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Identification of Superoxide Dismutase as a Cofactor for the *Pseudomonas* Type III Toxin, ExoU†

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ABSTRACT: *Pseudomonas aeruginosa* is an opportunistic pathogen that uses a type III secretion system and four effector proteins to avoid innate immune responses. ExoS, ExoT, ExoY, and ExoU all possess enzymatic activities that disrupt host cellular physiology and prevent bacterial clearance by host defense mechanisms. The specificity of these toxins for eukaryotic cells depends on the presence of substrate targets and eukaryotic cofactors responsible for effector activation. We used a combined biochemical and proteomic approach to identify Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD1) as a cofactor that activates the phospholipase activity of ExoU. Recombinant ExoU (rExoU) was activated in a dose-dependent manner by either bovine liver SOD1 or the yeast ortholog, Sod1p, but not by either Fe or Mn-containing SODs from *E. coli* or small molecule SOD mimetics. Inhibitor studies indicated that SOD enzymatic activity was not required for the activation of rExoU. The physical interaction between rExoU and SOD was demonstrated by capture techniques using either of the two proteins immobilized onto the solid phase. Identification of SOD as a cofactor allowed us to develop a new assay using a fluorescent substrate to measure the phospholipase activity of rExoU. The ability of SOD to act as a cytoplasmic cofactor stimulating ExoU phospholipase activity has significant implications for the biological activity of the toxin. Further elucidation of the structural mechanism of ExoU activation by this eukaryotic cofactor may provide a rational approach to the design of inhibitors that can diminish tissue damage during infection by ExoU-producing strains of *P. aeruginosa*.

Pseudomonas aeruginosa delivers four effector enzymes into eukaryotic cells via the type III secretion/injection system, ExoS, ExoT, ExoY, and ExoU. ExoS and ExoT are bifunctional enzymes that possess GTPase activating protein (GAP[1]) and ADP-ribosyltransferase activity (*1*−*7*). The GAP activity of ExoS/T inhibits phagocytosis and alters cytoskeletal structure in nonphagocytic cells. ExoT ADP-ribosylates CrkI and CrkII (*8*) and uncouples integrin signaling (*9*). ExoS ADP-ribosyltransferase activity targets members of the Ras superfamily and is correlated with cytotoxicity (*3*, *5*, *7*). The cellular toxicity of ExoS is postulated to be due to the inhibition of multiple signal transduction pathways and/or the disruption of actin cytoskeletal and receptor complexes (*10*−*12*). ExoY is an adenylyl cyclase that induces the accumulation of cytoplasmic pools of cAMP, which is associated with the disruption of intercellular junctions in endothelial cells and the leakage of fluids (*13*, *14*). ExoU is a potent member of the phospholipase A_2 family and hydrolyzes bilayer membrane lipids (*15*−*17*).

Type III-mediated intoxication of mammalian cells by *P. aeruginosa* strains producing only ExoU results in an acute cytotoxic response, rapid permeability, and necrotic death (*18*). ExoU is also toxic to yeast cells when protein expression constructs are induced (*15*, *17*, *19*). Motif alignment of *N*-terminal ExoU sequences with the plant phospholipase patatin, site-directed mutagenesis studies, and the visualization of a vacuolar fragmentation phenotype in yeast upon induction support a model in which ExoU is acutely cytotoxic through the cleavage of acyl side chains from membrane-associated phospholipids (*15*, *16*, *18*). ExoU phospholipase activity and resultant tissue necrosis perhaps explains the correlation between strains encoding ExoU, disease severity, and negative clinical outcome of both natural and model *P. aeruginosa* infections (*20*, *21*).

Changes in phospholipids and neutral lipids are easily detectable after in vivo induction of ExoU expression in either yeast or mammalian cells (*15*, *17*). In contrast, purified recombinant ExoU (rExoU) is inactive against liposome substrates (*15*). ExoS/T-encoded ADP-ribosyltransferase and ExoY-adenylyl cyclase are also relatively inactive in vitro unless extracts from eukaryotic cells are added to the reaction mixture. Coburn and Gill (*3*) and Fu et al. (*22*) identified members of the 14-3-3 family of proteins as cofactors for ExoS/T. These proteins are ubiquitously found in eukaryotes

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[1] Abbreviations: SOD1, Cu^{2+}, Zn^{2+}-superoxide dismutase; rExoU, recombinant ExoU; GAP, GTPase activating protein; LC/MS/MS, liquid chromatography and tandem mass spectroscopy; HA, hemagglutinin; TLC, thin-layer chromatography; POPC, 1-palmitoyl-2-oleoyl-phophatidylcholine; POPS, 1-palmitoyl-2-oleoyl-phophatidylserine; PED6, *N*-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; PLA_2, phospholipase A_2; RFUs, relative fluorescence units; SDS−PAGE, SDS-polyacrylamide gel electrophoresis; TBAP, tetra kis (4-benzoic acid) porphyrin; $cPLA_2$, cytosolic PLA_2.

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and function as molecular scaffolds facilitating signal transduction cascades. The cofactor for ExoY is currently unknown but appears to be present in mammalian cellular extracts and absent from yeast extracts (*13*). Sato et al. demonstrated that cytosolic extracts from either yeast or Chinese Hamster Ovary cells activate ExoU phospholipase activity in vitro (*15*, *16*). Noncatalytic forms of rExoU inhibit phospholipase activity of the wild-type enzyme, suggesting competition for a common factor (*16*). The activating factor is susceptible to heat and protease treatments and appears to be absent from prokaryotic soluble extracts (*16*). Combined, these data suggest that host factors are involved in the control of enzymatic activities of all of the known *P.* aeruginosa-encoded type III effectors.

