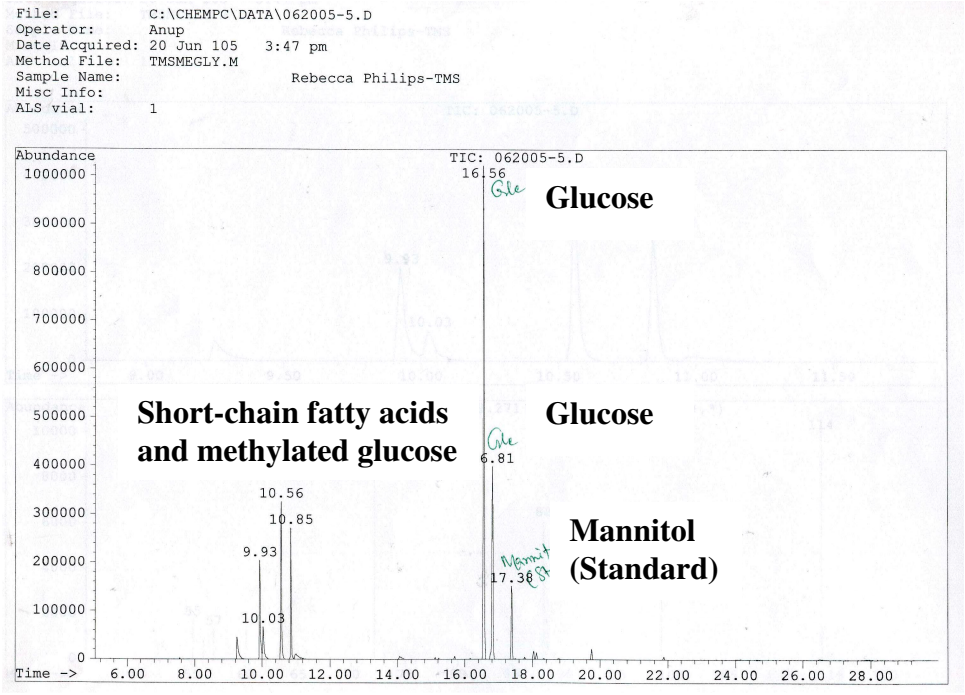
ExoU activator is composed mainly of glucose

Monosaccharide analysis was performed on purified ExoU activator to determine what types of saccharides might constitute all or part of the activator. The ExoU activator was purified using similar steps as that used for the NMR sample, except for additional second step of C-1 chromatography before the final sizing-exclusion chromatography was used. This additional step yielded higher purity, as determined by specific activity (data not shown). Monosaccharide analysis found the ExoU activator to be composed of approximately 90% glucose (Fig. 4.7A). Some of the glucose was found to be methylated. Interestingly, other peaks on the LC-MS trace were identified as a short-chain fatty acid (6 to 7 carbons). This method cannot detect whether these fatty acid chains were covalently linked to a saccharide or were contaminating molecules during purification.

ExoU activator is found in "purified" glycogen but is not likely glycogen itself

Because glucose appears to be a major part of the ExoU activator, many glucose-containing molecules and other saccharides were tested for their ability to activate ExoU in an *in vitro* phospholipase activity assay. Glucose, maltose, isomaltose, gangliosides, sulfatides, methylglucoside, and heparin did not have the ability to activate ExoU *in vitro* (data not shown). Interestingly, purified oyster glycogen (Sigma) was found to activate ExoU, although to a lesser extent compared to the same amount of yeast extract (Fig. 4.7B).

A.



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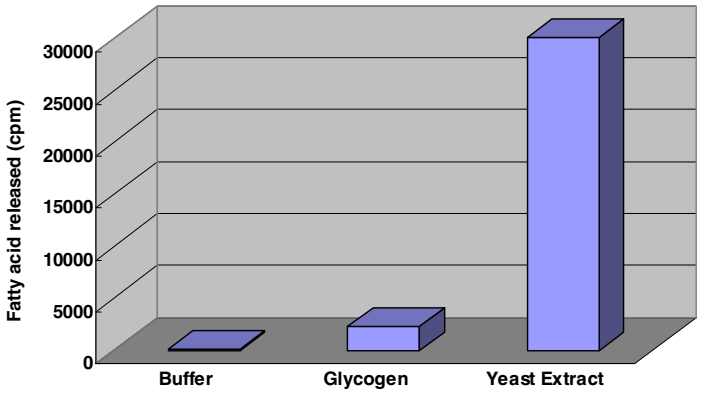

Figure 4.7. ExoU activator contains glucose and is found in 3 kDa-filtered glycogen. **A,** Monosaccharide analysis of purified ExoU activator. Peaks in chromatography trace were identified by mass spectroscopy. **B,** Activity assay results comparing the activity of an identical amount of yeast extract and glycogen.

These data surprised us for two reasons. First, glycogen is a very large and highly branched glucose polysaccharide that varies in size from 10^6 to 10^7 daltons. However, our data have suggested that the ExoU activator is less than 3000 daltons and is likely a single type of molecule. Second, our results so far suggest that the ExoU activator is a eukaryotic molecule, yet *E. coli* and *P. aeruginosa* have the ability to make glycogen. Interestingly, bacteria use a different mechanism to synthesize glycogen compared to that of eukaryotes. In bacteria, the synthesis of glycogen involves ADP-glucose as the glucosyl donor for elongation of the α-1,4-glucosidic chain. In eukaryotes, UDP-glucose is the glucosyl donor. Alas, this was not our solution, as UDP-glucose did not activate ExoU *in vitro* (data not shown).

To test whether the ExoU activator from oyster glycogen behaves similarly to activator from yeast extract, oyster glycogen was passed through a 3 kDa-cutoff membrane. Activity was found in the filtrate, just as ExoU activator in yeast extract (data not shown). ExoU activator purified from oyster glycogen was also found to elute from C-1 reversed phase and size-exclusion chromatography with similar retention times as ExoU activator from yeast extract, consistent with it being the same molecule (data not shown).

Finally, to determine whether the ExoU activator is simply a fragment of glycogen, various lengths of synthetic glucose polymers from 1 to 7 glucose units in length (glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose) were tested for the ability to activate ExoU *in vitro*, however none

was detected (data not shown). Additionally, β-cyclodextrin, a 7-glucose circular molecule previously used as a glycogen analog (Pinotsis et al., 2003), was unable to activate ExoU. Thus, we believe that the ExoU activator is an impurity in "purified" oyster glycogen.

Concavalin A-Sepharose beads cannot pull down ExoU's activator

Concavalin A (Con A) is a protein that binds α-D-mannose, α-D-glucose, and sterically related residues with available C-3, C-4, or C-5 hydroxyl groups. We tested whether ExoU activator could be pulled down by Con A covalently linked to Sepharose beads. The Con A beads were incubated with 3 kDa-filtered glycogen, and activity assays were performed from the unbound fraction, washes, and supernatant of boiled beads. All activity was found with the unbound fraction and first wash, suggesting that Con A is unable to bind ExoU activator (data not shown). This experiment further implies that ExoU activator either does not have α-linkages (consistent with NMR data) or has modified C-3, C-4, or C-5 hydroxyl groups.

Cleaner NMR spectra of ExoU activator from glycogen

We speculated that oyster glycogen was a less complex starting material for purification of ExoU activator and might yield cleaner NMR spectra compared to that from yeast extract. To see if ExoU activator purified from oyster glycogen has similar cleaner shifts and simpler NMR spectra compared to activator from yeast extract, oyster glycogen was passed through a 3 kDa-cutoff membrane, and found to elute with identical retention times on C-1 reversed phase (data not shown) and size-exclusion (Fig. 4.8A) chromatography columns. Purified ExoU activator was analyzed by proton NMR (Fig. 4.8B). Similar signature proton shift peaks suggest that the activator from glycogen is indeed the same molecule in yeast extract. Furthermore, the NMR spectra shows even simpler peaks (doublets, quadruplets, etc), indicating a high degree of sample purity. The purified ExoU activator was then analyzed by two-dimensional proton-proton NMR (Fig. 4.8C). These data reveal coupling between protons on the activator, such as the coupling between the proton with a doublet shift at 1.5 ppm and the proton with a quadruplet shift at 3.8 ppm. These data can also expose possible impurities, such as a likely impurity with proton shifts at 3.3 and 3.5 ppm that only couple to each other. The relatively stronger intensities in these peaks also suggest an impurity. Finally, the activator was analyzed by two-dimensional proton-carbon NMR (Fig. 4.8D). The small number of peaks in these data suggests that the signal-to-noise ratio was low, likely due to low sample amounts. Unfortunately, these data alone are not enough for structure elucidation, due to impurities and insufficient sample for carbon NMR. We concluded from these

experiments that another purification step and larger amounts of purified ExoU activator were required for structure elucidation.

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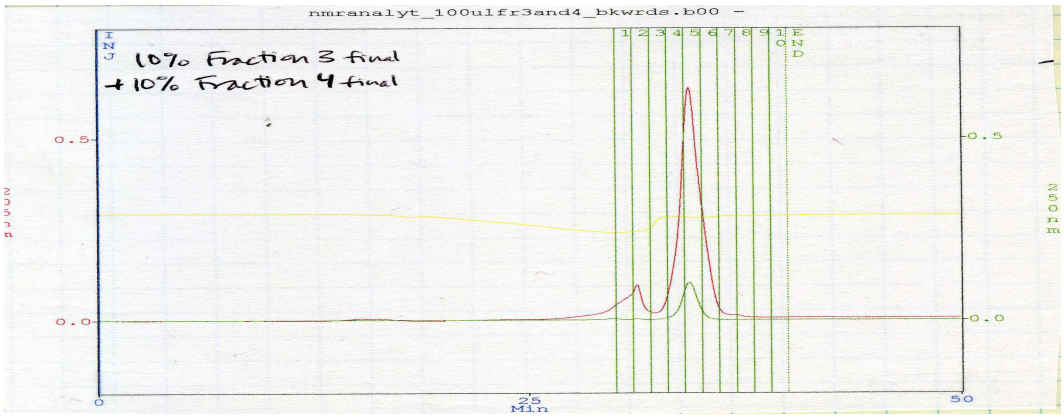

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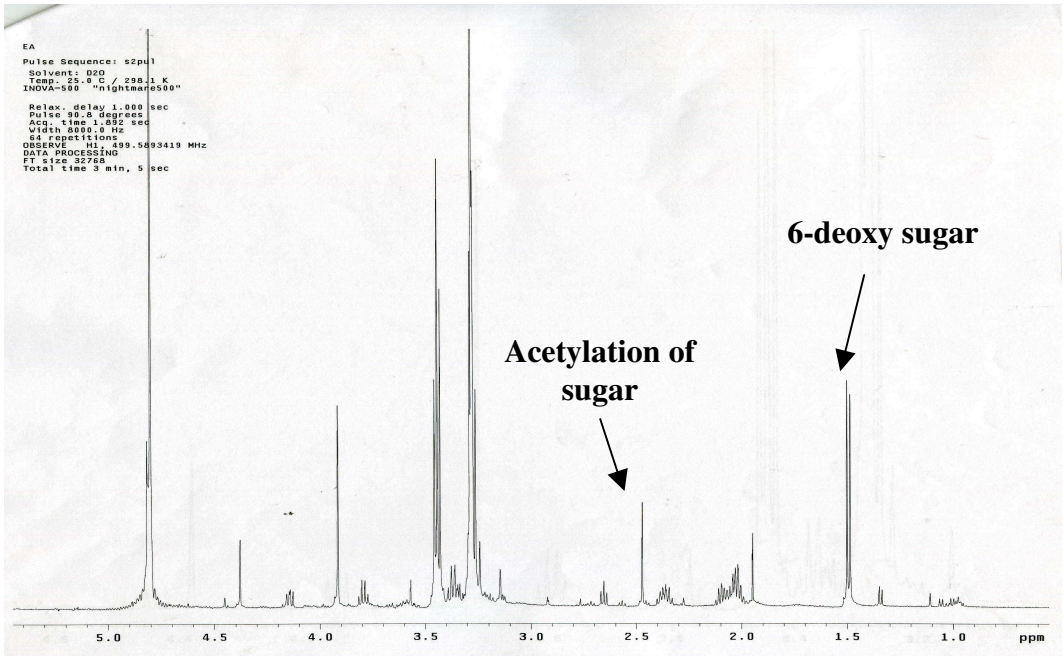
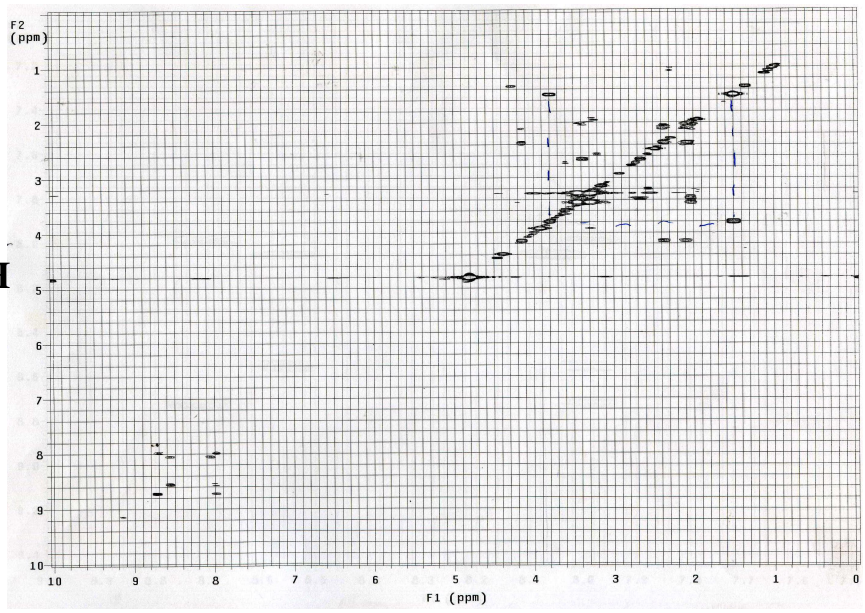


Figure 4.8. Pure ExoU activator size-exclusion trace and NMR. A, Size-exclusion trace of purified ExoU activator purified from 3 kDa-filtered glycogen by C-1 and size-exclusion column chromatographies. **B,** Proton NMR (in D_2O) of ExoU activator. The sample was verified to activate ExoU in a phospholipase assay.

C.



