Intracellular Localization of Type III-delivered *Pseudomonas* ExoS with Endosome Vesicles*

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ExoS (453 amino acids) is a bi-functional type III cytotoxin produced by *Pseudomonas aeruginosa*. Residues 96–219 include the Rho GTPase-activating protein (RhoGAP) domain, and residues 234–453 include the 14-3-3-dependent ADP-ribosyltransferase domain. Earlier studies also identified an N-terminal domain (termed the membrane localization domain) that comprises residues 51–77 and includes a novel leucine-rich motif that targets ExoS to the perinuclear region of cultured cells. There is limited information on how ExoS or other type III cytotoxins enter and target intracellular host proteins. Type III-delivered ExoS localized to both plasma membrane and perinuclear region, whereas ExoSΔMLD was localized to the cytosol. Plasma membrane localization of ExoS was transient and had a half-life of ~20 min. Type III-delivered ExoS co-immunoprecipitated 14-3-3 proteins and Rab9, Rab6, and Rab5. Immunofluorescence experiments showed that ExoS co-localized with Rab9, Rab6, and Rab5. Fluorescent energy transfer was detected between ExoS and 14-3-3 proteins but not between ExoS and Rab proteins. Together, these results indicate that type III-delivered ExoS localizes on the host endosomes and utilizes multiple pathways to traffic from the plasma membrane to the perinuclear region of intoxicated host cells.

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that causes urinary tract, respiratory system, and gastrointestinal infections as well as dermatitis and soft tissue, bone, and joint infections (1). *P. aeruginosa* is also responsible for a variety of systemic infections, particularly in patients with severe burns, cystic fibrosis, and cancer and AIDS patients who are immunocompromised (2). *P. aeruginosa* produces multiple cellular effectors that contribute to colonization, invasion, and cytotoxicity, including four type III cytotoxins: ExoS, ExoT, ExoU, and ExoY (3). ExoS is a bi-functional cytotoxin that encodes a Rho GTPase-activating protein (RhoGAP) domain (residues 96–219) and a 14-3-3-dependent ADP-ribosyltransferase domain (residues 234–453) (4). Iglewski *et al.* (5) first identified exoenzyme S as an ADP-ribosyltransferase that ADP-ribosylates Ras and several related GTPases (6). ExoS is a RhoGAP for Rho, Rac, and Cdc42 in vitro (7) and in vivo (8, 9). Subsequent studies showed that residues 51–77 (membrane localization domain, MLD) targeted ExoS to the perinuclear region of mammalian cells through a novel leucine-rich motif (10–12). Deletion of the MLD did not inhibit type III secretion of ExoS from *P. aeruginosa* or delivery of ExoS into mammalian cells, but it interfered with the ADP-ribosylation of Ras (11). This showed the importance of intracellular localization for efficient ADP-ribosylation of host proteins.

Upon internalization, many bacterial AB exotoxins exploit the mammalian cell retrograde trafficking systems and escape into the cytosol from different organelles, including endosomes, Golgi, and endoplasmic reticulum (ER) (13). Among these, the trafficking routes between endosomes and trans-Golgi network (TGN) are diverse and involve multiple pathways, including early, late, or recycling endosomes. Ricin is transported to the Golgi complex independent of recycling endosomes (Rab11), late endosomes (Rab7 and Rab9) (14–16), and appears to exploit more than one pathway. Similarly, shiga toxin B utilizes a direct early/recycling endosome to TGN trafficking that circumvents late endosomes (17), which is controlled by specific t-SNARE, early endosomal v-SNARE, and GTase Rab6A (18). Retrograde trafficking of choler toxin from the plasma membrane to the Golgi involves multiple endocytosis pathways to allow choler toxin access to the Golgi/ER (19). Inhibition of endocytosis by clathrin-, caveolin-, or ARF6-dependent mechanisms did not block choler toxin movement into the cell or attenuate toxicity (20, 21). In BSC1 cells, although inhibition with dynamin and ARF6 pathways together blocked transport of choler toxin into the cell by light microscopy, these inhibitors did not affect toxin potency. The molecular identity of this pathway remains to be identified, but it likely involves the membrane cholesterol and sphingomyelin homeostatic machinery (19). Recently, the retrograde trafficking of *Pseudomonas* exotoxin A was observed to involve both Rab9-dependent and Rab9-independent pathways (22). Partial localization of exotoxin A within the detergent-resistant membrane may permit a choice of trafficking routes, controlled by host lipid- and protein-sorting signals (22).