In this article, we describe the use of biochemical techniques to enrich the cofactor that activates the phospholipase activity of ExoU. Proteomic screening with liquid chromatography and tandem mass spectroscopy (LC/MS/MS) led to the identification of Cu^{2+}, Zn^{2+}-superoxide dismutase 1 (SOD1) from either yeast or mammalian sources as an activating cofactor for this type III-secreted cytotoxin. Identification of the cofactor allowed us to develop a new assay to measure the phospholipase activity of ExoU. SOD is the first cofactor identified for ExoU and the first observation of a proteinaceous cofactor for known phospholipases. In addition, bacterial superoxide dismutases were unable to activate ExoU phospholipase activity, which suggests that the cofactor is eukaryotic cell specific. The identification of SOD as an ExoU cofactor carries intriguing implications for the biological role of the toxin and is consistent with the requirement of a eukaryotic cofactor for all known type III effector proteins from *P. aeruginosa.*

MATERIALS AND METHODS

Materials. All enzymes used for Figure 2A were purchased from Sigma, except for yeast proteins. *S. cerevisiae* strain (Y258) with a plasmid (BG1805) containing *SOD1* was purchased from Open Biosystems. Sod1p was induced on the basis of the manufacturer's protocol and purified using metal affinity chromatography. Protein expression was confirmed by SDS−PAGE and Western blot analyses using antibodies against hemagglutinin (HA) and histidine tags.

Ion Exchange Chromatography. A cationic exchanger SP sepharose was used to enrich for cofactor activity in fractions of yeast extract. Yeast proteins (60 to 120 mg) were loaded onto the ion exchange column, and the resin was washed with 50 mM MOPS. The bound proteins were eluted with 50 mM MOPS with various concentrations of NaCl as indicated. All eluants were dialyzed against 50 mM MOPS/50 mM NaCl (pH 6.3) prior to the phospholipase assay (*16*).

Sizing Proteins in Eukaryotic Cell Extracts Using SDS-Polyacrylamide Gel Electrophoresis (PAGE). SDS−PAGE (13.5% polyacrylamide gels) was used to narrow the size range of the protein cofactor. Cell extracts (100 to 200 μg per well) suspended in SDS sample buffer were loaded in several wells flanked by wells of prestained molecular weight markers. Protein samples were reduced but were not heat denatured before electrophoresis. After gel electrophoresis, proteins corresponding to different size ranges were excised from the gel on the basis of the molecular weight markers and extracted from the matrix by diffusion in 50 mM MOPS (pH 6.3). To remove the inhibitory effect of SDS on rExoU activity, gel eluants were dialyzed in 50 mM MOPS/50 mM NaCl (pH 6.3) to attain the optimal buffer conditions for the rExoU phospholipase assay.

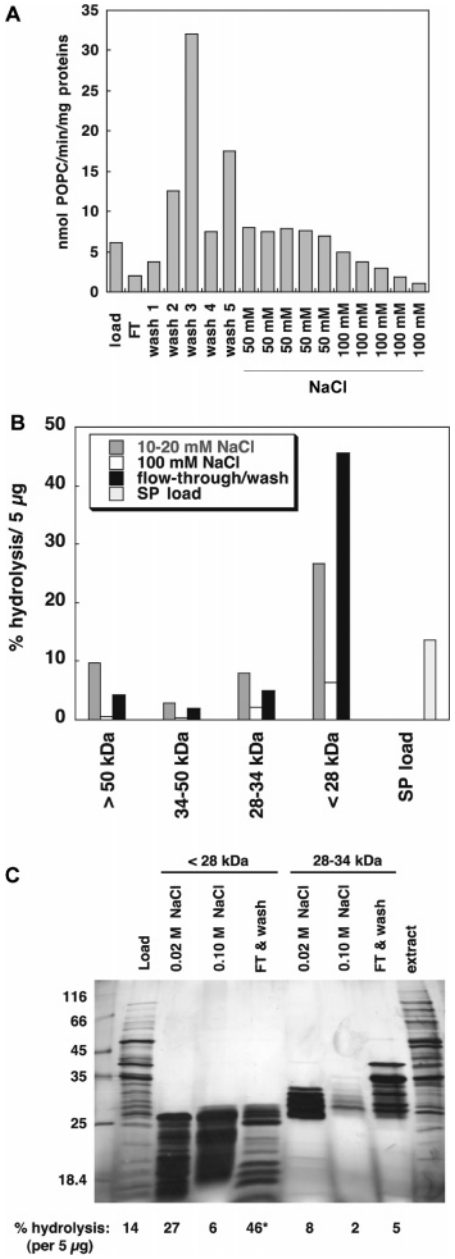

FIGURE 1: Enrichment of the cofactor. (A) Crude yeast extract dialyzed against 50 mM MOPS (pH 7), applied to an SP cation exchange column, and eluted with MOPS buffer containing varying NaCl concentrations (50 or 100 mM). Column fractions were tested for phospholipase activity and subsequently pooled and subjected to SDS−PAGE. (B) Cofactor activity of pooled samples from SP column elution followed by SDS−PAGE size-fractionation. Bands were excised, and 5 μg of the extracted proteins were assayed for phospholipase activity upon addition of 5 μg of rExoU. (C) Analytical SDS−PAGE following size fractionation of SP column fractions. rExoU phospholipase activity in the presence of 5 μg of protein from each given fraction is shown below each lane. (*) A majority of the cofactor activity eluted in the <28 kDa flow-through (FT)/no-salt washes. Proteins in this fraction were subjected to analysis by LC/MS/MS (Table 1).