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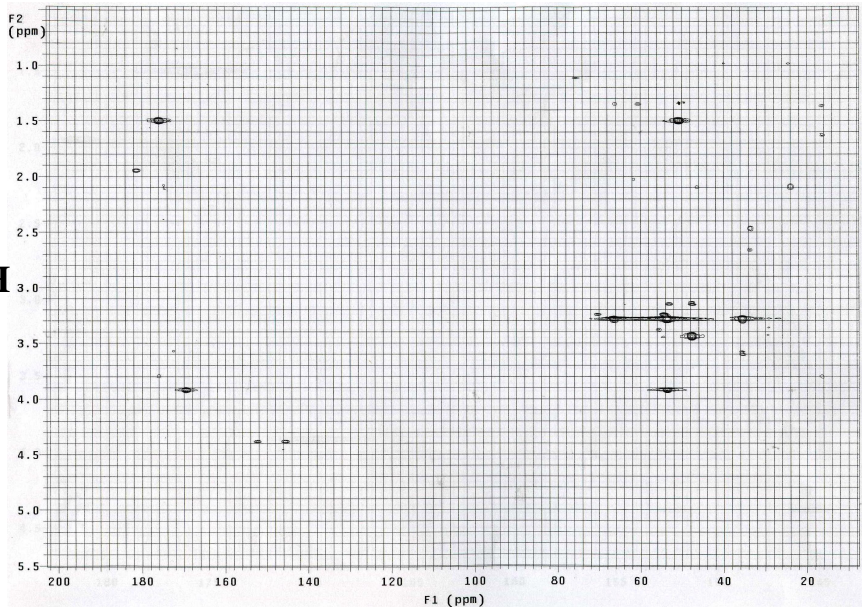
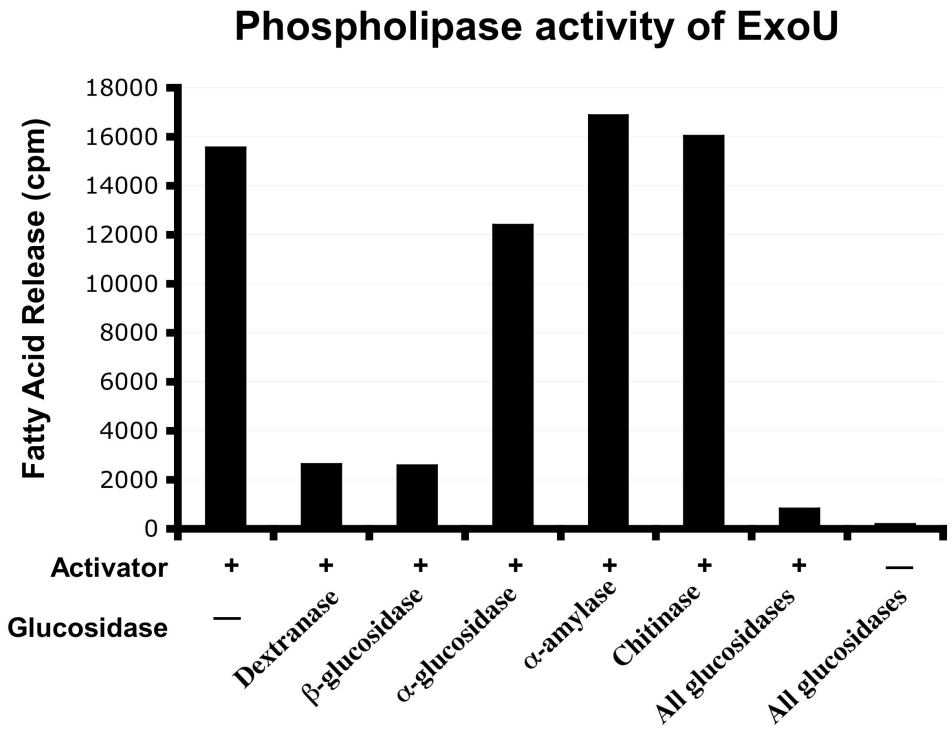


Figure 4.8 continued. Pure ExoU activator size-exclusion trace and NMR. **C,** Two-dimensional proton-proton (gCOSY) NMR of the same sample of ExoU activator. **D,** Two-dimensional carbon-proton (gHMBC) NMR.

ExoU activator is degraded by specific glycosidases

Up to this point, our data suggest that the ExoU activator is an oligosaccharide. However, it is still formally possible that saccharide components in the purified ExoU activator sample are a contamination. To test the idea that the ExoU activator is a saccharide, we sought to destroy the activity with specific glycosidases, enzymes that cleave glycosidic linkages. The identification of glucose as a major component led to the simple prediction that the small-molecule activator should be destroyed by specific glucosidases, or glucose-specific glycosidases. Indeed, this is what we find. ExoU activator from 3 kDa-filtration of glycogen was subjected to overnight digestion with various glycosidases, and tested for the loss of ability to activate ExoU *in vitro*. As shown in Figure 4.9, both dextranase and β-glucosidase decrease the ability to activate ExoU substantially, as compared to α-glucosidase, α-amylase, and chitinase. The capability of β-glucosidase but not α-glucosidase to degrade the activator is consistent with NMR evidence of β-linkage at the anomeric carbon. Notably, the degradative effects of dextranase and β-glucosidase are additive, as seen by the combination of glucosidases (+ Activator, All glucosidases) resulting in further loss of the activator as compared to the enzymes added individually. Chitinase was tried as it degrades components of the yeast cell wall, and the last column (- Activator, All glucosidases) is a control showing that the glucosidases themselves have no phospholipase activity.



Enzyme	Specificity
Dextranase	1,6-α-D-glucosidic linkage
β-glucosidase	1,4-β-D-glucosidic linkage of terminal, nonreducing end
α-glucosidase	1,4-α-D-glucosidic linkage of terminal, nonreducing end
α-amylase	1,4-α-D-glucosidic linkage in oligo/polysaccharide
Chitinase	1,4-β-N-acetyl-D-glucosamine

Figure 4.9. Effect of glycosidases on ExoU activator. ExoU activator was subjected to overnight digestion by one or all of the listed glycosidases. The digests were then subjected to 3 kDa-filtration and the filtrates were tested for ability to activate ExoU *in vitro* phospholipase activity.

Linkage analysis of purified ExoU activator

A variety of glycosidic linkages may be formed in polysaccharides, depending on the location of the former hydroxyl groups on each monosaccharide involved in the linkage, and whether the linkage forms with an alpha or beta anomer. To determine the types of glycosidic linkages in ExoU activator, linkage analysis was performed. Linkage analysis involves methylation of the free hydroxyl groups of the saccharide, then cleavage of the glycosidic linkages to produce individual monosaccharides with new free hydroxyl groups at the positions that were previously involved in a linkage. These partially methylated monosaccharides are then derivatized to produce volatile molecules and analyzed by GLC-EIMS (Glycosyl-Linkage Composition Electrospray Ionization Mass Spectrometry (GLC EIMS). Identification is achieved by using a combination of retention times (as compared to those of known standards analyzed under the same conditions) and EIMS patterns (mass spectra).

ExoU activator was purified from oyster glycogen using consecutive purification steps of 3 kDa-filtration, C-1 and size-exclusion chromatography, and subjected to linkage analysis (Fig. 4.10). Consistent with monosaccharide analysis of ExoU activator purified from yeast extract, the only type of sugar found in activator sample was glucose. Interestingly, linkage analysis revealed modification mainly at the 1 and 3 positions, suggestive of glucose polymers connected by 1,3-linkages. These data are inconsistent with glycosidase digests suggesting 1,4-β and 1,6-α linkages, although promiscuity of dextranase and β-glucosidase may be possible.

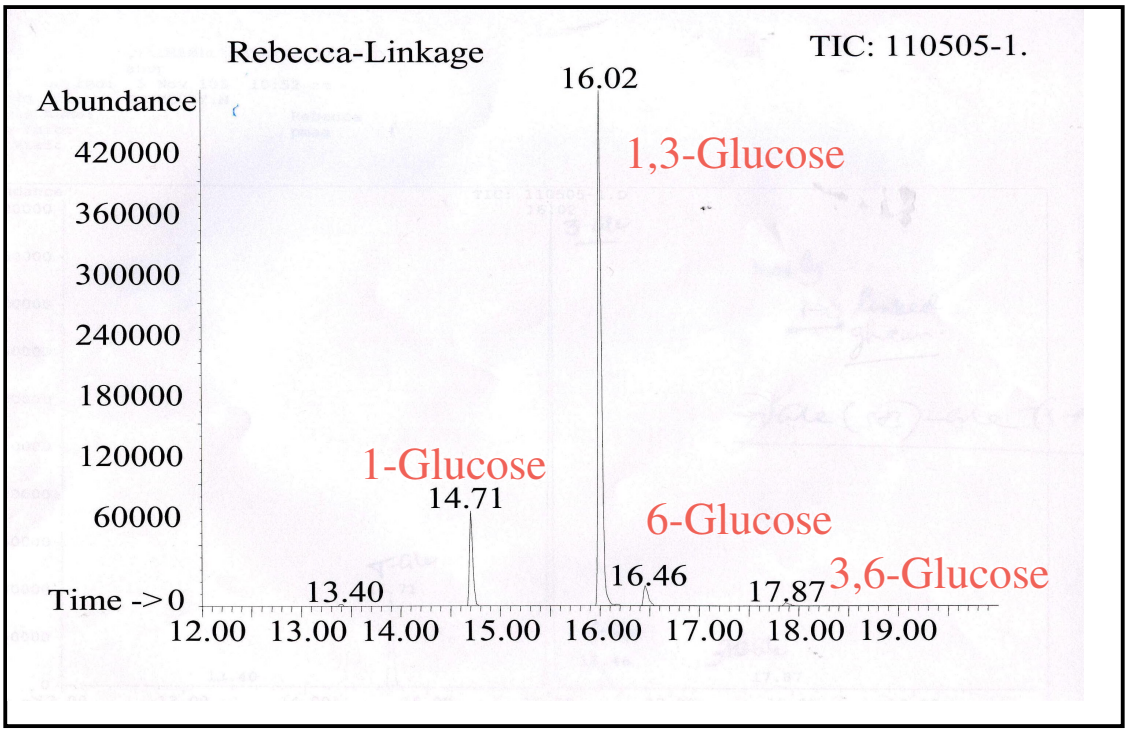


Figure 4.10. Chromatography trace of linkage analysis products. Peaks were identified by comparing to the retention times and mass spectra of standards.

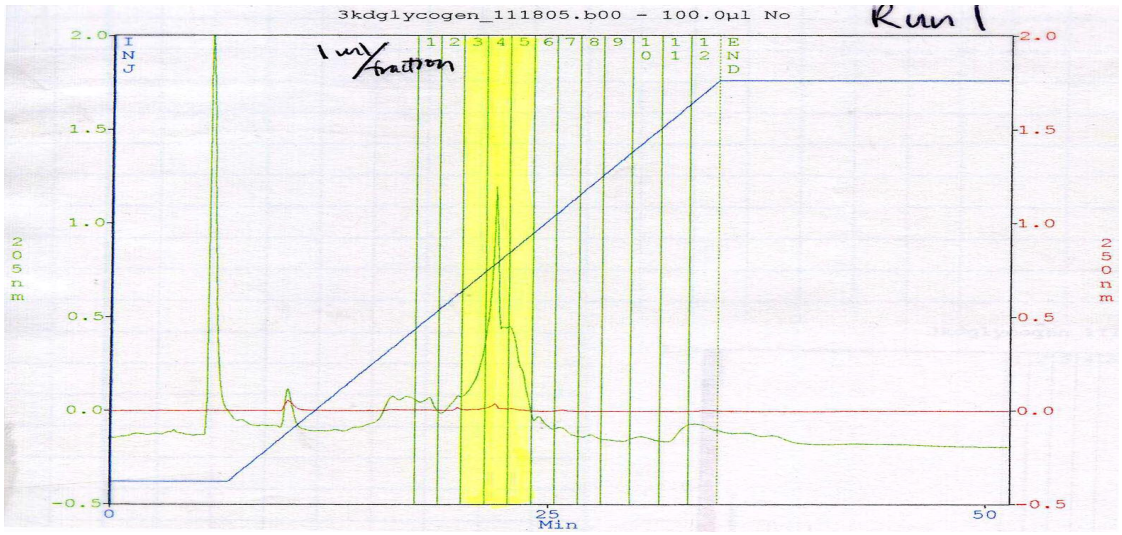
Further purification of ExoU activator by normal phase chromatography

Because the data so far were still not sufficient for structure elucidation party due to sample purity, we sought another purification step. We turned to the Carbohydrate ES column (Alltech), a normal phase chromatography column, which binds hydrophilic molecules and has the ability to separate oligosaccharides from 1 to 25 units in length. Glycogen (3 kDa-filtered) was applied to the Carbohydrate ES column, and a water:acetonitrile (10:90) to 100% water gradient was applied to elute samples from the column. Fractions were then assayed for the presence of the ExoU

activator. As shown in Figure 4.11A, the ExoU activator binds the Carbohydrate ES column and is eluted at ~50% water. To test whether this column provided additional purification to that which we had already established, a sample of ExoU activator purified by previous methods was applied to the Carbohydrate ES column. The chromatography trace shows contaminant peaks outside that of the ExoU activator, indicating that further purification is achieved with the Carbohydrate ES column.

Consecutive purification steps of 3 kDa-filtration, C-1 reverse-phase, Carbohydrate ES normal phase, and size-exclusion chromatography were used to purify ExoU activator from oyster glycogen. The sample elutes as a single peak on the size-exclusion column (Fig. 4.11B) and also forms a single peak when reapplied, for analytical purposes, to the Carbohydrate ES column during that same day (Fig. 4.11C). Unfortunately, the purified ExoU activator is not stable when stored overnight in neutral pH water or as a speed-vac dried solid. The evidence for this is seen by the loss in its ability to activate ExoU (data not shown) and the change in elution trace on the Carbohydrate ES column (Fig. 4.11D). Similar results are seen after purification on the Carbohydrate ES column only, in column elution solution or as a dried pellet. Attempts at storing the sample under nitrogen gas or vacuum yielded similar results. Thus, purified ExoU activator is only stable for hours (less than 18 hours), making a major roadblock in the purification of sufficient sample for obtaining carbon NMR data.

A.



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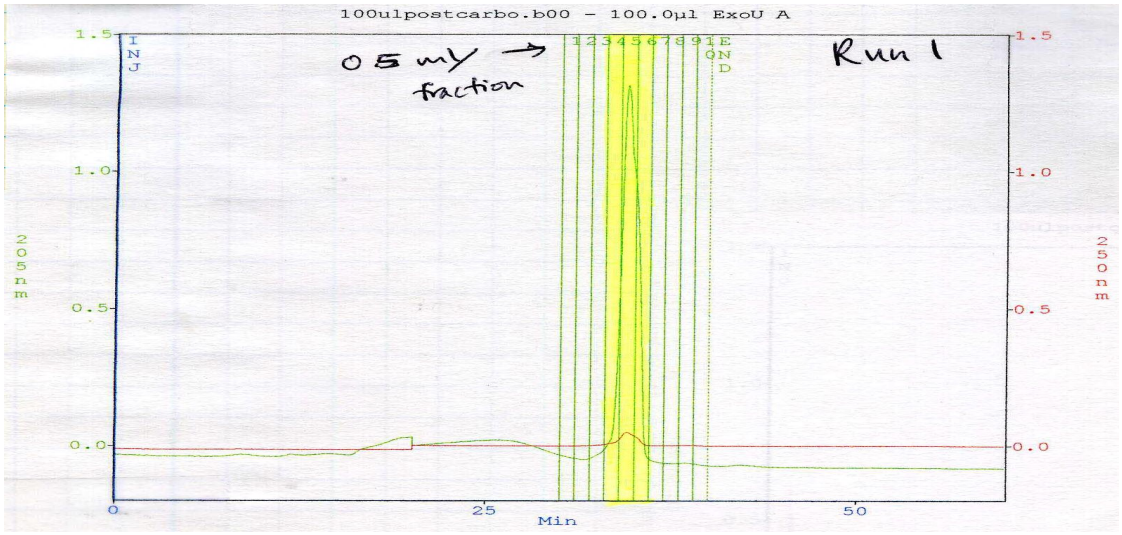
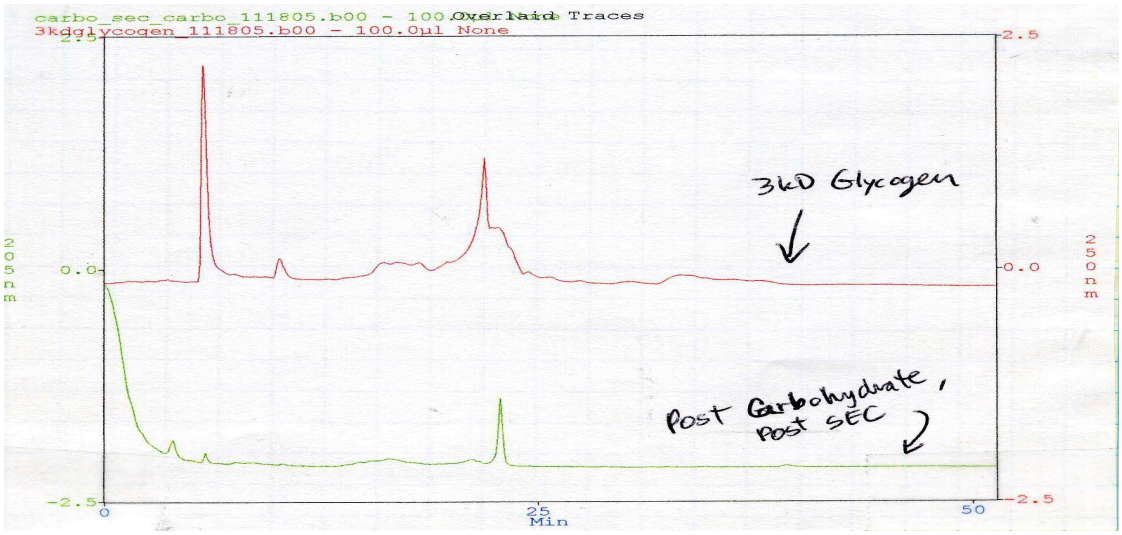


Figure 4.11. Purification of ExoU activator using normal-phase chromatography. **A,** Carbohydrate ES column normal-phase chromatography trace of 3 kDa-filtered glycogen. ExoU activator is highlighted in yellow. **B,** Size-exclusion chromatography trace of ExoU activator following normal-phase chromatography. ExoU activator is highlighted in yellow.