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2 The abbreviations used are: MLD, membrane localization domain; FRET, fluorescence resonance energy transfer; co-IP, co-immunoprecipitation; IP, immunoprecipitation; DN, dominant negative; PNS, postnuclear supernatant; m.o.i., multiplicity of infection; CFP, cyan fluorescent protein; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; YFP, yellow fluorescent protein; ER, endoplasmic reticulum; TGN, trans-Golgi network; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PIPE, 1,4-piperazinediethanesulfonic acid; siRNA, small interference RNA; HA, hemagglutinin; SNARE, soluble NSF attachment protein receptors; IEF, isoelectric focusing; ICQ, intensity correlation quotient.
Intracellular localization of several type III cytotoxins has been observed previously. YopM localizes to the nucleus and stimulates the activity of PKR2 and RSK1 kinases (23, 24). YopH localizes to the focal adhesion complexes and is essential for anti-phagocytosis and virulence (25). YopE localizes to cytoplasmic granules and the perinuclear region (26, 27). However, how type III toxins traffic in intoxicated cells has not been established. This study shows that type III-delivered ExoS localizes temporally on the plasma membrane and associates with markers of early and late endosomes.

**EXPERIMENTAL PROCEDURES**

**Materials**—HeLa cells (CCL-2) were from the ATCC. Tissue culture media and sera were from Invitrogen. Reagents for molecular and cellular biological techniques were from New England Biolabs or Invitrogen, and chemicals were from Sigma, unless noted. DNA primers were from Operon Technologies.

**Bacterial Strains and Construction of Vectors**—*P. aeruginosa* strain PA103 (ΔexoU, exoT::Tc) with a pUCP derivative to express the indicated form of ExoS was cultured as described previously (10, 11, 28). ExoS-KDD and ExoSΔMLD-KDD were generated by QuikChange using ExoS R146K, E381D (29) as a template. Primers are as follows: positive, 5′-AGCGGATATGGAACATCAAGAATGACAGATCTCTTATACATGTA-3′, and negative, 5′-ATGATGTGTACGGGATTATCGAGATCAGGAAAGACATGATCTCTTATACATGTA-3′.

**Construction of RasWT and Ras(C181S,C184S)**—pET16b c-H-Ras vector was a gift from H. Fu (Emory University) (30). A c-H-Ras vector was digested with KpnI and BamHI restriction sites. The PCR product was cloned into pEGFP-C1 vector with KpnI and BamHI restriction sites. The PCR product was sequenced to confirm mutations.

**Cell Culture Growth**—HeLa cells were cultured in complete medium (minimum essential medium + 10% fetal calf serum, nonessential amino acids, sodium pyruvate, sodium bicarbonate and penicillin/streptomycin) and maintained in a 37 °C humidified 5% CO₂ (v/v) incubator.

**Transfection and Cellular Fractionation of HeLa Cells**—HeLa cells were seeded in 85-mm dishes with 3 × 10⁵ cells the day before use. Cells were grown to 70% confluency and transfected with Lipofectamine and Plus reagent-mediated liposome transfection system (Invitrogen), as described by manufacturer, using 1 µg of indicated DNA (31). Total DNA was normalized with pCMV10Luc (luciferase) (Kent Wilcox, Medical College of Wisconsin). After 18–24 h, transfected cells were washed twice with PBS, suspended in 10 ml of homogenization buffer (HB1) (250 mM sucrose, 3 mM imidazole, pH 7.4), washed in 300 µl of HB1, suspended in 300 µl of HB2 (HB1 plus 1% mammalian protease inhibitor mixture set III (Sigma) and 0.5 mM EDTA). Cells were lysed by passage 20 times through a 25-gauge needle. The whole-cell lysate was centrifuged for 5 min at 2000 rpm in a microcentrifuge at 4 °C, and the pellet (nuclei and unbroken cells) and postnuclear supernatant (PNS) were collected. The PNS was centrifuged for 30 min at 100,000 × g, and the pellet (membrane) and supernatant (cytosol) were collected. Samples were normalized to volume equivalent with SDS-PAGE sample buffer, boiled, and stored at −20 °C.