Thin-Layer Chromatography (TLC) Assay of Phospholipase Activity. Phospholipase activity was determined by TLC as described previously (*16*), using 5 μg of rExoU and 5 μg

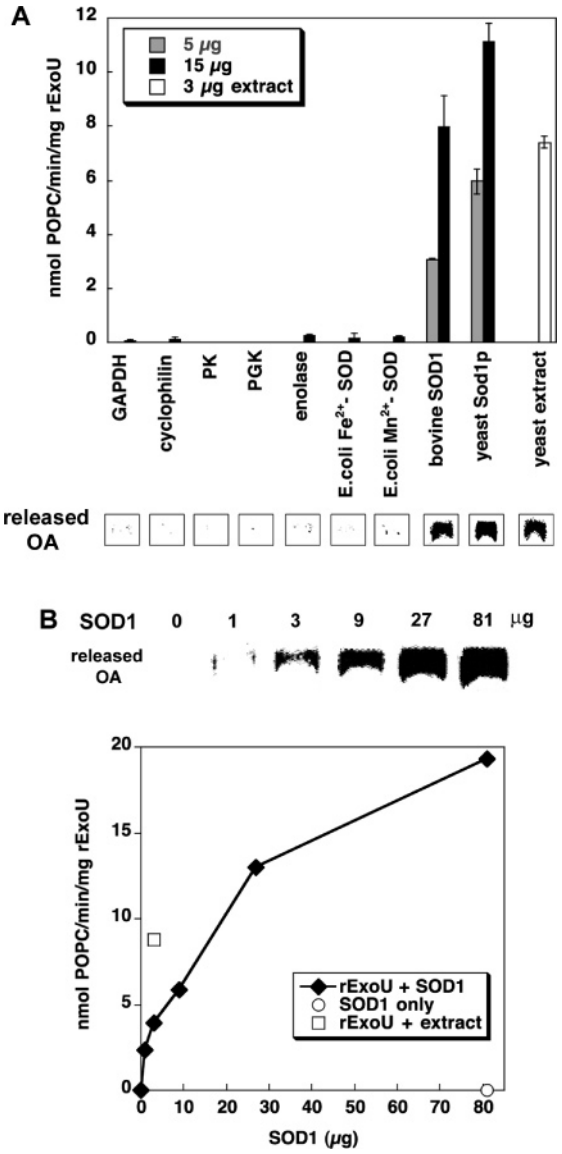

FIGURE 2: Phospholipase activity of rExoU in vitro in the presence of cofactor candidates (A) and SOD1 dose-dependence (B). (A) The cofactor candidates (either 5 or 15 μg) were tested for activation of rExoU (5 μg) using the TLC assay. Only bovine liver SOD1 and its yeast ortholog Sod1p were able to activate rExoU enzymatic activity. The TLC band of hydrolyzed oleic acid (OA) from POPC substrates is shown below the graph for each cofactor candidate. Glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and phosphoglycerate kinase are indicated as GAPDH, PK, PGK, respectively. (B) Phospholipid hydrolysis as a function of SOD1 concentration: TLC bands of the released oleic acid (OA) product (top) and specific activity of rExoU (bottom). Note that 80 μg of bovine SOD1 alone (○) had no detectable activity; 3 μg of yeast extract protein (□) was used as a positive control.

of candidate cofactor protein in the presence of large unilamellar vesicles containing the radiolabeled substrate. The composition of liposomes was a 1:1 molar ratio of 1-palmitoyl-2-oleoyl-phophatidylcholine (POPC) to 1-palmitoyl-2-oleoyl-phophatidylserine (POPS) containing 0.5 mol % 1-palmitoyl-2-[^{14}C]oleoyl-phophatidylcholine. After 1.5 h of incubation at 30 °C, the release of radiolabeled fatty acids (^{14}C-oleic acid from POPC) was quantitated using InstantImager Electronic Autoradiography (Packard). The reported values are the mean and standard deviation of assays done in triplicate.

Inhibition and Quantification of SOD Activity. Inhibition of bovine liver SOD1 (4 μg, Sigma) by 5 mM EDTA or 32 mM azide (*23*) was accomplished by preincubation for 15 min at room temperature. The treatment of SOD1 with 0.014 M HCl was performed at room temperature for 10 min, and the pH of the sample was neutralized by the addition of 0.014 M NaOH (*24*). Chelated SOD1 was prepared by dialyzing the protein against 5 mM EDTA/50 mM MOPS (pH 3.8) overnight (*25*), followed by a secondary dialysis against 50 mM MOPS (pH 6.3) to remove EDTA and released metals and to adjust the pH. SOD1 activity was measured by the oxidation of 5,6,6a,11b-tetrahydro-3,9, 10-trihydroxybenzo-[*c*]fluorene using a superoxide dismutase assay kit (Calbiochem). rExoU phospholipase activity was determined as described previously (*16*).