C.



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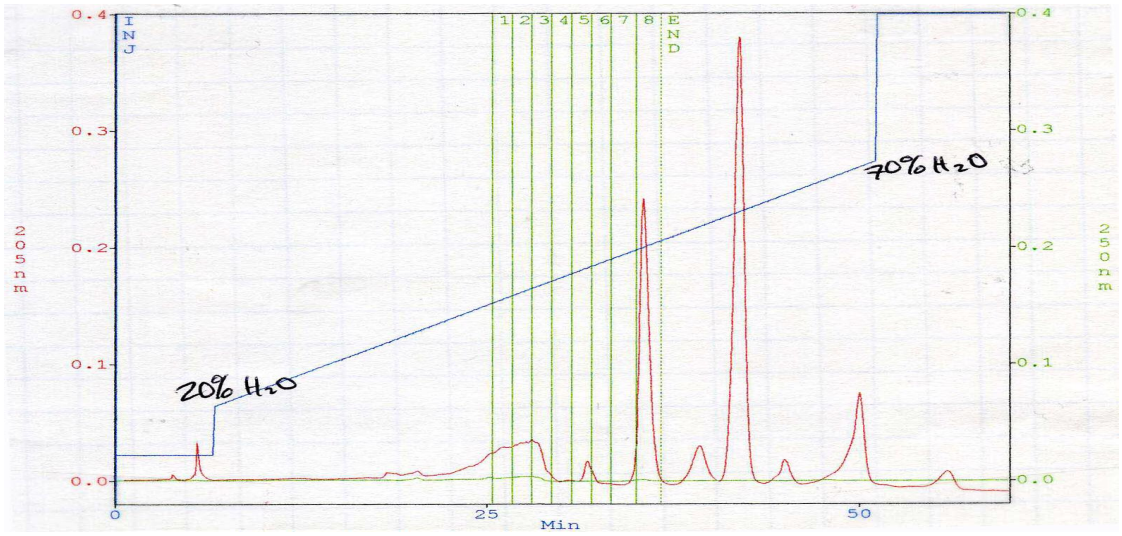

Figure 4.11 continued. Purification of ExoU activator using normal-phase chromatography. C, Comparison of normal-phase chromatography traces of 3 kDa-filtered glycogen (green line) versus purified ExoU activator (following normal-phase and size-exclusion purifications) (red line). **D,** Normal-phase chromatography trace of purified ExoU activator following overnight storage of dried sample under nitrogen gas at -20°C. Similar results were seen under other storage conditions.

***Rickettsia prowazekii* RP534 has phospholipase activity, and has the same mechanism of activation as ExoU**

BLAST searches showed that ExoU is most closely related to a gene product from *R. prowazekii* called RP534 (68 kDa). To address whether RP534 has a similar mechanism of action to ExoU, this protein was expressed recombinantly in *E. coli* as a soluble protein with an introduced N-terminal his-tag, and purified by metal-chelation (MC/M) and size-exclusion chromatographies (Fig. 4.12A). Purified RP534 was assayed for phospholipase activity in a mixed micelle assay, and exactly like ExoU, has no activity in the absence of yeast extract, but has substantial activity in the presence of yeast extract (Fig. 4.12B). These results indicate the likelihood that ExoU and RP534 share a common mechanism of activation.

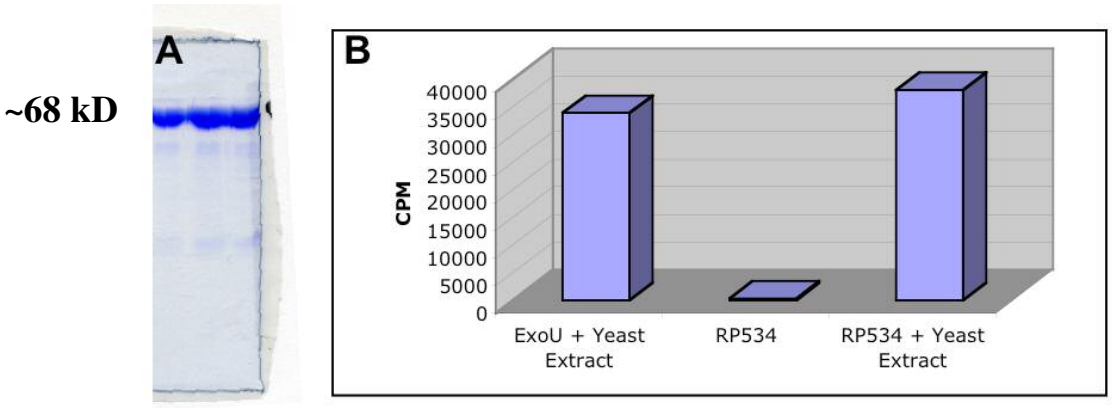

Figure 4.12. Phospholipase activity assay with ExoU homolog RP534. A, SDS-PAGE of purified RP534, with fractions from size-exclusion chromatography shown. **B,** Mixed micelle phospholipase assay, showing RP534 has no activity in the absence of yeast extract, but has substantial activity in the presence of yeast extract.

Discussion

Our research demonstrates that ExoU has *in vitro* phospholipase A_2 activity only in the presence of a host cellular extract, consistent with reports published at the time of these experiments (Sato et al., 2003; Tamura et al., 2004). Because yeast are susceptible to ExoU toxicity (see Chapter 3), we used yeast lysates for activation of ExoU, and we found that commercial powdered yeast extract also activates ExoU. We show that bacterial lysates cannot activate ExoU, indicating that the activator of ExoU is likely eukaryote-specific. Furthermore, ExoU has activity on a variety of phospholipid substrates, indicating that ExoU is likely a non-specific phospholipase. Here, we used classical biochemical purification techniques to fractionate yeast extract and identify the host-cell factor involved in the activation of ExoU.

To begin, we sought initial characterization of the ExoU activator. We tested the sensitivity of ExoU activator to temperature, organic extraction, filtration, and enzyme digestion. Remarkably, the ExoU activator was stable to boiling and flowed through a 3 kDa cutoff filter, suggesting a small, non-proteinaceous molecule. Furthermore, RNase, DNase, protease, and organic extraction were unable to destroy or deplete the activator.

Various chromatography columns were used to further characterize the ExoU activator and isolate it for its identification. We found that ExoU activator binds a C1

but not C18 column, and only binds the C1 column with acid in the mobile phase. This is suggestive of a polar molecule with anionic property, which is likely masked with the acid. The possibility of anionic character was confirmed by the ability of the ExoU activator to bind an anion exchange column. Finally, the ExoU activator eluted as a ~750 dalton molecule on a size-exclusion column, consistent with filtration experiments suggesting a size between 500 and 3000 daltons.

These purification steps were used to prepare a sample of ExoU activator for proton NMR analysis. Interestingly, observed proton resonances between 3 and 4 ppm are consistent with an oligosaccharide. Resonances also suggest a β-linkage at the anomeric carbon, a 6-deoxy group, and acetylation. This led us to the hypothesis that the ExoU activator is a eukaryotic-specific oligosaccharide. As a result, we were surprised by the results from monosaccharide analysis revealing that glucose was the only saccharide in the ExoU activator sample.

How could a eukaryotic-specific oligosaccharide contain only glucose? To answer this, we sought to ask whether any glucose-containing molecule found in both prokayotes and eukaryotes would activate ExoU. We tested over 20 different saccharide-containing molecules, among others (Table 4.1). Besides yeast and bovine extracts, only one substance tested, glycogen, a glucose polymer found in both prokayotes and eukaryotes, was able to activate ExoU. Because the ExoU activator in glycogen flowed through a 3 kDa-filter, we suspected that ExoU activator is an impurity in glycogen. All of our control experiments supported this theory.

Table 4.1. Molecules tested for their ability to activate ExoU *in vitro* phospholipase activity.

Chemical	**ExoU activation?**
Yeast extract (powdered extract)	Yes
Brain-heart infusion (powdered extract)	Yes
Oyster glycogen	Yes
Yeast synthetic media	No
Bacterial minimal media	No
Vitamin mixture	No
Divalent ions: Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}	No
Inositol and PIP2	No
Glucose	No
Bile salts	No
Cholesterol	No
Gangliosides from bovine brain	No
Sulfatides from bovine brain	No
Methylglucoside	No
Maltose	No
Isomaltose	No
Maltotriose	No
Maltotetraose	No
Maltopentaose	No
Maltohexaose	No
Maltoheptaose	No
UDP Glucose	No
Heparin	No
Beta-cyclodextrin	No
Trehalose	No

We next sought to destroy the ExoU activator with specific glycosidases. We found two enzymes, β-glucosidase and dextranase, that were able to significantly destroy the activator, and the two enzymes together decreased activity even further than either of the enzymes alone. This was confirmation of our hypothesis that the ExoU activator contains saccharide properties.

The ExoU activator purified from oyster glycogen was purer than that purified from yeast extract, as evidenced by simpler NMR spectra. However, structure elucidation of ExoU activator was still not attainable, for two reasons. First, a likely impurity, as evidenced by intense proton shifts at 3.3 ppm and 3.5 ppm that couple only to each other, may be covering up data important for structure elucidation. Second, the small number of peaks in the two-dimensional proton-carbon NMR data suggested that the sample amounts were too low. We concluded from these experiments that another purification step and larger amounts of purified ExoU activator were required for structure elucidation.

We sought another method for purification of ExoU activator and found that a normal-phase column designed for saccharide purification was able to further purify ExoU activator. Unfortunately, after using this step, we found that the sample cannot be stored and seems to quickly become unstable and degraded. We reason that the ExoU activator instability is not a result of the normal-phase purification step, since it uses the same solvents (water and acetonitrile) as those in reverse-phase chromatography. Instead, we suspect that the sample is stable over time with

impurities, which might offer protection from degradation, but not stable for more than a day as a pure molecule. As a result, the amount purified in one day (without storage) is not sufficient for two dimensional NMR analysis.

An intriguing aspect of these experiments is the fact that no activity can be seen without long (~12 hour) pre-incubation times with ExoU and activator. The time-course of ExoU activity suggests that activation is not a slow and steady event. Rather, activation appears to happen like a nucleation event, where no activity can be measured until ~12 hours, after which swift and linear activity is measured. Because these data contradict published data demonstrating immediate ExoU activity in the presence of a yeast lysate (Sato et al., 2003; Tamura et al., 2004), we hypothesized that another molecule might play a role in accelerating the activation of ExoU by the small molecule, perhaps by forming a platform for the nucleation-like activation. We decided to continue with the small molecule activator purification because this small molecule is a sufficient activator of ExoU. Alongside, we began experiments to identify the "ExoU accelerator," as described in Chapter 5, and we sought to answer whether the ExoU activator co-purifies with the accelerator.

Finally, we found that the ExoU-related protein RP534 from the bacterial pathogen *Rickettsia prowazekii* is also a host-factor activated phospholipase A_2. RP534 was active only in the presence of yeast extract, demonstrating a mechanistic conservation between ExoU and RP534. Furthermore, these data suggest that phospholipase activity is likely the cause of the sick or slow-growth phenotype

observed when RP534 is expressed in yeast. Whether RP534 is the phospholipase A_2 implicated in escape of *R. prowazekii* from phagosomes has yet to be proven (Ojcius et al., 1995).

Overall, these data strongly suggest that the ExoU activator is a eukaryotic-specific, anionic, and glucose-containing saccharide. These data have broader implications, in that the ExoU-related protein RP534 appears to have the same mechanism of activation. ExoU-homologs from other bacterial pathogens, such as *Legionella pneumophila, Treponema palladium, Bacillus anthracis,* and *Mycobacterium tuberculosis*, and others (Banerji and Flieger, 2004) may also share the same mechanism of activation. Unfortunately, obtaining purity to the level of structure determination of the activator has proven a difficult task, as the purest sample is unstable over short times. Interestingly, long-incubation times between ExoU and the activator suggest that another molecule might play a role in the *in vivo* activation mechanism of ExoU. We investigate this hypothesis in the following chapter.

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V.

Biochemical approach to characterize the ExoU accelerator

Abstract

The *Pseudomonas aeruginosa* cytotoxin ExoU acts a membrane-lytic and host-cell activated phospholipase A_2. We and others have demonstrated that the soluble fraction of a *Saccharomyces cerevisiae* lysate contains one or more factors involved in ExoU activation. Our results in Chapter 4 demonstrate that ExoU phospholipase activity is activated *in vitro* by a small glucose-containing saccharide. However, lack of detectable activity by the saccharide until approximately 12 hours is inconsistent with immediate detection of ExoU cytotoxicity *in vivo*, and suggests that another molecule plays a role in accelerating the activation of ExoU by the saccharide, or another molecule is entirely sufficient for rapid activation. Here, we sought to isolate and characterize the molecule responsible for rapid activation of ExoU by using biochemical means to fractionate it from a yeast cell lysate, and following it by detection of *in vitro* phospholipase activity at short incubation times. Our results suggest that this molecule, termed the ExoU accelerator, purifies as a heat-labile, anionic, and extremely large (>670 kD) RNA-protein complex. This complex, however, is susceptible to RNase digestion, which causes the ExoU accelerator to migrate on a size-exclusion column as a ~18 kD molecule. Surprisingly, we find that the small-molecule activator does not co-purify with the ExoU accelerator, which we show is capable of activating ExoU without supplementation with the saccharide activator. This result suggests that yeast either contain two completely different ExoU activators, or that the saccharide activator is an unusual glycosylation modification on the ExoU accelerator.

Introduction

Reports from our lab and others demonstrate that the highly cytotoxic protein ExoU from *Pseudomonas aeruginosa* acts as a membrane-lytic phospholipase A_2 and requires one or more host cell factors for activation (Phillips et al., 2003; Sato et al., 2003). In Chapter 4, we revealed that a small, glucose-containing saccharide is sufficient to activate ExoU phospholipase activity. Puzzlingly , ExoU activation by the saccharide is undetectable until approximately 12 hours, and then proceeds in linear fashion. In addition, these results are not consistent with our finding of rapid *in vivo* phospholipase activity (Phillips et al., 2003), nor with data published at the time of these studies that demonstrate immediate activation of ExoU by a yeast cell lysate (Sato et al., 2003; Tamura et al., 2004). It has also been reported that a heat-labile protein or complex greater than 100 kD is responsible for ExoU activation (Sato et al., 2005).

The data taken together lead us to propose an activator-accelerator hypothesis, which suggests that another molecule, an "ExoU accelerator," might be involved in accelerating the process of ExoU activation by the saccharide *in vivo*. Here, we sought to test this hypothesis by isolating and characterizing the molecule or molecules in a yeast cell lysate that activate ExoU in an *in vitro* phospholipase activity at short incubation times (~3 hours). Our results suggest that the ExoU accelerator purifies as a heat-labile, anionic, and extremely large (>670 kD) RNA-protein complex. We find that this complex is degraded by RNase, which results in the ExoU accelerator migrating as a ~18 kD molecule on a size-exclusion column. Surprisingly, we find that the small-

molecule activator does not co-purify with the ExoU accelerator, which we show is active without supplementation with saccharide activator. This result suggests that yeast either contain two unique ExoU activators, or that the saccharide activator is an unusual glycosylation modification on the ExoU accelerator.

Experimental Procedures

Preparation of yeast cell lysate

Yeast cell lysate was prepared as described in Chapter 4, except a protease-deficient strain of *Saccharomyces cerevisiae* (LPY0312, *MATa leu2 pep4-3 prb1-1122 prc1-407 trp1 ura3-52*) was used to minimize protease activity during activity assays. Boiling and 3 kDa-filtration experiments were carried out as described in Chapter 4.

Phospholipase A_2 activity assays

In vitro phospholipase A_2 activity assays were carried out as described in Chapter 4, although the mixed micelles (400 µl/assay) were incubated with ExoU and fractionated yeast lysate at 37°C for only 3 hours (except where designated as 18 hours). As described in Chapter 4, the specific activity (measured activity for the ExoU accelerator at a certain time-point per the protein quantity of fractionated yeast lysate) was used to determine the purest fractions. Protein quantity of lysate was determined with a Bradford assay. SDS-PAGE with Coomassie or silver staining was used to assess protein purity.

Pull-down of yeast lysate with ExoU-biotin

ExoU with an N-terminal 6XHis-tag was cloned into the pAC6 vector (Avidity) to express a C-terminal AviTag-protein fusion for *in vivo* modification with biotin. DNA sequencing verified the integrity of this construct. His-ExoU-biotin was expressed in

AVB101 *E. coli* cells containing an IPTG (isopropyl-1-thio-β-D-galactopyranoside)-inducible birA gene to over-express biotin ligase (pBirAcm). Cell growth and expression was carried out as described previously (Cull and Schatz, 2000). Briefly, cells were grown at 37°C to OD_{600} ~0.8 in TYH media containing 0.5% glucose, 100 μg/ml ampicillin, and 10 μg/ml chloramphenicol. Expression was induced with 1.5 mM IPTG, and cells were supplemented with 50 μM biotin and shaken overnight at 25°C. His-ExoU-biotin was purified by metal chelation and size-exclusion chromatographies as described previously (Phillips et al., 2003).

His-ExoU-biotin (100 ug) in 1 mL binding buffer (50 mM Tris·Cl, pH 8.0, 150 mM NaCl, 5% glycerol) was incubated with 100 μl packed streptavidin beads (Pierce) that had been previously washed with binding buffer for 45 minutes at room temperature with gentle agitation. Beads were washed 8 times with binding buffer, and then were incubated with 1 mL yeast lysate for 1 hour at room temperature with gentle agitation. The flow-through was collected, and beads were washed with binding buffer 10 times using the column method. Bound proteins were eluted with 200 μl 8 M Urea. Beads were then boiled in SDS-PAGE loading buffer. All samples were analyzed by SDS-PAGE. A negative control experiment with beads alone (no ExoU-biotin) was performed.

Depletion assays were used to assess loss of ExoU accelerator after pull-down with the ExoU-biotin-loaded beads. Activity assays were performed to compare the

activity of pre-column lysate with the flow-through of both negative control and ExoU-biotin pull-down samples.

Ammonium sulfate precipitation

Standard methods were used to determine the ammonium sulfate precipitation concentration at which ExoU accelerator precipitates. Solid ammonium sulfate was added slowly (equal amounts over 30 minutes) to a known volume of yeast lysate while stirring rapidly on ice. The sample was centrifuged (35,000 x g, 30 min, 4°C) and the pellet was resuspended in phospholipase activity reaction buffer and used in activity assays, Bradford assays, and SDS-PAGE analysis.

Anion exchange chromatography

The pellet from 40% ammonium sulfate precipitation of yeast lysate was resuspended in buffer (30 mM Tris·Cl, pH 8.0), filtered through a 0.4 μm filter, and fractionated on a strong anion exchange column (Poros HQ/M, 10 mm x 100 mm) in 30 mM Tris·Cl, pH 8.0, at a flow rate of 15 ml/min. A 20-1000 mM NaCl gradient followed by a step to 3 M NaCl over 20 column volumes was used for sample elution. To assess specific activities, selected fractions were precipitated with 85% ammonium sulfate and centrifuged (35,000 x g, 30 min, 4°C). Pellets were resuspended in phospholipase activity reaction buffer and used in activity and Bradford assays.

A weak anion-exchange column (Poros PI, 4.6 mm x 100 mm) was used to further fractionate yeast lysate. The column was run in 50 mM N-methylpiperazine, pH 5.0, at a

flow rate of 5 ml/min. A 20-1000 mM NaCl gradient followed by a step to 3 M NaCl over 20 column volumes was used for sample elution.

Size-exclusion chromatography

The 85% ammonium sulfate pellet following anion exchange chromatography was resuspended in size-exclusion column buffer (50 mM Tris·Cl, pH 8.0, 150 mM NaCl, 5% glycerol), filtered through a 0.4 μm filter (Millex, Millipore), and fractionated on a size-exclusion column (Superdex 200, Pharmacia, 16mm x 600mm) at a flow-rate of 1.0 ml/min. Purity of fractions was assessed by specific activity and SDS-PAGE as above.

A Superose 6 column (Pharmacia, 10mm x 300mm) was used at a flow rate of 0.35 ml/min to fractionate extremely large biomolecules and complexes in 40% ammonium sulfate precipitated yeast lysate, and purity of fractions was determined as above. On both size-exclusion columns, gel filtration standards (BioRad) were used to calibrate molecular mass with elution times.

Hydrophobic interaction chromatography

The pellet from 40% ammonium sulfate precipitation of yeast lysate was resuspended in pre-column buffer (50 mM Tris·Cl, pH 7.0, 1.5 M NaCl), filtered through a 0.4 μm filter, and fractionated on a hydrophobic interaction column (Poros PE [phenyl ether], 4.6 mm x 100 mm) in 30 mM Tris·Cl, pH 8.0, at a flow rate of 5 ml/min. A

decreasing 1200 to 0 mM ammonium sulfate gradient over 20 column volumes was used for sample elution. Purest fractions were determined as above. Other hydrophobic interaction columns with higher hydrophobicity (HP2, phenyl) or lower hydrophobicity (ET, ether) were also used.

Mass spectrometry analysis

Standard mass spectrometric techniques were used to identify proteins in fractions with highest specific activities. Controls included fractions with low or no activity. Samples were prepared by reducing proteins with 10 mM reducing agent dithiothreitol (DTT) at 37°C for 30 minutes, alkylating with 50 mM iodoacetimide at room temperature for 30 minutes, and then digesting with trypsin (1:100 mass trypsin: mass sample) overnight at 37°C. Tryptic peptides were identified by sequencing through liquid chromatography followed by tandem mass spectrometry (LC/MS/MS). The genome sequence of *S. cerevisiae* was used for protein identification via peptide fingerprinting.

Activity assays with URA2, FAS1 and FAS2

A viable strain of *Saccharomyces cerevisiae* (*ura2::G418*) with the *ura2* gene deleted and replaced by a G418-resistance cassette was used to prepare lysates as above for 3-hour activity assays. Because deletion of *fas1* or *fas2* renders yeast inviable, *Saccharomyces cerevisiae* strains containing these genes fused endogenously to a TAP-tag (FAS1-TAP and FAS2-TAP) were used to prepare yeast lysates. Lysates were prepared as above, except in TBS-NP40 (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2% NP40). Lysates were incubated with pre-washed Sepharose-IgG beads

(Amersham) overnight at 4°C with gentle agitation. Beads were washed 5 times with TBS-NP40, and protein was eluted by cleavage with TEV protease and 0.1 mM DTT for 3 hours at room temperature with gentle agitation. Eluted protein was analyzed by SDS-PAGE with silver staining, and bands at expected size were excised, digested in-gel with trypsin, and identified by mass spectrometry as above. Approximately 10 μg of FAS1, FAS2, or FAS1 and FAS2 together were tested for the ability to activate ExoU in 3-hour activity assays. The yeast strains used for these experiments were a gift from the Huilin Zhou lab (UCSD).

Construction of *S. cerevisiae* (FAS1-TAP, *ura2::G418*)

Homologous recombination with a PCR product was used to replace the *ura2* gene with a G418- resistance cassette in *S. cerevisiae* (FAS1-TAP). Primers were designed for the genome regions flanking *ura2* (Forward primer: CCTGTCGACAACAACCACAAACAAAAGG and reverse primer: GTGGTAGATGATGCATTGTCTCTGC) and were used to amplify a PCR product from the genomic DNA of *Saccharomyces cerevisiae* (*ura2::G418*). The PCR product was transformed by standard procedures into *S. cerevisiae* (FAS1-TAP) and plated onto YPD media containing 0.5 mg/mL geneticin sulfate (G148 plates). Insertion of the G418-resistance cassette was confirmed by PCR.

Results

A yeast cell extract activates ExoU immediately as a phospholipase

Our data with the small molecule ExoU activator demonstrated that no *in vitro* phospholipase activity was detected until approximately 12 hours of incubation of ExoU, small molecule activator, and mixed micelles. These data suggest that another molecule might play a role in accelerating the activation of ExoU by the small molecule activator, or be sufficient to activate ExoU in a more rapid manner.

To address our hypothesis that another molecule might accelerate the process of ExoU activation, we sought to test whether ExoU is activated by a yeast cell lysate immediately by testing for activity after 3 hours (Fig. 5.1, gray bars). We found that the soluble fraction of a yeast cell lysate was able to cause immediate ExoU phospholipase A_2 activity (Fig. 5.1, 'Lysate', gray bar), as has been reported in the literature (Sato et al., 2003). As seen in previous overnight assays, the yeast cell lysate by itself has no phospholipase activity (Fig. 5.1) and neither did ExoU by itself (data shown in Chapter 4). We hypothesize from this data that another molecule, operationally named the ExoU accelerator, must play a role in causing rapid activation of ExoU phospholipase activity by the activator.

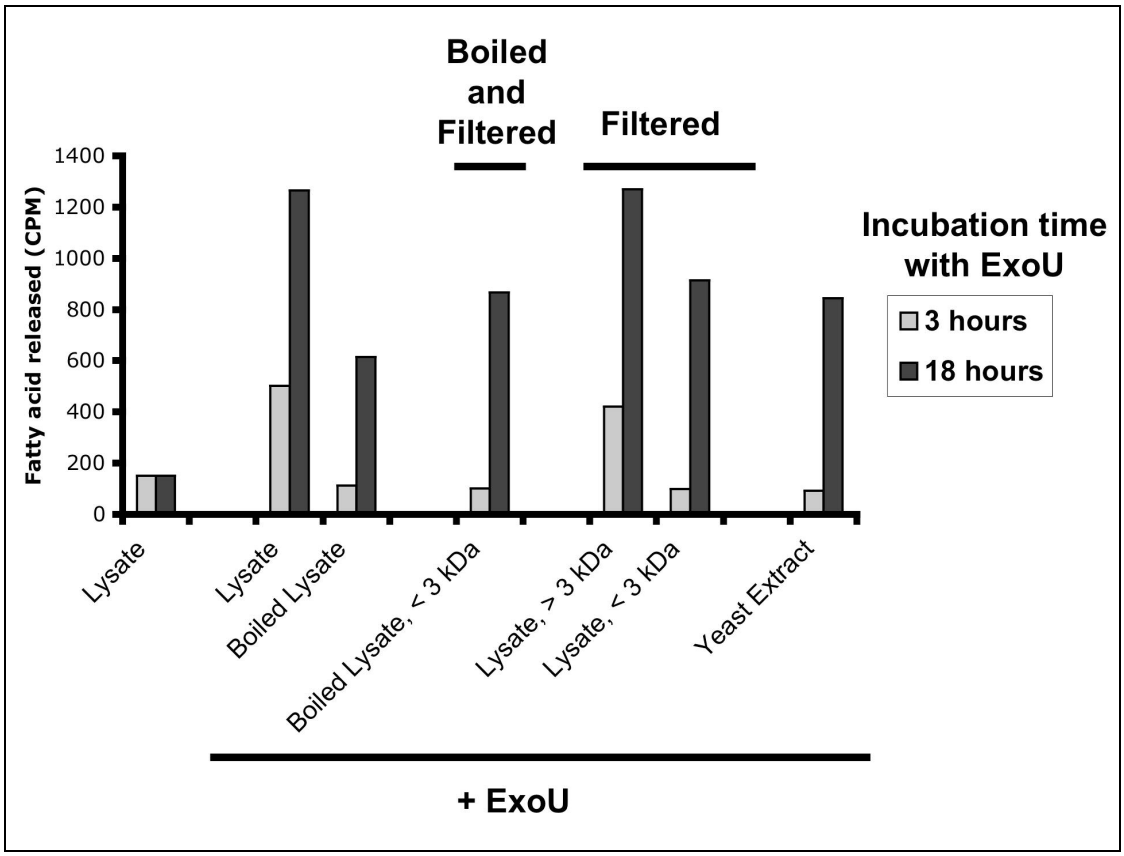


Figure 5.1. Phospholipase assays testing for immediate/accelerated activity (3 hours) or delayed/no acceleration activity (18 hours). Mixed micelles were incubated with buffer alone, or ExoU supplemented with yeast cell lysate that was untreated or treated as indicated, or yeast cell extract.

ExoU accelerator is heat-labile and retained by a 3kD-filter

To provide initial characterization of the ExoU accelerator, a yeast cell lysate was boiled for 10 minutes at 100°C, and insoluble material was removed by centrifugation. Interestingly, the supernatant of the boiled extract was found to lose its immediate phospholipase activity, suggesting that the ExoU accelerator is a heat-labile molecule (Fig. 5.1, 'Boiled Lysate', gray bar). This is consistent with a report published during the course of this research that phospholipase activity is lost with boiling of the yeast lysate (Sato et al., 2005).

Furthermore, the yeast cell lysate was subjected to filtration with a 3 kDa-cutoff membrane, and immediate phospholipase activity with ExoU was found only with the retentate (i.e., portion retained by the membrane) (Fig. 5.1, 'Lysate, >3kDa', gray bar). This indicates that the ExoU accelerator is larger than 3 kDa, which is also consistent with the report published during the course of this research that suggested a molecular weight >100 kDa (Sato et al., 2005).

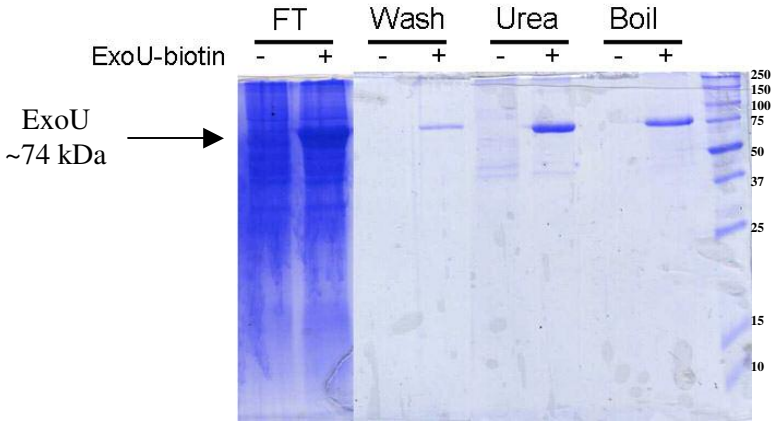
ExoU pull-down of yeast lysate

A pull-down assay was used to determine if ExoU could pull-down the heat-labile ExoU accelerator, as evidenced by SDS-PAGE, or if the ExoU pull-down could deplete the lysate of ExoU accelerator, as evidenced by activity assays. ExoU with a C-terminal biotin tag was purified and bound to streptavidin-linked beads. Beads alone, or ExoU -coated beads, were incubated with yeast lysate for 1 hour at room temperature. Beads were washed, and urea was used to elute bound proteins. Finally, beads were boiled to

release strongly bound proteins. No protein band, besides ExoU, was evident in the urea or boiling elutions of ExoU-coated beads, as compared to the beads alone (Figure 5.2A). Because some ExoU-biotin is eluted in these steps, it was not possible to visualize a bound protein with a similar size to ExoU.