Capture of rExoU by Immobilized Yeast Sod1p. An anti-HA IgG antibody (130 nmol, Babco) was bound to protein G beads, washed, and incubated with 20 nmol of HA-tagged yeast Sod1p. Unbound Sod1p was removed by washing with 50 mM MOPS/50 mM NaCl (pH 6.3), and rExoU (4 nmol) was added to the column and incubated in the presence or absence of 0.2 mM liposomes at room temperature for 1 h. The column was washed with the above MOPS buffer, and the proteins were eluted with ImmunoPure IgG Elution Buffer (Pierce). The eluants were subjected to SDS−PAGE and Western blot using anti-ExoU monoclonal IgG.

rExoU−SOD Binding Assay Using Cobalt Affinity Column. rExoU (4 nmol) was immobilized on cobalt affinity sepharose, washed, and incubated with bovine SOD1 (20 nmol) in the presence or absence of 0.2 mM liposomes (POPC/POPS, 1:1) at room temperature for 1 h. The beads were carefully washed with 50 mM MOPS/50 mM NaCl (pH 6.3) and 15% of each sample was tested for in vitro phospholipase activity with the addition of free rExoU (7 μg). Histidine-tagged PcrV and the matrix itself were used as negative controls.

Phospholipase Assay Using Fluorescent substrate. A fluorescent phospholipase substrate PED6 (*N*-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt, Molecular Probes) was diluted to a concentration of 0.0297 mM with 50 mM MOPS/50 mM NaCl (pH 6.3). For rExoU titration, indicated amounts of rExoU and 10.5 μg of SOD1 were added to 50 μL of 0.0297 mM PED6 in a 96-well plate and incubated at room temperature for 30 min. A noncatalytic form of rExoU (D344A-rExoU) and honeybee venom phospholipase A_2 (PLA_2, Biomol) were used as controls. Relative fluorescence units (RFUs) were measured using a SpectraMax M2 microplate reader (Molecular Devices) with an excitation wavelength at 488 nm and an emission wavelength at 511 nm. The emission cutoff filter was set at 495 nm.

RESULTS

Enrichment of ExoU-Activation Factor. In our initial efforts to capture and identify proteins that supported rExoU phospholipase activity, we subjected yeast and Chinese hamster ovary cell extracts to several biochemical techniques, for example, immunoprecipitation, cross-linking, and Far Western blot. Proteins captured by these methods, however,

Table 1: Cofactor Candidate Proteins in the Less than 28 kDa Sample

Protein
enolase
aconitase
transketolase
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
cytoplasmic peptidyl-prolyl *cis*−*trans*-isomerase (cyclophilin)
glutaredoxin (thioltransferase)
(glutathione reductase)
branched-chain amino acid aminotransferase
protein for mitochondrial maintenance
transaldolase
peripheral membrane protein that interacts with a mating protein
Cu−Zn superoxide dismutase (SOD1)
nucleoside diphosphate kinase
translational elongation factor
DMRL (6,7-dimethyl-8-ribityllumazine) synthase
ubiquitin peptide
pyruvate decarboxylase
phosphoglycerate kinase (PGK)
salt induced protein (Sip18p)
proteinase
alcohol dehydrogenase
acetohydroxy-acid isomeroreductase
mitotic protein
protein disulfide isomerase
protein with an unknown function (7 proteins)

appeared to be bound nonspecifically and did not activate rExoU (data not shown). These results supported our previous observation (*16*) that the interaction between the cofactor and rExoU is apparently weak and/or transient. Additional methods for the enrichment of the activation factor included the use of sepharose column chromatography (anionic or cationic exchanger and phenyl or octyl hydrophobic beads), isoelectric focusing, sucrose density gradient centrifugation, or ammonium sulfate precipitation. None of these preparations significantly enriched the cofactor for rExoU (data not shown). Significant enrichment was detected, however, when we fractionated a soluble yeast extract by using a combination of ion exchange chromatography and SDS−polyacrylamide gel electrophoresis (SDS−PAGE). Briefly, a cytosolic extract from the yeast *Saccharomyces* cerevisiae was applied to an SP cation exchange column, and the ability to activate rExoU activity was measured. Fractions containing the highest specific activity eluted in the washes without NaCl (Figure 1). This enriched preparation was concentrated and subjected to SDS−PAGE under reducing and nondenaturing conditions. A series of bands, corresponding to proteins of different molecular weight ranges, were excised, extracted from the gel, dialyzed to remove SDS, and tested for their ability to activate rExoU in vitro (*16*). We found that the yeast cofactor was consistently associated with proteins in the SP column flow-through/no-salt wash that were smaller than 28 kDa (Figure 1B,C). Liquid chromatography and tandem mass spectroscopy (LC/MS/MS) analysis of this sample identified 30 primary candidates as putative ExoU cofactors (Table 1). We then subjected the SP column wash sample to further fractionation by SDS−PAGE. The ability to stimulate rExoU phospholipase activity was distributed among proteins in the 15−20 kDa range (data not shown), suggesting that different isoforms, protein modification, or multiple proteins may act as the ExoU cofactor. LC/MS/MS analysis of these fractions narrowed the list to 17 candidates, several of which overlapped with candidates from the primary LC/MS/MS analysis (Table 2).