To see if ExoU beads depleted the accelerator from the lysate, activity assays were used to compare lysate with the flow-through fractions of beads alone or ExoU-coated beads. Despite the presence of protease inhibitors in the lysate, ExoU seemed to be cleaved from the beads, as seen by the appearance of an ExoU band in the flow-through sample in Figure 5.2A. This resulted in an increase in phospholipase activity, as compared to pre-bead lysate or lysate incubated with beads alone (Figure 5.2B). These data suggest that ExoU accelerator must be purified and identified by other means.

A.



B.

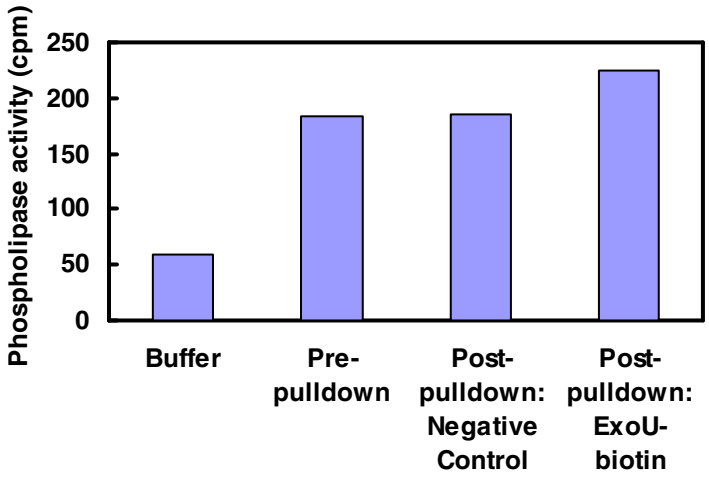


Figure 5.2. Pull-down of yeast lysate and resulting activity assays. A, SDS-PAGE with Coomassie staining. Beads alone, or ExoU-biotin immobilized on strepatvidin beads, were used for pull-down assays with yeast lysate. FT = flow-through or unbound fraction, Wash = final wash of beads, Urea = 8 M urea elution step, Boil = boiled beads. **B,** Activity assays of ExoU incubated with pre-pulldown yeast lysate, FT of beads (Negative Control), or FT of ExoU-beads.

ExoU accelerator purification by ammonium sulfate fractionation

Standard methods were used to determine the concentration of ammonium sulfate required to precipitate or "salt out" the ExoU accelerator. The advantage of this method is that proteins are precipitated in folded form, meaning that we can resuspend the precipitated pellet and test for the presence of ExoU accelerator. As seen in Figure 5.3A, most of ExoU's accelerator precipitated between 40 and 60% ammonium sulfate. However, we sought to isolate the purest fraction of ExoU accelerator, that is, the fraction with the highest specific activity. To determine this, we used a Bradford assay to quantify the amount of protein in each fraction, and then plotted the specific activity (measured activity at a certain assay time-point per quantity of protein in the fractionated yeast lysate). Protein content was also visualized by SDS-PAGE (Fig. 5.3B). Specific activity calculations showed that the purest fraction was the 0 to 20% ammonium sulfate cut (Fig. 5.3C). We believe that this was somewhat deceiving, however, since this fraction contained nearly background levels of activity and very little protein, making the specific activity of the sample not very dependable. Notably, the 20-40% cut had nearly 5-fold higher specific activity than the 40-60% cut, indicating that while a larger proportion of the ExoU accelerator was in the 40-60% cut, a much purer sample of ExoU accelerator could be obtained in the 20-40% cut. Based on these results, we chose to use a 0-40% ammonium sulfate cut as the first step in future purification techniques.

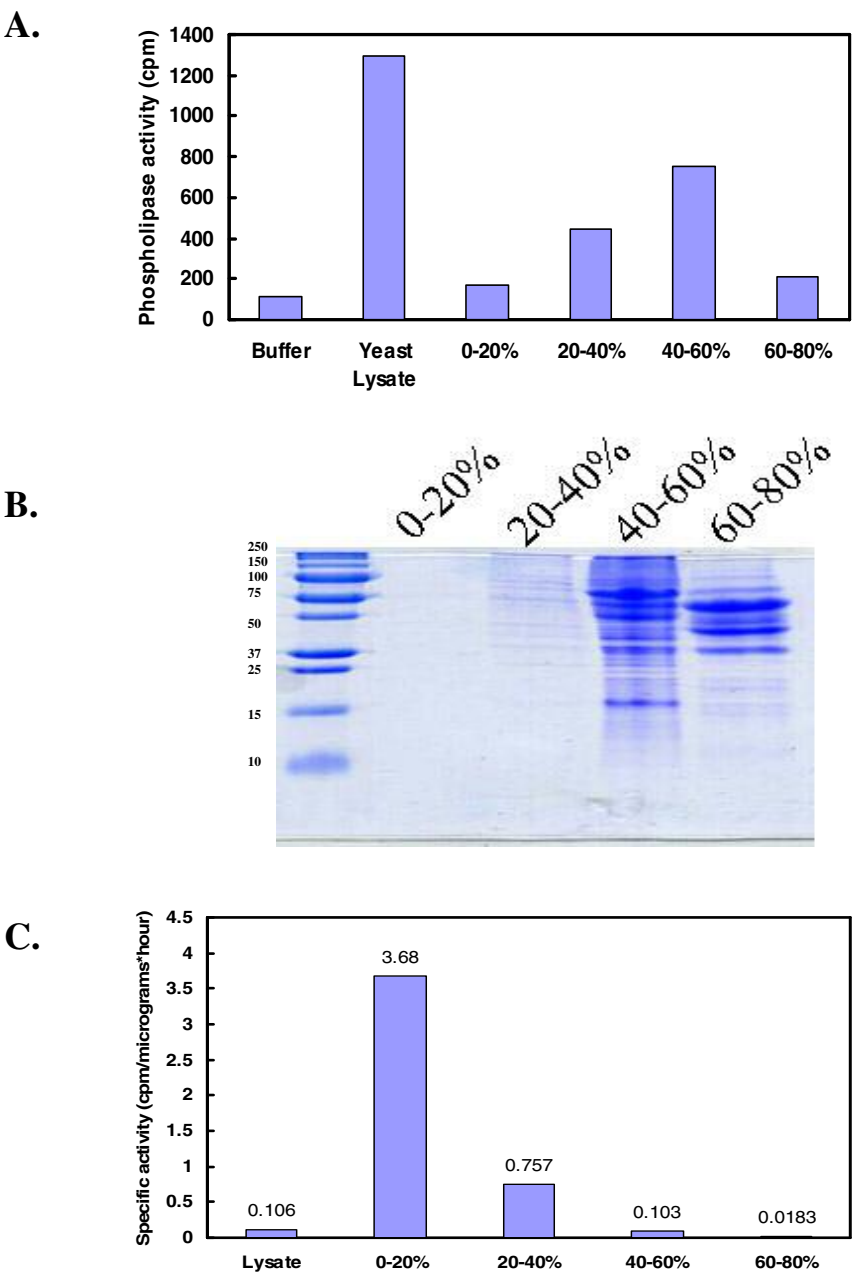


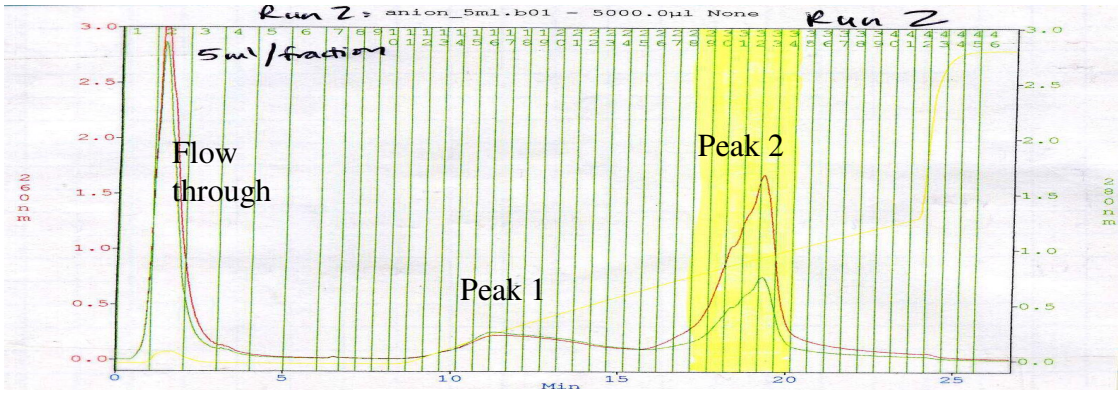
Figure 5.3. Purification of ExoU accelerator with ammonium sulfate precipitation. **A,** Activity assay using the pellets from each of the specified fractions. **B,** SDS-PAGE with Coomassie staining of the specified ammonium sulfate precipitation fractions. **C,** Specific activity for each of the specified fractions, as determined by the following calculation: [(activity sample- activity buffer)/ (μg sample used in activity assay)·(hours of activity assay)]

ExoU accelerator is anionic

We used strong anion exchange (Poros HQ/M) chromatography to purify the ExoU accelerator. The 40% ammonium sulfate precipitated cut of yeast lysate was resolubilized and fractionated on the anion exchange column. Fractions were then precipitated with 85% ammonium sulfate, and pellets were resuspended in buffer and tested in activity assays. As seen in Figure 5.4A, the ExoU accelerator eluted from this column at ~600-700 mM NaCl, suggesting that it is a strongly anionic molecule or complex. SDS-PAGE analysis of fractions eluting from this column demonstrate that the sample is significantly purer after this step.

Because of the late elution from the strong anion column, we sought to better resolve the ExoU accelerator purification using low pH and a weak anion column (Poros PI). However, even after lowering the pH with N-methylpiperazine buffer (pH 5.0) to mask some negative charge on the ExoU accelerator, no better resolution could be seen using this column (Fig. 5.4C). Only one peak eluted from the column, at a similar concentration of salt, and activity was found only under this peak.

A.



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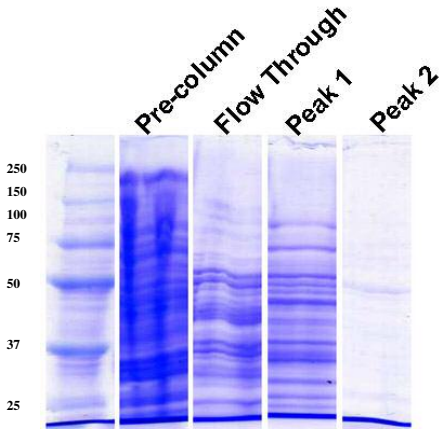


C.

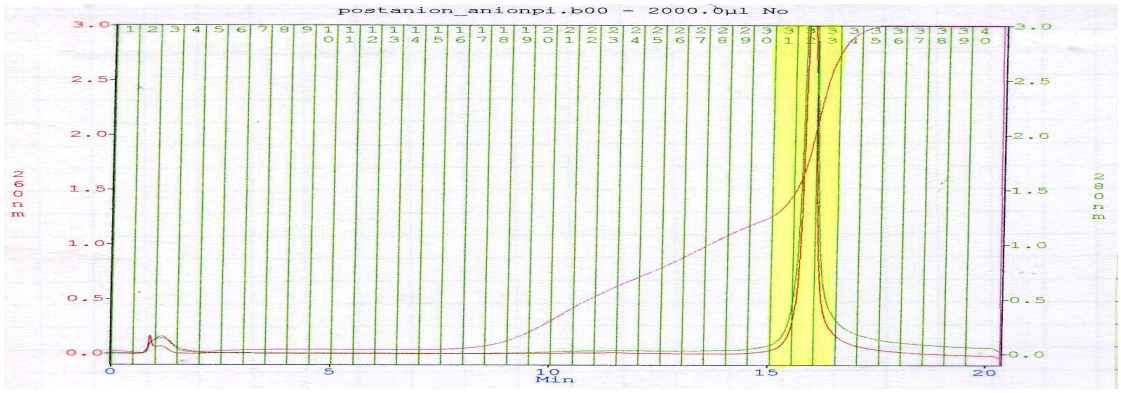


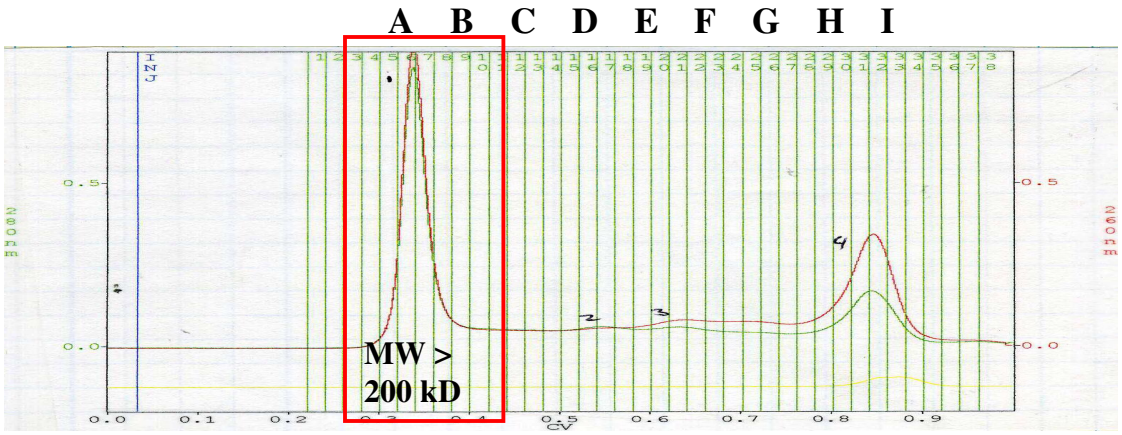
Figure 5.4. Purification of ExoU accelerator by anion exchange chromatography. A, Strong anion exchange chromatography trace. Fractions found to activate ExoU are highlighted in yellow, and NaCl conductivity gradient is shown as the yellow line. **B,** SDS-PAGE with Coomassie staining of peaks eluted from anion-exchange column. **C,** Weak anion exchange chromatography at pH 5.0. Fractions found to activate ExoU are highlighted in yellow, and NaCl conductivity gradient is shown as the pink line.

ExoU accelerator elutes as a very large molecule or complex on a sizing column

The post-anion ExoU accelerator was further fractionated on a Superdex 200 sizing column which resolves molecules between 10 and 600 kDa. Fractions were tested as before in an activity assay. As seen in Figure 5.5A, ExoU was activated only by fractions in the void volume, or unresolved volume, of the column. These data suggest that the ExoU accelerator is likely a very large protein or part of a complex of molecules. A large proportion of proteins run in the included volume of the column, as evidenced by SDS-PAGE (Fig. 5.5B) and Bradford assays. Thus, highest specific activities were also found in the void volume fractions (data not shown).

To better separate a molecule or complex of this size, we turned to a Superose 6 size-exclusion column, which resolves very large molecules up to 5000 kDa. The 40% ammonium sulfate precipitated cut of yeast lysate was fractionated on a Superose 6 column, and fractions were tested as before in an activity assay. Interestingly, the ExoU accelerator was still seen in the void volume of the column, indicative of a very large complex (Fig. 5.5C).

A.



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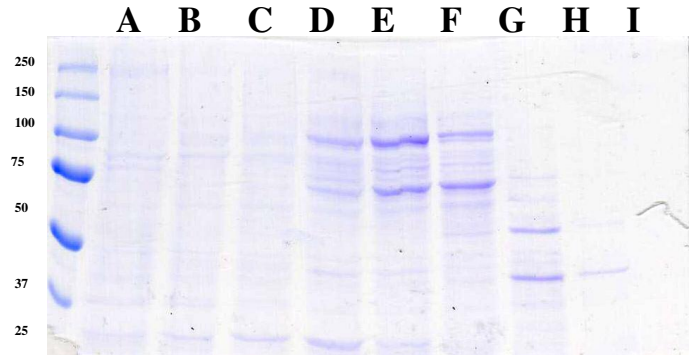


C.