Table 2: Cofactor Candidate Proteins in the 15 to 20 kDa Samples

Protein
enolase
pyruvate kinase (PK)
transaldolase
DMRL (6,7-dimethyl-8-ribityllumazine) synthase
transketolase
chromatin remodeling protein
Cu−Zn superoxide dismutase (SOD1)
phosphoglycerate kinase (PGK)
phosphoglucose isomerase
triosephosphate isomerase
heat shock protein
translational elongation factor
myosin-like protein
meiosis-specific protein
protein with an unknown function (3 proteins)

Activation of ExoU by Purified Cofactor Candidates. ExoU is a hydrophilic protein, and it is unclear as to how it interacts with membrane substrates. One candidate, Sip18p (Table 1), was considered as a likely cofactor for ExoU because it possesses phospholipid-binding activity (*26*). Sip18p transcription is induced approximately 20−25-fold upon the addition of NaCl to yeast growth medium (*27*). To determine the potential involvement of Sip18p in rExoU activation, yeast cells were grown in either a synthetic complete minimal medium or a rich YPD medium in the presence or absence of 0.5 M NaCl. After 1 h of NaCl induction (*27*), yeast cells were lysed, and a soluble extract was prepared. NaCl induced and uninduced extracts (at various concentrations) were tested for the activation of rExoU enzymatic activity (data not shown). Hydrolysis of substrate phospholipids by rExoU was not influenced by yeast extract made from salt-induced cells, suggesting that Sip18p is unlikely to be the required cofactor.

Purified proteins identified in the LC/MS/MS screen were obtained from commercial sources and tested for their ability to activate rExoU, in vitro, at two concentrations. Many of these candidate proteins, including phosphoglucose isomerase, triosephosphate isomerase, and several others, did not activate rExoU (Figure 2A and data not shown). Of the candidate proteins tested, only Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD1) from bovine liver significantly activated ExoU phospholipase activity (Figure 2A). Activation of rExoU by bovine SOD1 was dose-dependent (Figure 2B). SOD1 alone exhibited no phospholipase activity, even at high concentrations (Figure 2B, open circle). rExoU containing a mutation at either catalytic site, S142A or D344A, did not hydrolyze phospholipid substrates even in the presence of SOD1 (data not shown). To further confirm the ability of eukaryotic SOD1 to support rExoU phospholipase activity, we expressed the yeast ortholog Sod1p from a high copy plasmid under control of the *GAL1* promoter in *S. cerevisiae* and purified the histidine-tagged protein by affinity chromatography. Purified yeast Sod1p also activated rExoU enzymatic activity (Figure 2A). In contrast, neither *E. coli* Fe^{2+}-SOD nor *E. coli* Mn^{2+}-SOD activated rExoU (Figure 2A), consistent with the eukaryotic cell specificity of the cofactor (*15*, *16*, *18*).

Purity of Bovine Liver SOD1. To eliminate the possibility that a contaminant in the bovine liver preparation contributed

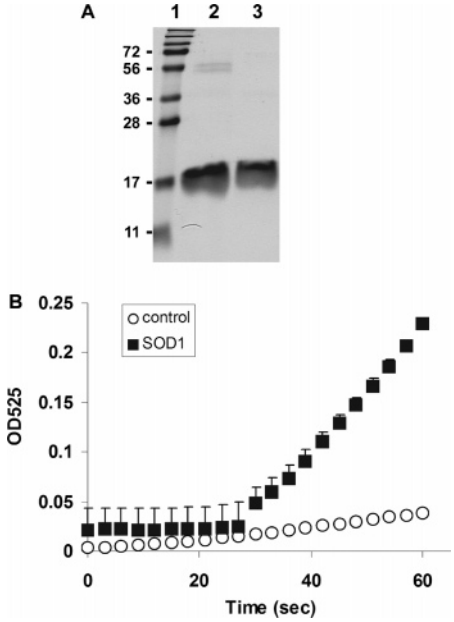

FIGURE 3: Further purification of the bovine liver SOD1 preparation (A) and superoxide dismutase activity of SOD1 (B). (A) Monomer of bovine liver SOD1 was further purified by extraction from SDS−PAGE gels. The isolated SOD1 (lane 3) activated rExoU as well as the original preparation. The original preparation of SOD1 is shown in lane 2, and the size of molecular weight markers are indicated in units of kDa (lane 1). (B) The oxidation of the SOD substrate by autoxidation (control, ○) or by 4 μg of bovine SOD1 (■) was measured at 525 nm.

to the activation of rExoU, we assessed purity of this protein by SDS−PAGE. According to this analysis, the preparation is estimated to be more than 94% pure (Figure 3A). The monomer form of SOD1 was further purified by excising a band corresponding to the appropriate molecular weight from a SDS−PAGE gel run under reducing and nondenaturing conditions (Figure 3A), and SDS removed as previously described. Purified SOD1 monomers (>99% purity) activated rExoU phospholipase activity with the same dose-dependence as that of the original preparation (data not shown).

SOD Enzymatic Activity Is Not Required for the Activation of ExoU. SOD catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen with extremely high specific activity (*25*).

$$2O^{\bullet}_2 + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

The enzymatic activity of the bovine liver SOD1 preparation was measured on the basis of the acceleration of oxidation of a superoxide dismutase substrate, 5,6,6a,11b-tetrahydro-3,9, 10-trihydroxybenzo[*c*]fluorene. The linear range of enzymatic reaction rates of SOD1 (2.8 μg) was detected within 60 s (Figure 3B).