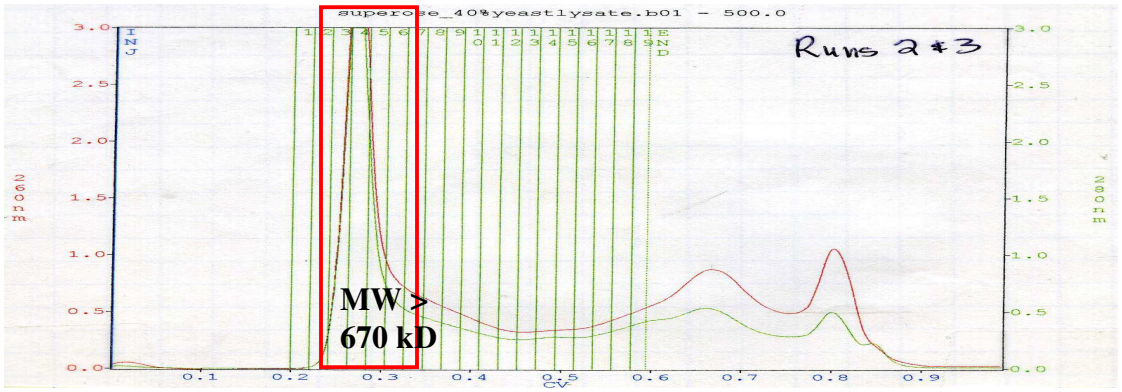


Figure 5.5. Purification of ExoU accelerator by size-exclusion chromatography. Size-exclusion traces of 40% ammonium sulfate precipition cut of yeast lysate purified on (A) Superdex 200 column or (C) Superose 6 column. Fractions with majority of activity are boxed. **B,** SDS-PAGE with Coomassie staining of fractions eluted from Superdex 200 column.

ExoU accelerator does not bind a hydrophobic interaction column

Three different types of hydrophobic interaction columns (Poros HP2 (phenyl), PE (phenyl ether), or ET (ether)) were tested for their ability to further purify the ExoU accelerator. The 40% ammonium sulfate precipitated cut of yeast lysate was suspended in high-salt buffer (see methods) and injected onto a hydrophobic interaction column. Protein was eluted with a decreasing 1200 to 0 mM ammonium sulfate gradient. Fractions were tested as before in an activity assay. As seen in Figure 5.6, the ExoU accelerator eluted in the flow-through of the PE column, as it did for the HP2 and ET columns also (data not shown). Although an impurity was found to bind the column, we found that this impurity is purified away by the anion exchange column (data not shown), suggesting that combination of hydrophobic and anion exchange offer no advantage in purification of the ExoU accelerator.

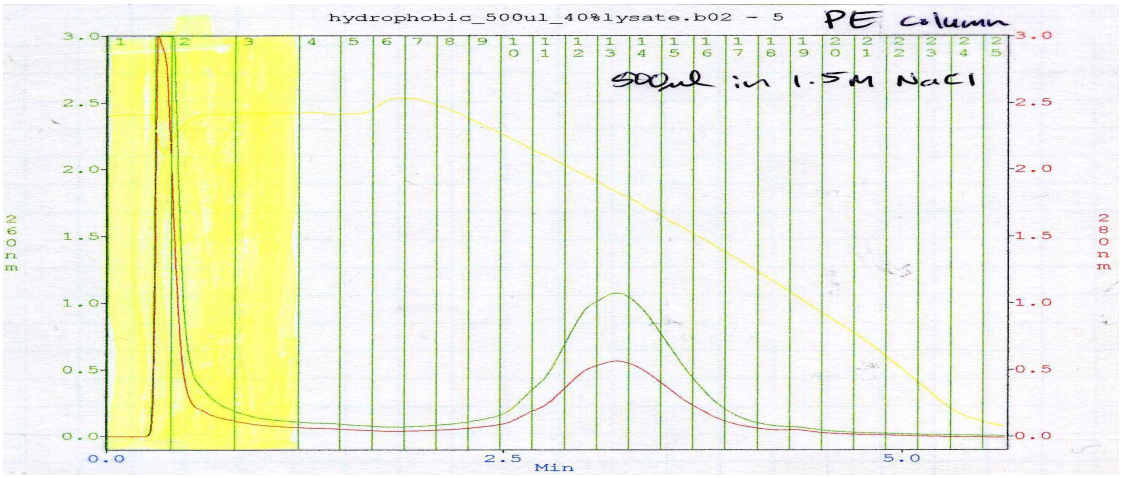


Figure 5.6. Hydrophobic interaction chromatography trace. Yellow line indicates decreasing ammonium sulfate gradient. Fractions found to activate ExoU are highlighted in yellow.

URA2, FAS1 and FAS2 are major contaminations in the purified ExoU accelerator

Consecutive purification steps of 40% ammonium sulfate precipitation, strong anion exchange chromatography and Superdex 200 size-exclusion chromatography were used to purify ExoU accelerator (data not shown). We used the Superdex 200 column because, at the time, the Superose 6 column was not functioning. Fractions with high, low, and no specific activity were used for mass spectroscopy identification. Samples were trypsinized overnight and tryptic peptides were analyzed by sequencing through liquid chromatography followed by tandem mass spectrometry (LC/MS/MS). The genome sequence of *S. cerevisiae* was used for protein identification via peptide fingerprinting.

Sequencing results identified three major proteins in the high specific activity fraction: URA2, which catalyzes the first two enzymatic steps in the biosynthesis of pyrimidines, and fatty acid synthetases 1 and 2 (FAS1 and FAS2), which form a large multimeric complex. To determine whether any of these genes were involved in ExoU activation, we considered whether they were essential or non-essential genes.

First, URA2 is a non-essential gene (assuming uracil is provided in growth media). As a result, a yeast mutant with a deletion of the non-essential gene URA2 (*ura2::G418*) was already tested previously for its survival during ExoU expression, and it was not found to survive (see SGA screen in Chapter 3). However, to confirm that URA2 is not involved in ExoU activation, we made a yeast lysate from the *ura2::G418* mutant and asked whether it could activate ExoU in a 3-hour activity assay. As expected,

lysate from this mutant yeast was able to activate ExoU as efficiently as wild-type yeast (data not shown). This implies that URA2 is a contaminating protein during the purification of ExoU accelerator.

Next, we considered FAS1 and FAS2. Both genes are essential, so analysis of mutant yeast with a *fas1* or *fas2* gene deletion was not possible. Instead, we turned to a library of yeast where each open reading frame is tagged from its natural chromosomal location with a tandem affinity purification tag (TAP-tag) (Ghaemmaghami et al., 2003). The TAP-tag consists of two calmodulin binding peptides, a TEV protease cleavage site, and two IgG binding domains of *S. aureus* protein A, which allow for a simple purification of any yeast protein. Yeast containing FAS1-TAP or FAS2-TAP were lysed and TAP-tagged fusions were pulled down with Sepharose IgG beads. FAS1 and FAS2 were removed via TEV protease cleavage, and proteins were analyzed by SDS-PAGE with silver staining (Fig. 5.7). Bands at the expected molecular weight were excised and in-gel trypsinized, and LC/MS/MS confirmed the presence of FAS1 and FAS2 in each pull-down elution (data not shown). Furthermore, LC/MS/MS data revealed that FAS1 was able to pull-down FAS2, and vice versa, as expected since these two proteins have been reported to form a complex (Krogan et al., 2006). We then tested whether FAS1, FAS2, or both proteins together purified by the above means could activate ExoU. No activity was detected, suggesting that FAS1 and FAS2 are also contaminating proteins in the purification of the ExoU accelerator.

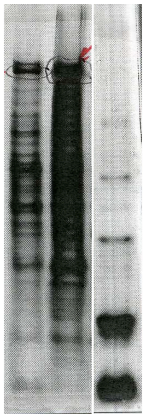


Figure 5.7. SDS-PAGE with silver staining of FAS1 and FAS2 used for activity assays. Lane 1 = FAS2 (~207). Lane 2 = FAS1 (~229). Bands in each lane were cut out, in-gel trypsin digested, and verified by mass spectroscopy. In each case, both FAS1 and FAS2 tryptic peptides were seen in mass spectroscopy data, suggesting association of FAS1 and FAS2.

To eliminate these three contaminating proteins from the yeast lysate, we used homologous recombination to delete the *ura2* gene in the FAS1-TAP yeast strain (data not shown). We chose the FAS1-TAP fusion yeast strain because FAS1 is produced in higher quantities than FAS2 in yeast, and FAS2 co-purifies with FAS1 during pull-downs.

Purification of ExoU accelerator from *S. cerevisiae* (FAS1-TAP, *ura2::G418*)

Consecutive purification steps of 40% ammonium sulfate precipitation, strong anion exchange chromatography, Sepharose-IgG pull-down of FAS1-TAP, and Superdex 200 size-exclusion chromatography were used to purify ExoU accelerator from *S. cerevisiae* (FAS1-TAP, *ura2::G418*) lysates (data not shown). Fractions with high, low, and no specific activity were used for mass spectroscopy identification as before.

Sequencing results revealed over 100 proteins in the high specific activity fraction (data not shown). This fraction was collected from the void volume of the sizing-column, indicative of very large proteins or protein complexes. Our sequencing data confirmed this, with the majority of proteins sequenced being ribosomal or ribosome-associated. Due to the high abundance of ribosomes in cells, we believed that they were likely contaminants in the ExoU accelerator purified sample. The only protein identified in both the high and low specific activity fractions was an essential 14-3-3 protein called BMH2. This caught our interest immediately because a 14-3-3 protein has been implicated in activation of the *P. aeruginosa* effector ExoS (Fu et al., 1993). Another candidate that caught our interest, SEC26, is involved in endoplasmic reticulum-to-Golgi (ER-to-Golgi) protein trafficking. This interested us because the ExoU-related protein VipD from *Legionella pneumophila* has been reported to interfere with ER-to-Golgi trafficking (Shohdy et al., 2005). However, further investigation of BMH2 and SEC26 revealed that they were not likely the ExoU accelerator. We tested this with depletion assays, where partially purified lysates with TAP-tagged versions of the candidate proteins (following 40% ammonium sulfate precipitation and anion exchange) were tested for loss of activity after pull-down of the TAP-tag containing molecule. No decrease in activity was detected (data not shown).

RNase digestion causes a major change in size-exclusion retention time

The previous mass spectroscopy data suggest that ribosomal proteins are complicating identification, because they are likely to be overwhelming in abundance compared to that of the ExoU accelerator. To break down ribosomes or other nucleotide-

containing complexes, purified ExoU accelerator was subjected to digestion with RNase A and DNase. While no change in activity was detected (Fig. 5.8A), a significant change in size-exclusion retention time was observed following digestion (Fig. 5.8B). Activity assays reveal that the ExoU accelerator has a retention time of a ~18 kD molecule (Fig. 5.8C), and significant purification is observed, as evidenced by SDS-PAGE (Fig. 5.8D). Notably, this significant change is retention time seems attributable only to RNase A digestion, as similar effects are seen in the absence of DNase (Fig. 5.8B). Unfortunately, mass spectroscopy sequencing data was complicated by the presence of excess RNase A (~13.7 kD), which elutes slightly after the ExoU accelerator, and in one initial attempt no proteins were identified that are different from those in the "no activity" negative control in previous sequencing experiments.

A.

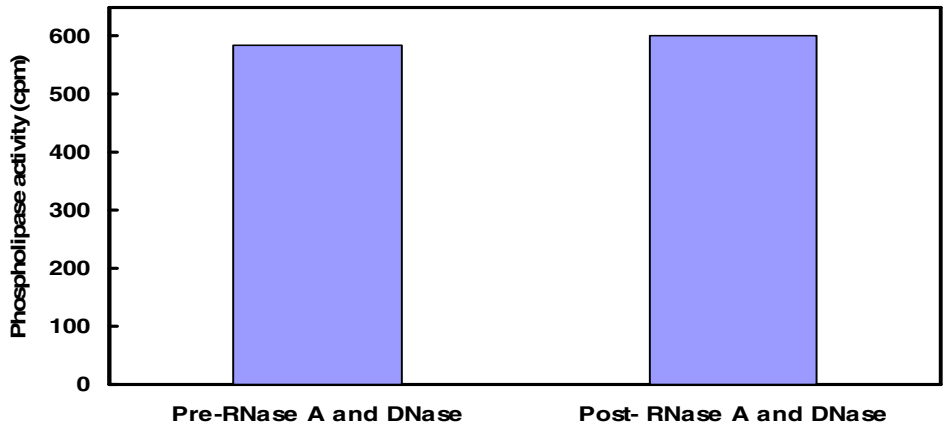
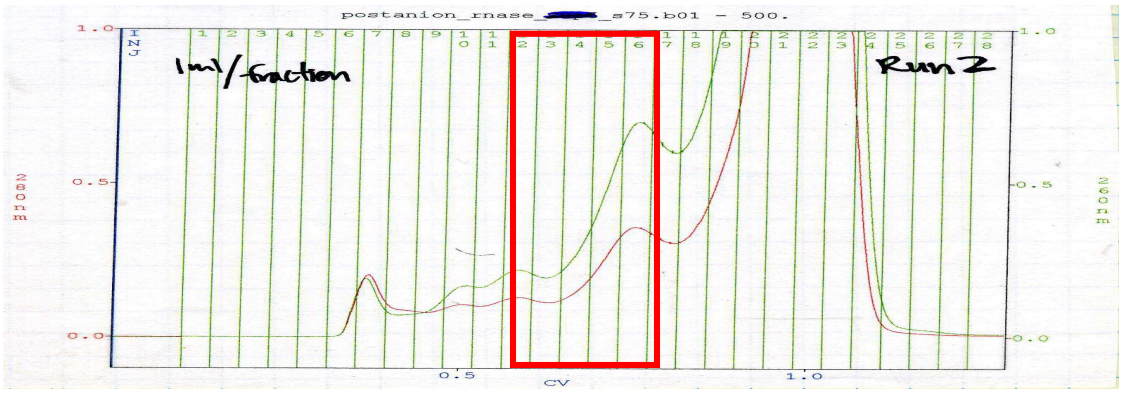
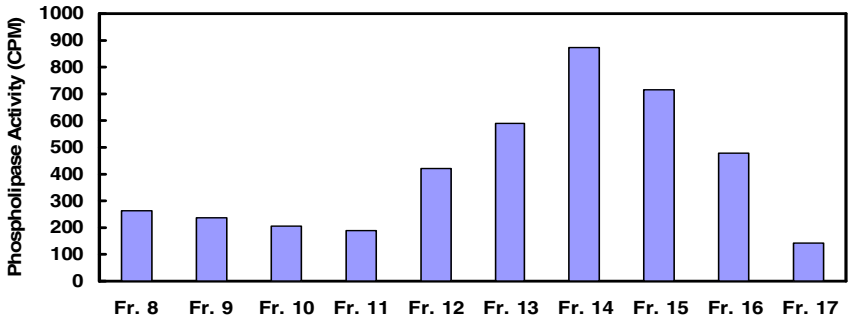


Figure 5.8. Effect of RNase A on ExoU accelerator. ExoU accelerator for these experiments was first purified by 40% ammonium sulfate precipitation and anion exchange chromatography. **A,** Activity assay testing effect of digestion of ExoU accelerator with RNase A and DNase.

B.



C.



D.