To determine if rExoU activation was dependent on the enzymatic activity of SOD1, a variety of inhibitors were used to decrease SOD enzymatic activity, and their effects on the activation of rExoU were tested (Figures 4A and B). As shown in Figure 4A, SOD1 dismutase activity could be inhibited by azide (*23*), treatment with HCl (*24*), or metal ion chelation (*25*) up to 80% of full activity. However, none of the SOD inhibitors significantly affected rExoU phospholipase activity (Figure 4B). SOD mimetics, Mn(III)-tetra kis (4-benzoic acid) porphyrin (Mn-TBAP) and Fe-TBAP, also did not activate rExoU (data not shown). In addition, galactose oxidase, another Cu^{2+}-containing oxidase, did not activate rExoU phospholipase activity (data not shown). Thus, we conclude that activation of ExoU by SOD1 is not related to the dismutase activity of the latter enzyme.

Interaction of ExoU and SOD. These results suggest a novel role of SOD1, independent of its enzymatic activity, which may arise because of the physical interaction between the two proteins. SOD1 has a demonstrated capacity for self-association to form high MW oligomers and has been shown to interact with heat shock proteins (*28*) and the anti-apoptotic protein, Bcl-2 (*29*). It was recently reported that SOD1 binds to SK-N-BE neuroblastoma cell membranes and activates phospholipase C in a manner that does not depend on its dismutase activity (*30*). Our previous study demonstrated that the addition of a noncatalytic form of rExoU-S142A to the wild-type protein inhibited rExoU phospholipase activity, suggesting competition for a common cofactor (*16*). We postulate that the ability of SOD1 to associate with other proteins and membranes may provide an activation mechanism for rExoU.

To test for physical interaction between yeast Sod1p and rExoU, HA-tagged Sod1p was immobilized to protein G sepharose via an anti-HA IgG antibody and examined for its ability to capture rExoU. The sepharose-bound Sod1p was incubated with rExoU in the presence or absence of liposomes. After thorough washing, the protein was eluted from the beads with low pH, and the eluants were subjected to SDS−PAGE followed by Western blot using an anti-ExoU monoclonal antibody (Figure 5A). rExoU was captured by matrix-bound Sod1p, either in the presence or in the absence of liposomes (Sod1p + rExoU and Sod1p + rExoU + lipo in Figure 5A). Background binding of rExoU was not observed in negative controls (Matrix + anti-HA IgG and Matrix alone in Figure 5).

The physical interaction of rExoU with SOD1 was also analyzed in the converse fashion by capturing bovine SOD1 with immobilized rExoU. Histidine-tagged rExoU was bound to a cobalt affinity column and examined for its ability to capture bovine liver SOD1. Immobilized rExoU was incubated with SOD1 in the presence or absence of liposomes, and the beads were carefully washed and then directly tested for in vitro phospholipase activity using radioactive substrates. Because immobilized rExoU demonstrated low phospholipase activity relative to that of unbound rExoU (data not shown), the in vitro assay was supplemented with 7 μg of free rExoU. As shown in Figure 5B, strong activity was observed following incubation of SOD1 and liposomes (POPC/POPS, 1:1) with matrix-bound rExoU (rExoU + SOD1 + lipo). Activity was somewhat weaker but still well above background when immobilized rExoU was incubated with SOD1 in the absence of liposomes (rExoU + SOD1). Only weak background activity was observed for negative controls, which included another protein of the type III secretion system, PcrV, bound to the cobalt matrix via a histidine tag and the matrix alone incubated with SOD1 in the presence of liposomes (Figure 5B, rPcrV + SOD1 + lipo and SOD1 + lipo, respectively). Thus, the data in Figure 5B demonstrate the capture of SOD1 by immobilized rExoU. We also examined the effects of preincubating rExoU with SOD1 and/or liposomes prior to measuring phospholipase activity and confirmed our earlier observation with crude

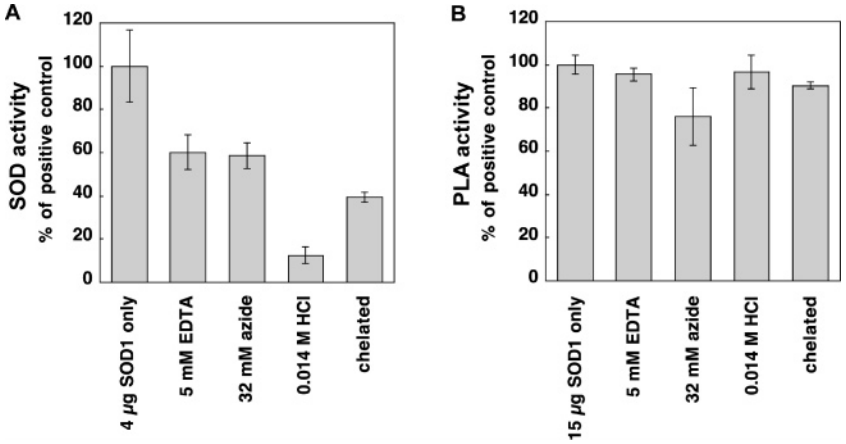

FIGURE 4: Effect of various SOD inhibitors on the activation of rExoU phospholipase. (A) SOD activity was measured following a variety of inhibitory treatments as described in Materials and Methods, and (B) similarly inhibited SOD was added to rExoU (15 μg of SOD, molar ratio SOD/rExoU, 5:1) for the determination of phospholipase activity. This concentration of SOD results in half-maximal activation of rExoU. The reduction in the superoxide dismutase activity of SOD did not affect the activation of ExoU phospholipase activity. Prior to the measurement of SOD1 or rExoU activity, EDTA was removed from the chelated SOD1 preparation (chelated) by dialysis.