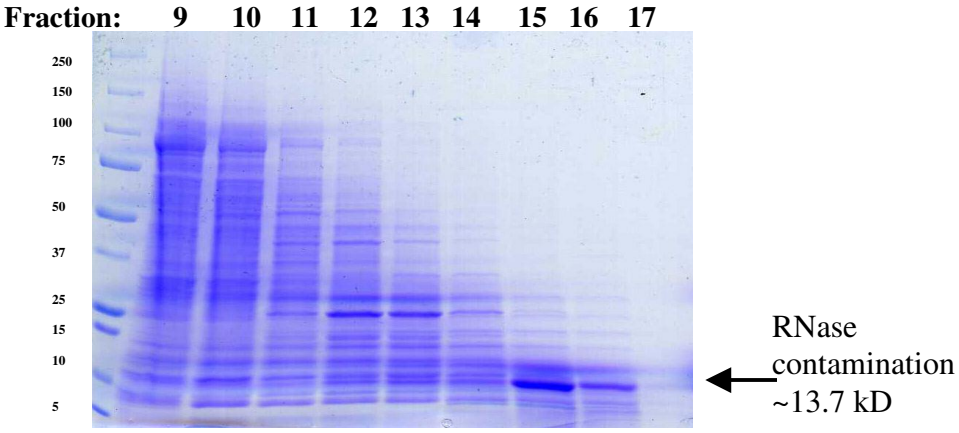


Figure 5.8 continued. Effect of RNase A on ExoU accelerator. ExoU accelerator for these experiments was first purified by 40% ammonium sulfate precipitation and anion exchange chromatography. **B,** Superdex 75 column chromatography trace. Fractions with majority of activity are boxed. **C,** Activity of each of the indicated fractions. **D,** SDS-PAGE with Coomassie staining of size-exclusion column fractions.

Boiling purified ExoU accelerator does not release small-molecule activator

In Figure 5.1, we addressed the differences between *in vitro* phospholipase activities during a short incubation (3 hours) and a long incubation (18 hours). While immediate activity is seen only with a heat-labile factor in yeast lysate, long incubations reveal that a small, heat-labile molecule is able to activate ExoU as a phospholipase. This data led us to the hypothesis that the small molecule activator is the only factor required for activity, and that the heat-labile factor accelerates this activation process. However, we did not rule out other models, such as one where two separate activators exist: one heat-labile molecular species that activates 'rapidly' *in vitro* after 3 hours of co-incubation, and a second heat-stable and small molecular species that activates slowly after 12 hours co-incubation.

To distinguish between these models, we asked whether the small-molecule activator co-purified with the accelerator. First, we tested whether the addition of small-molecule activator (from 3 kDa-filtered yeast extract) was able to increase the activity of the ExoU accelerator at short activity assay times. No increase in activity was detected after any purification step (data not shown) suggesting that, if our model is correct, the small-molecule ExoU activator co-purifies with the ExoU accelerator.

We tested the idea of co-purification directly by boiling and 3 kDa-filtering the ExoU accelerator after the ammonium sulfate precipitation and anion exchange purification steps. If our model was correct, we reasoned that we would detect small-molecule activity in an 18-hour activity assay. As seen in Figure 5.9, 18-hour activity

was detected in the boiled, 3 kDa-filtered ammonium sulfate precipitation purified ExoU accelerator, consistent with our hypothesis. However, following anion exchange chromatography, the boiled, 3 kDa-filtered ExoU accelerator was no longer active after 18 hours. Before boiling and filtering, this sample did have 3-hour activity, demonstrating the presence of ExoU accelerator. These data suggest that our accelerator-activator hypothesis is not correct, or may be more complicated than originally proposed.

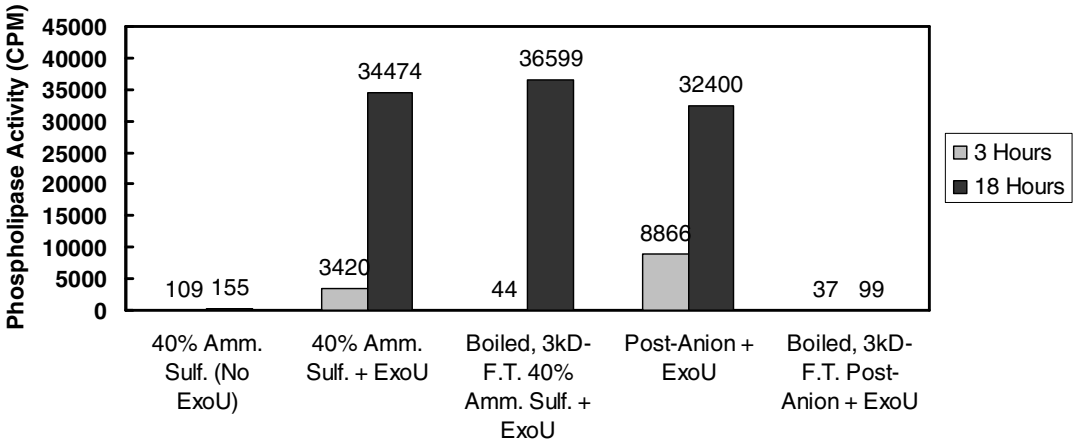


Figure 5.9. Phospholipase assays testing for presence of small-molecule activator in purified ExoU accelerator sample. Mixed micelles were incubated with ExoU supplemented with the 40% ammonium sulfate cut of yeast lysate or the same sample further purified by anion exchange. The samples were untreated to test for presence of functional ExoU accelerator (3 hours) or boiled and 3 kDa-filtered to test for presence of small molecule activator after 18 hours.

Discussion

We approached the experiments in this chapter with the hypothesis that an accelerating molecule was involved in activation of ExoU by the saccharide activator, but that the ExoU accelerator alone would be unable to activate ExoU. We tested our hypothesis by isolating the factor in a yeast lysate required to activate ExoU *in vitro* phospholipase activity at short-incubation times, and asking whether the ExoU activator co-purifies with this factor. Our results suggest that the ExoU accelerator purifies as a heat-labile, anionic, and extremely large (>670 kD) RNA-protein complex. We report intriguing results that this complex is degraded by RNase, resulting in the ExoU accelerator migrating on a size-exclusion column as a ~18 kD molecule. Most importantly, we find that the small-molecule activator does not appear to co-purify with the ExoU accelerator. Purified accelerator is able to activate ExoU without supplementation of the saccharide activator, and heat-denaturation of the purified accelerator did not release a heat-stable activator.

One possible explanation for these results, among many, is that the saccharide activator is an unusual glucose-only glycosylation modification of the ExoU accelerator. In this model, we would not expect to see release of the saccharide activator upon heat-denaturation of the ExoU accelerator, because the saccharide activator is covalently attached. This type of "glucosylation" has been reported in a few cases for extracellular proteins involved in blood clotting (factor VII, factor IX, protein Z, and thrombospondin) and a receptor involved in cellular development, Notch 1 (Bjoern et al., 1991; Moloney et

al., 2000; Peter-Katalinic, 2005). Testing this hypothesis before identification of the ExoU accelerator may be challenging, because this type of glucosylation is found to be O-linked, and no general O-glycosidase exists to digest partially purified ExoU accelerator and test for the loss of ExoU activation or release of saccharide activator. Our initial tests of incubation of purified ExoU accelerator with the N-glycosidase PNGase F showed no decrease in ExoU rapid activation (data not shown). Furthermore, complete removal of glycosylation modifications on native proteins is often difficult due to steric hindrance. Once we identify the ExoU accelerator, we will investigate the presence of any type of glycosylation modification using standard methods.

While we have not ruled out the possibility that yeast contain two unique ExoU activators, a heat-labile fast-acting activator and a heat-stable slow-acting activator, it seems unlikely that a toxin would have a slow-acting activator when it can be quickly activated with another molecule. Furthermore, the exquisite specificity of the saccharide activator, as evidenced by the numerous molecules that were unable to activate ExoU, suggests an important role for this saccharide in ExoU activation.

Overall, our experiments offer significant progress in the purification of the heat-labile molecule involved in ExoU activation. Whether this molecule is a glycosylated activator or an activator alone has yet to be determined and will likely require its identification.

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VI.

Appendix A.

ExoU crystallization trials

Summary

Reports from our lab and others demonstrate that the highly cytotoxic protein ExoU from *Pseudomonas aeruginosa* acts as a membrane-lytic phospholipase A_2 and requires one or more host cell factors for activation (Phillips et al., 2003; Sato et al., 2003). Visualization of the molecular structure of ExoU would be invaluable for understanding the mechanism of ExoU host cell activation and construction of ExoU-specific antimicrobial inhibitors. Here, we carried out X-ray crystallographic studies of purified full-length ExoU, ExoU truncation fragments, and ExoU-related proteins. Protein constructs were set up in vapor-diffusion crystallization trials using rational (McPherson, 1985) and sparse matrix (Jancarik and Kim, 1991) screening methods. Limited proteolysis digestion of full-length ExoU was used to identify protease-resistant truncation fragments. ExoU-related proteins RP534 from *Rickettsia prowazekii* and VipD from *Legionella pneumophila* were also investigated. We report that crystallization attempts from nine protein constructs yielded no crystals for further investigation.

Experimental Procedures

Purification of ExoU, RP534, and VipD Constructs

Full-length ExoU and ExoU truncation fragments were expressed and purified as described previously (Phillips et al., 2003). For removal of the His-tag, purified His-ExoU or His-ExoU (45-687) (both containing a thrombin cleavage site for His-tag removal) were incubated with purified thrombin (Sigma) at a ratio of 1:200 (mg thrombin: mg His-ExoU). Reactions were carried out in cleavage buffer (50 mM phosphate buffer, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$) at room temperature for 20 minutes with gentle agitation. Reactions were stopped by the addition of 5 mM EDTA and 2 mM phenylmethylsulphonylfluoride (PMSF). Loss of His-tag was monitored by western blotting. ExoU or ExoU (45-687) was purified from cleaved His-tag and thrombin by size-exclusion chromatography.

To produce purified ExoU/SpcU-His, *Pseudomonas aeruginosa* PA103 chromosomal DNA was used a template in PCR amplification of both the *exoU* open reading frame and the downstream *spcU* open reading frame, whose start codon overlaps with the stop codon of *exoU* (Finck-Barbancon et al., 1998). The PCR product was cloned into pET28b (Novagen) so that SpcU (cloned without the stop codon) was in-frame with a C-terminal His-tag. ExoU/SpcU was expressed and purified as described for His-ExoU (Phillips et al., 2003)., except purified protein was stored in 10 mM Tris (pH 8) and 10 mM βME.

RP534 was expressed and purified as described in Chapter 4. VipD from *Legionella pneumophila* cloned into the pET15b (Novagen) expression vector was a gift of Dr. Howard Shuman (Columbia University Medical Center). VipD was expressed and purified in the same way as RP534.

Activity Assays

Activity was assessed by toxicity to mammalian cells in a syringe-loading assay, as described previously (Phillips et al., 2003), for the following constructs: His-ExoU, ExoU, ExoU (45-687), His-ExoU (104-687), His-ExoU (45-667), His-ExoU (403-687), and ExoU/SpcU-His. *In vitro* phospholipase A_2 activity assays were carried out, as described in Chapter 4, to determine activity for the following constructs: His-ExoU, His-RP534, and His-VipD.

Limited Proteloysis

Papain protease (Boehringer Mannheim) was activated by incubating 1 mg/ml papain in activation buffer (10 mM Tris (pH 8.0), 1 mM dithiothreitol (DTT), and 1 mM EDTA) for 5 minutes at 37°C. Immediately after papain activation, ExoU was incubated with papain at a ratio of 1:1000 (mg papain: mg ExoU) in reaction buffer (100 mM MES buffer (pH 6.5), 150 mM NaCl, 2 mM DTT) at room temperature. Samples of digestion reaction were inactivated at specific timepoints by boiling in SDS-PAGE sample buffer containing 50 mM iodoacetic acid (Sigma). Samples were

separated by SDS-PAGE, transferred to a nitrocellulose membrane, and stained with Coomassie blue. Bands were cut out and used for N-terminal sequencing analysis.

Limited-proteolysis digestion of ExoU for mass spectroscopy analysis was carried out by incubating ExoU with papain at a ratio of 1:10,000 (mg papain: mg ExoU) at room temperature for 2 hours. Papain was inactivated by the addition of 100-fold molar excess of E-64 inhibitor (Roche). Proteolysis fragments were separated on a Superdex 75 (Pharmacia) size-exclusion column. Mass spectrometric analysis of purified digested ExoU fragment (~66 kD) was carried out by Dr. Ross Hoffman at the Department of Chemistry and Biochemistry Mass Spectrometry Facility.

Crystallization Trial Conditions

Purified protein constructs were set up in vapor-diffusion crystallization trials using rational (McPherson, 1985) and sparse matrix (Jancarik and Kim, 1991) screening methods. Approximately 400 conditions were screened for each protein at room temperature, and trials were monitored over approximately 2 months.

Purified ExoU (45-687) at 50 mg/ml was sent to the High-Throughput Crystallization Laboratory at the Hauptman-Woodward Medical Research Institute for automated screening of 1536 crystallization conditions at room temperature. Digital photos were taken to monitor the screen over one month.

Results

Crystallization trials

Nine protein constructs (Fig. 6.1A) were expressed, purified, and set up in vapor-diffusion crystallization trials of ~400 conditions. Activity of each construct was determined by toxicity to mammalian cells or *in vitro* phospholipase activity. Purity of each construct was assessed by SDS-PAGE (Fig. 6.1B). In most cases, the protein constructs were found to be highly soluble and exist in a monomeric and monodispersed state (as indicated by size-exclusion chromatography and dynamic light scattering). The exception was ExoU/SpcU-His, which eluted from a size-exclusion column (Superdex 200) in two peaks that both contained ExoU and SpcU-His (data not shown). The first peak eluted in the void volume of the column, suggesting very large heteromeric complexes or soluble aggregates. The second, later-eluting peak elutes as a ~110 kD molecule (as compared to globular protein standards), which is similar to the predicted molecular weight of ExoU and a dimer of SpcU-His (~105 kD). The protein in this second peak was pursued for crystallography.

Rational fragments were designed by inspecting the predicted secondary structure of ExoU. Comparison with the structure of human cPLA$_2$α, whose crystal structure has been determined (Dessen et al., 1999), revealed that ExoU residue 104 is the first residue with predicted homology to that of the human cPLA$_2$α catalytic core domain. Thus, His-ExoU (104-687) was investigated. Further inspection of the ExoU

predicted secondary revealed highly helical structure in the second half of ExoU, with the first residue of the first predicted helix in the second half of ExoU being residue 403. Because the C-terminal region of ExoU is essential for its activity, as shown by our lab and others (Finck-Barbancon and Frank, 2001; Phillips et al., 2003), His-ExoU (403-687) was investigated.

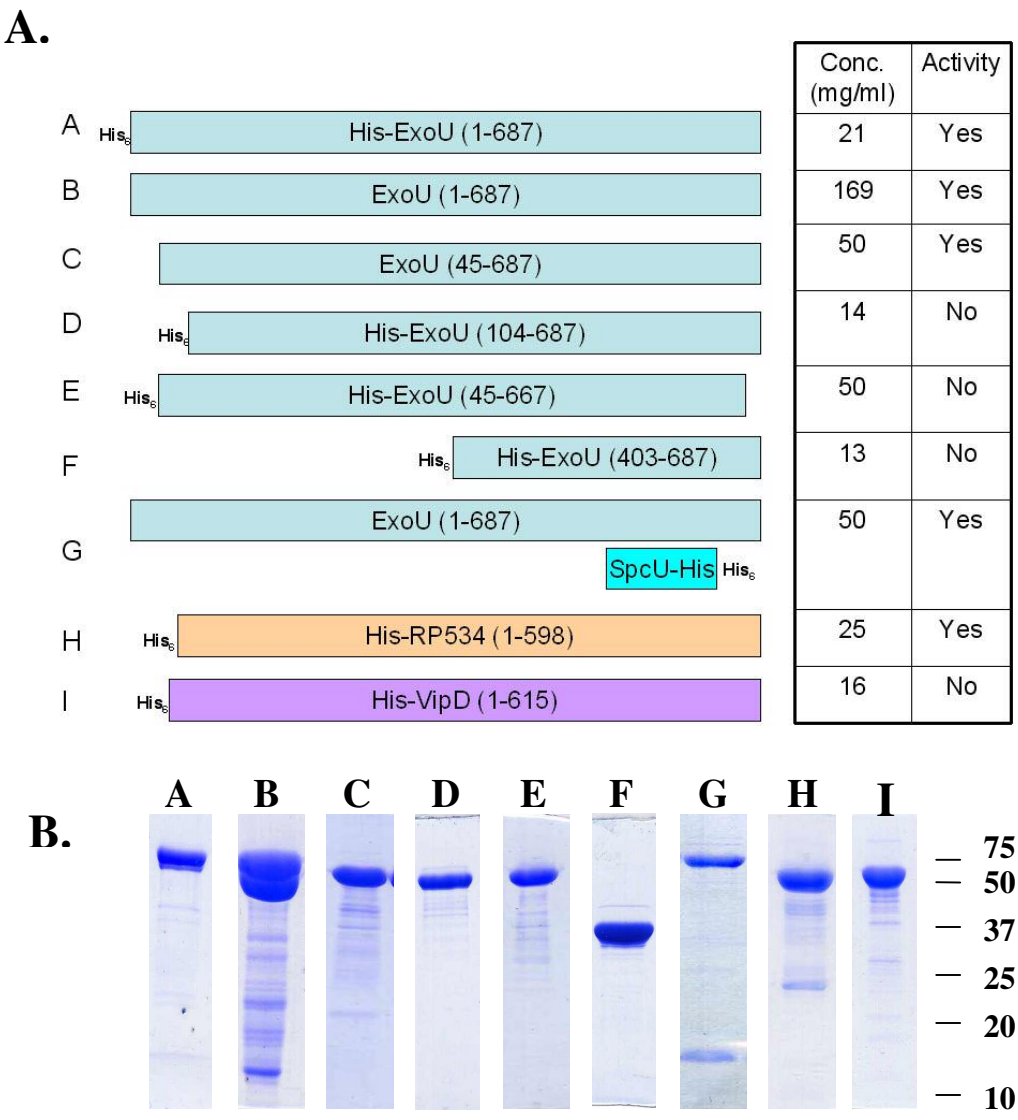

Figure 6.1. Protein constructs used in crystallization trials. **A,** Protein constructs used in crystallization trials, final concentration of each construct, and activity of the purified product, as assessed by toxicity to mammalian cells (A, B, C, D, E, F, G) or *in vitro* phospholipase activity (A, B, H, I). In all cases, crystallization trials were set up with 1 µl protein and 1 µl crystallization solution. **B,** SDS-PAGE with Coomassie staining of purified proteins.

Limited proteolysis experiments were carried out to identify fragments of ExoU more amenable to crystallization. Papain was used to digest full-length ExoU and SDS-PAGE was used to visualize digestion timepoints (Fig. 6.2). A slightly smaller fragment at ~65 kD was observed, and N-terminal sequencing revealed that the first 44 amino acids of ExoU were cleaved (data not shown). Mass spectroscopy analysis of the purified fragment was unable to resolve an exact molecular weight, suggesting that the fragment may be heterogeneous with regard to the C-terminal digestion site. An estimate of the ExoU fragment size was determined by SDS-PAGE, and two constructs were made for crystallographic analysis: His-ExoU (45-687) and His-ExoU (45-667).

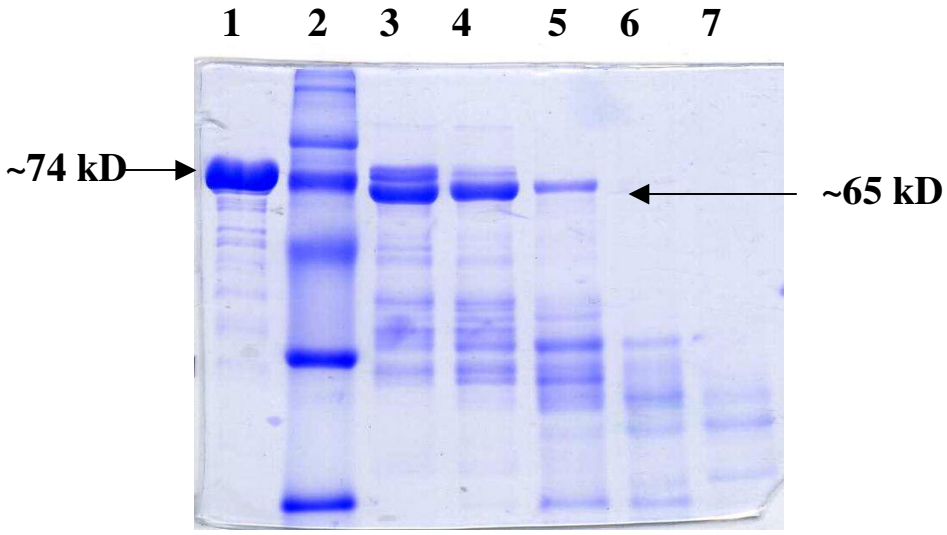

Figure 6.2. Time course of limited proteolysis digestion of ExoU. SDS-PAGE analysis of ExoU digestion with papain. Lane 1, undigested ExoU. Lane 2, molecular weight markers. Lanes 3 through 10 indicate time-points after digestion (2, 5, 15, 30, 60, 120, and 180 minutes, respectively).

Finally, ExoU-related proteins were investigated in crystallization trials. RP534 from *Rickettsia prowazekii* has ~37% sequence homology to ExoU, and VipD from *Legionella pneumophila* has ~33% sequence homology to ExoU.

Crystallization trials of ~400 conditions were set up for these nine protein constructs. In addition, the smallest active ExoU fragment investigated, ExoU (45-687), was purified and sent to the High-Throughput Crystallization Laboratory at the Hauptman-Woodward Medical Research Institute for automated screening of 1536 crystallization conditions. In all cases, we report that no protein crystals were grown.

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VII.

Appendix B.

Investigation of interactions between YopE and SycE

Summary

The type III secretion system (TTSS) of Gram-negative bacterial pathogens delivers effector proteins required for virulence directly into the cytosol of host cells. Delivery of many effectors depends on association with specific cognate chaperones in the bacterial cytosol. The mechanism of chaperone action is not understood. Here we present biochemical results on the *Yersinia pseudotuberculosis* SycE-YopE chaperone-effector complex that contradict previous models of chaperone function that chaperones maintain effectors in an unfolded and secretion-competent state. We demonstrate that the SycE-YopE chaperone-effector complex has RhoGAP catalytic activity, suggesting that the complex is not unfolded. Furthermore, we show specific association between purified SycE and GST-YopE fusion protein *in vitro*, which demonstrates that association does not depend on the process of folding or require unfolding of preexisting structure. These data supported the model that chaperones function to maintain general, three-dimensional TTSS secretion signals.

Experimental Procedures

Protein expression and purification

GST-RhoA was purified as previously described (Buetow et al., 2001), as were SycE (Birtalan and Ghosh, 2001) and GST-YopE (Black and Bliska, 2000), except for the addition of a size-exclusion chromatography step (Superdex 200, Pharmacia) in the case of GST-YopE. GST was purified by size-exclusion chromatography (Superdex 200) from cleaved GST-YopE fusion protein. SycD cloned from *Yersinia pseudotuberculosis* by PCR was expressed in pET28b (Novagen) with a thrombin-cleavable N-terminal histidine tag. His-SycD expression was induced with 1mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 25°C in *Escherichia coli* BL21 (DE3). Bacteria were lysed by sonication in 50 mM phosphate buffer (pH 8.0), 150 mM NaCl, 10 mM β-mercaptoethanol, 20 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.02 mg/mL DNase. Lysate was clarified by centrifugation (35,000 *g*) and soluble His-SycD was purified by metal chelation chromatography (Poros MC/M). The histidine tag was removed by thrombin digestion, and SycD was further purified by size-exclusion chromatography (Superdex 75, Pharmacia). Pure SycD was dialyzed into 10 mM Tris (pH 8.0) and 10 mM βME, and stored at -80°C.

GTPase Activity Assay

GTPase activity assays were performed as described previously (Black and Bliska, 2000), except radioactive quantification was carried out using a 96-well mixed cellulose filtration system (Millipore) and phosphoimaging. GST-RhoA (0.9 μg) was preloaded with 10 μCi [γ-^{32}P]-GTP (>2000 Ci/mmol, 10 μCi/μl, ICN Pharmaceuticals) by incubation at room temperature for 15 minutes in 40 μl total in 20 mM Tris (pH 7.6), 5 mM EDTA, 1 mM DTT. Preloading was stopped by the addition of 10 mM $MgCl_2$ and incubation on ice. Three microliters (0.13 μg) of the pre-loaded RhoA-GST was diluted into 30 μl of 20 mM Tris (pH 7.6), 0.1 mM DTT, 1 mM GTP, 1 mg/mL BSA. A 2.5 μl sample was removed (time = 0) and diluted into 250 μl ice-cold assay buffer (50 mM Tris (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$). In the appropriate reactions, 10 nM YopE-GST, YopE/SycE, or SycE was added at this time. Reactions were incubated at room temperature, and 2.5 μl samples were removed and diluted into 250 μl ice-cold assay buffer at 2.5, 5, 10, and 15 minutes thereafter. Samples were filtered through a 96-well mixed cellulose filtration system (Millipore) pre-wetted with ice-cold assay buffer. Filters were washed twice with 250 μl ice-cold assay buffer and then air dried. The amount of radioactivity bound to the filter was determined by phosphoimaging.

Binding Assay

Protein mixtures, containing 8 μM SycE, 4 μM GST-YopE, 4 μM GST, or 10 μM SycD, were incubated for 1 hr at room temperature in 150 mM NaCl, 50 mM Tris (pH 8.0), and a protease-inhibitor cocktail. Samples were then incubated with glutathione-agarose beads at 4°C for 10 min. Beads were washed four times with 150 mM NaCl and 10 mM phosphate buffer (pH 7.4). Bound proteins were eluted using 50 mM Tris (pH 8.0) and 10 mM reduced glutathione, and immediately precipitated with trichloroacetic acid.

Results

Catalysis by Chaperone-Effector Complexes

The TTSS needle has a diameter (20–30 Å) (Blocker et al., 2001) that appears too small for the transport of folded proteins, for example, of the YopE RhoGAP domain, which has a minimum dimension of 25 Å (Evdokimov et al., 2002). This raises the suggestion that chaperones act to keep effectors in an unfolded and secretion-competent state (Stebbins and Galan, 2001). We reasoned that if this were the case, chaperone-effector complexes would be expected to lack catalytic activity. To test this, *Y. pseudotuberculosis* SycE-YopE complexes, expressed and purified from *Escherichia coli*, were assayed for RhoGAP activity. As in *Yersinia*, YopE produced in *E. coli* without SycE forms aggregates (data not shown). In contrast, SycE-YopE complexes are highly soluble and form heterotrimers, with a single YopE bound to a SycE dimer, as determined by sedimentation equilibrium centrifugation (data not shown) and in agreement with previous results (Cheng and Schneewind, 1999). SycE-YopE is highly stable ($K_d \sim 0.3$ nM) (Cheng and Schneewind, 1999) and not observed to dissociate except by denaturation.

In contradiction to the secretion-competency hypothesis, SycE-YopE complexes are found to be catalytically active (Fig. 7.1). The RhoGAP activity of SycE-YopE is similar to that of uncomplexed YopE (Black and Bliska, 2000), as represented by a glutathione S-transferase fusion of YopE (GST-YopE), which is

more biochemically tractable than aggregation-prone YopE. The increase in the rate of GTP hydrolysis by GST-RhoA as elicited by SycE-YopE is almost identical to that elicited by uncomplexed YopE, and SycE by itself has no RhoGAP activity (Fig. 7.1). The high SycE-YopE affinity and the tendency of free YopE to aggregate indicate that the catalytic activity observed for SycE-YopE derives from the complex and not from dissociated YopE. This result shows that the Ef domain is folded and functional while YopE is bound to SycE, which makes it unlikely that chaperones maintain effectors in an unfolded and secretion-competent state. This result also rules out the possibility that chaperones inhibit potential effector activity in the bacterial cytosol. Similar results have recently been reported for *E. coli* CesT-Tir and *Salmonella* SigE-SigD TTSS chaperone-effector complexes (Luo et al., 2001).

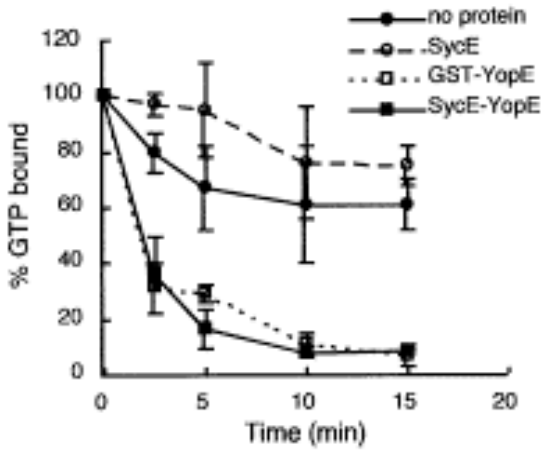

Figure 7.1. GAP activity assays. The effect of SycE, GST-YopE, or SycE-YopE on the GTPase activity of GST-RhoA was measured in a filter binding GAP assay. GST-RhoA (90 nM, preloaded with [γ-^{32}P]-GTP) was either incubated alone (no protein) or mixed with 10 nM GST-YopE fusion protein, SycE-YopE complex, or SycE, and samples were removed at specified time points for quantification of GTP bound to the filter. Data represent the mean ± standard deviation of three separate experiments.

YopE and SycE Can Associate in the Native State

The structure of free YopE(Cb) is not known but is unlikely to resemble the SycE-stabilized structure observed in the complex. It is possible that free Cb is structured and that association with SycE occurs during folding in vivo and would therefore require unfolding *in vitro*. Alternatively, free Cb may be unstructured and take on secondary structure only in association with SycE, and would therefore associate with SycE *in vitro* without an unfolding step. The short length of YopE(Cb), paucity of core-forming hydrophobic residues, and tendency to aggregate argue for free YopE(Cb) being unstructured. To distinguish between these possibilities, we examined *in vitro* binding between purified SycE and GST-YopE fusion protein. The two were incubated under native conditions, and association was assessed using a glutathione pull-down assay (Fig. 7.2). GST-YopE but not GST associates with SycE, and the association is specific as GST-YopE does not associate with SycD, a *Yersinia* TTSS chaperone specific to other effectors (Neyt and Cornelis, 1999). This experiment demonstrates that association does not depend on the process of folding or require unfolding of preexisting structure.

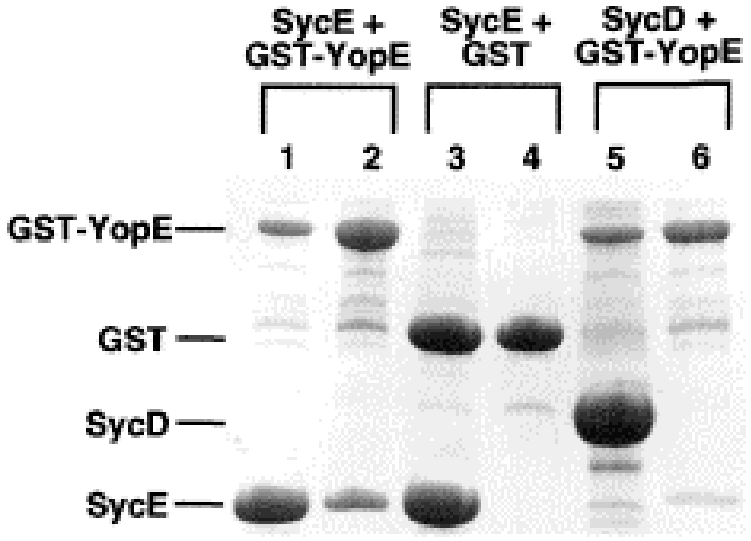

Figure 7.2. Specific binding between YopE and SycE under native conditions. SycE and GST-YopE fusion protein (lane 1), SycE and GST (lane 3), or SycD and GST-YopE (lane 5) were mixed, and association was detected with a glutathione pull-down assay (lanes 2, 4, and 6) and visualized by 15% SDS-PAGE.

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