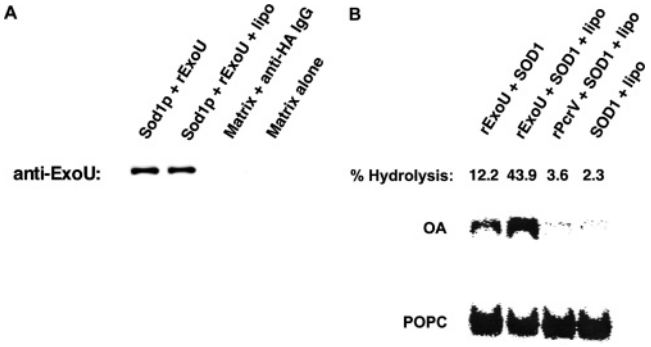

FIGURE 5: (A) Capture of rExoU by yeast Sod1p. HA-tagged Sod1p was immobilized to protein G sepharose via an anti-HA IgG antibody and incubated with rExoU (Sod1p + rExoU) or rExoU and liposomes (Sod1p + rExoU + lipo). Following elution, the samples were subjected to SDS−PAGE, and rExoU was detected by Western blot with a monoclonal antibody. No rExoU was detected from samples incubated with either matix-bound anti-HA IgG (Matrix + anti-HA IgG) or matrix alone (Matrix alone). (B) Bovine SOD1 captured by immobilized rExoU supported rExoU phospholipase activity. His-tagged rExoU was immobilized on a cobalt-affinity column and incubated with SOD1 (rExoU + SOD1) or SOD1 and liposomes (rExoU +SOD1 + lipo). Negative controls included immobilized His-tagged PcrV incubated with SOD1 and liposomes (rPcrV + SOD1 + lipo) and cobalt resin incubated with untagged SOD1 and liposomes (SOD1 + lipo). Following incubation, the resin was washed, supplemented with free rExoU, and mixed with liposomes containing radiolabeled substrate POPC. The reaction mixture was subjected to TLC to separate cleaved fatty acids (oleic acid, OA) from residual phospholipids (POPC). Percent hydrolysis of POPC substrate is indicated above each lane.

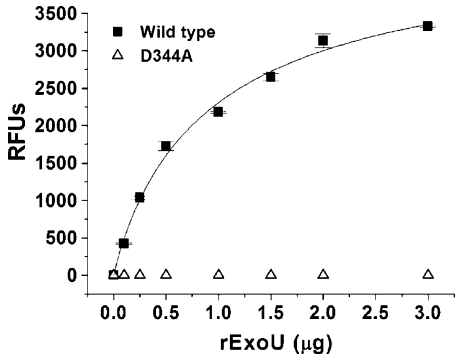

FIGURE 6: Quantitation of rExoU phospholipase activity by an optimized fluorescence-based assay. Various amounts of wild-type rExoU (■) in the presence of 10.5 μg of SOD1 increased relative fluorescent units (RFUs) by cleaving the self-quenched PED6 substrate. The hydrolysis of PED6 was dose-dependent on rExoU. Data were fitted to a nonlinear Hill sigmoidal model (solid line). Note that the noncatalytic form of rExoU (D344A, △) in the presence of SOD1 did not increase RFUs.

yeast extract (*16*) that preincubation does not enhance activity (data not shown), that is, SOD1 must be present during the phospholipase assay for activity to be observed.

Development of a Fluorescence-Based Assay for rExoU Phospholipase Activity. Our optimized TLC assay has been an important tool to quantitate phospholipase activity of rExoU in the presence of crude eukaryotic cell extracts (*15*, *16*) and has allowed us to identify the eukaryotic cofactor SOD1. However, there are several disadvantages to using the assay, for example, the usage of radioactive materials, requirement of special equipment, slow development, and limitation of the number of samples that can be analyzed in a given experiment. Prior to the identification of SOD1 as a rExoU cofactor, our attempts to develop fluorescent or colorimetric assays were unsuccessful because of an increase in background fluorescence upon the addition of crude yeast or CHO cell extracts. Using purified SOD1 eliminated the problematic background fluorescence and allowed us to measure the phospholipase activity of rExoU without radiolabeled substrates and TLC procedures. The fluorescent phospholipase substrate PED6 is a sensitive substrate for both secreted and cytosolic PLA_2 (*31*). The *sn*-2 acyl chain of PED6 contains a BODIPY FL dye, which is quenched by a dinitrophenyl quencher group positioned in the phosphoethanolamine headgroup. Our previous studies of enzymatic activity demonstrated that rExoU preferred phospholipid substrates containing either phosphatidylethanolamine and phosphatidylcholine (*16*). The addition of up to 56 μg of SOD1 to 0.0297 mM PED6 did not increase background fluorescence (data not shown). Using this fluorescent substrate, the titration of rExoU in the presence of 10.5 μg of SOD1 indicated that phospholipase activity was dose-dependent (filled squares, Figure 6). In contrast, the noncatalytic D344A-rExoU mutant did not demonstrate any enzymatic activity with PED6 even in the presence of SOD1 (open triangles, Figure 6).

Using PED6 as the substrate, the specific activity of rExoU (769.6 μmol min^{-1} mmol^{-1}) was approximately 10-fold higher than that of honeybee venom PLA_2 (77.9 μmol min^{-1} mmol^{-1}). In our TLC assay, the release of free fatty acids from radiolabeled phospholipid substrates by rExoU was 2-fold lower than that by honeybee venom PLA_2 on a per mole basis (*16*). These data suggest either that rExoU prefers PED6 as a substrate or that bee venom PLA_2 activity against PED6 is diminished relative to phospholipid.

DISCUSSION

A number of important pathogens utilize a type III secretion system to directly deliver effector proteins into the cytosol of host cells. Effector proteins encode activities that generally allow bacteria to evade host defense systems and break down barriers for replication and dissemination. In addition to direct vectorial delivery, the requirement of a host-derived cofactor is an important mechanism by which the invading bacterium is protected from the effects of its own toxin. The *P. aeruginosa* type III toxin ExoU is a potent phospholipase, capable of rapidly destroying both intracellular and plasma membranes and responsible for acute cytotoxicity (*13*−*16*). rExoU readily hydrolyzes liposome bilayers in vitro, whose composition mimics the inner membrane of Gram-negative bacteria (*16*). This reaction only occurs when soluble eukaryotic cell extracts are added to the rExoU/substrate mixture (*15*). These results suggest that the specificity for eukaryotic cellular damage by ExoU is not due to the availability of a suitable substrate but is limited by the availability of the cofactor (*18*). Similarly, all of the other known *P. aeruginosa*-encoded type III effectors require a host factor for enzymatic activity (*3*, *13*). In this study, we have used a combined biochemical/proteomic approach to identify eukaryotic type 1 superoxide dismutase (SOD1) as a proteinaceous cofactor capable of supporting in vitro ExoU phospholipase activity. Our analyses further demonstrate that neither Fe^{2+}-SOD nor Mn^{2+}-SOD from *E. coli* activate ExoU. Thus, these studies reinforce the general principle that the requirement for a eukaryotic cofactor, in conjunction with vectorial transfer, ensures that *P. aeruginosa* can deliver a potent toxic protein with little or no modification of its own substrates.

Our data indicate an important role for physical interaction between ExoU and its cofactor. Preincubation of ExoU with SOD1 does not produce an activated ExoU upon removal of SOD, and there is no evident change in the physical properties of ExoU (molecular weight or isoelectric point) following incubation with either SOD1 or crude eukaryotic cell lysates. One model consistent with our observations is that SOD1 produces a conformational change in ExoU either exposing or stabilizing its catalytic site. Interfacial activation, in which association with a bilayer or micelle displaces an inhibitory lid domain, is a well-established mechanism of phospholipase activation (*32*, *33*). Domain mapping (*34*), mutagenesis (*35*), and yeast suppressor analyses (*15*) all support a two-domain concept for the molecular organization of ExoU (*18*) and implicate the importance of the *C*-terminal domain for cytotoxic activity, even though all components of the catalytic site are located in the *N*-terminal domain (*15*). Alternatively, SOD1 may facilitate the association of ExoU with its membrane substrate. SOD1 has been reported to bind to SK-N-BE neuroblastoma cell membranes, activating phospholipase C in a manner that does not depend on its dismutase activity (*30*). Identification of SOD1 as a cofactor will facilitate more detailed studies of the structural interactions that contribute to the expression of ExoU phospholipase activity, and a rigorous structural analysis of ExoU with and without SOD and substrate liposomes will be required to fully understand the mechanistic aspects of activation.

The ability of Cu^{2+}, Zn^{2+}-SOD to act as a cytoplasmic cofactor stimulating ExoU phospholipase activity has significant implications for the biological activity of the toxin. SOD is widespread in nature and is particularly important for protecting oxygen-metabolizing cells against the harmful effects of superoxide free-radicals. It is an essential component of phagocytic cells that utilize a respiratory burst to combat invading pathogens. Mammalian lungs also represent a unique environment exposed to oxidant stress. In lung and other tissues, there are three types of SOD enzymes, cytosolic copper−zinc, mitochondrial manganese, and extracellular SOD, that function to prevent the accumulation of superoxide radicals and their metabolites (*36*). Lung epithelium is particularly susceptible to injury caused by *P. aeruginosa* infections (*37*). Multiple organ failure, the development of sepsis, and poor prognosis is linked to intoxication by the *P. aeruginosa* type III system (*38*) and specifically to the expression of ExoU (*21*, *38*). Our discovery of Cu^{2+}, Zn^{2+}-SOD as a cofactor of ExoU may explain in part the susceptibility of the lung to injury and the subsequent sudden release of inflammatory mediators that define sepsis (*38*). It will be important to determine whether ExoU can be activated by mitochondrial or extracellular SODs. ExoU, which is released into extracellular space, may be active under conditions that are especially relevant to an inflammatory environment, if extracellular SOD is able to activate this type III cytotoxin.

In summary, we have identified the first eukaryotic cofactor capable of activating phospholipase activity of the *P. aeruginosa* type III toxin, ExoU. SOD1 is also the first proteinaceous cofactor shown to activate a PLA_2-type phospholipase. The activation of ExoU by SOD provides a powerful weapon by which *P. aeruginosa* can damage host neutrophils and macrophages to facilitate the survival of the bacterium during infection and degrade the plasma membrane of lung epithelia to promote dissemination. Identification of SOD as a cofactor allows the use of fluorescence-based phospholipase assays, which should greatly facilitate in vitro studies of ExoU activity, including high-throughput screening of potential inhibitors. Further elucidation of the structural mechanism of ExoU activation by protein cofactors may provide a rational approach to the design of inhibitors that can diminish tissue damage during infection by ExoU-producing strains of *P. aeruginosa*.

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