**Type III Delivery of ExoS into HeLa Cells**—HeLa cells were cultured in 6-well plates to 70% confluence and transfected with 200 ng of the indicated plasmid. After 18–24 h, cells were infected at a multiplicity of 8:1 (bacteria:HeLa cells) of *P. aeruginosa* PA103 ΔexoU, exoT::Tc (pUCP) (32) as control, and the indicated ExoS construct, including pUCPExoS (33) or pUCPExoS (33) or pUCPExoS R146K, E379D, E381D, termed KDD. After 3.5 h, HeLa cells were washed with PBS and treated with 100 µg/ml gentamicin and 200 µg/ml ciprofloxacin to kill extracellular bacteria. At the indicated times, cells were washed twice with PBS, and lysates were prepared and subjected to subcellular fractionation or cells were treated with 1% paraformaldehyde and examined by direct fluorescence, subjected to immunofluorescence analysis, or phase-contrast microscopy.

**Immunoﬂuorescence Microscopy**—HeLa cells seeded on coverslips in 6-well plates were infected with *P. aeruginosa* PA103 ΔexoU, exoT::Tc (pUCP-ExoS-KDD) at an m.o.i. of 8:1 (bacteria:HeLa). Four hours postinfection, cells were washed three times with PBS and fixed with 1% paraformaldehyde for 1 h at 4 °C. Cells were permeabilized with 0.1% Triton X-100 in 4% formaldehyde (15 min at room temperature), which was followed by three washes with PBS. Cells were blocked with 1% bovine serum albumin in PBS for 20 min at room temperature and then incubated with individual antibodies. For Rab6/ExoS double staining, cells were incubated with rabbit α-Rab6 IgG (1:500) and mouse α-HA IgG (1:1,000) for 1 h at room temperature, washed three times with PBS, incubated with goat α-rabbit IgG-Alexa-488 (1:500) and goat α-mouse IgG-Alexa-568 (1:500). For Rab5 and Rab9 staining, cells were first stained with either mouse α-Rab5 IgG (1:500) or mouse α-Rab9 IgG (1:200), followed by incubation with goat α-mouse IgG-Alexa-488. Next, cells were fixed with paraformaldehyde, washed with PBS, and stained with prelabeled mouse α-HA IgG-(Fab)₂.
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Alexa-568 according to the manufacturer’s protocol (Zenon prelabeling kit, Molecular Probes). Cells were then washed, mounted, and observed under a ×60 oil objective. Images were captured with a Roper camera using Metamorph software and cropped in Adobe Photoshop. Confocal images were obtained on a Leica TCS SP2 laser scanning confocal microscope at the Bryant Imaging Facility, Medical College of Wisconsin. Incidence of fluorescence between two fluorochromes was determined as described in ImageJ software. Briefly, ICQ numbers of the boxed regions of analyzed figures were calculated by ImageJ plugin intensity correlation analysis (National Institutes of Health, Bethesda, MD). Typical values are as follows: random (or independent) staining ICQ = −0; dependent staining 0 < ICQ ≤ +0.5; and segregated (or exclusive) staining 0 > ICQ ≥ −0.5. For quantification of the percentage plasma membrane localization, random fields of HeLa cells were scored by a blinded observer. Plasma membrane association of ExoS was scored by focusing through the entire cell. Mouse α-hemagglutinin (HA) antibody was from Covance (catalog number MMSS101P); mouse α-Rab9 antibody was from Calbiochem (catalog number 552101). Rabbit α-Rab6 antibody was from Santa Cruz Biotechnology (C-19). Mouse α-Rab5 antibody was from BD Transduction Laboratories (catalog number R60020-050). Mouse α-Rab4 antibody was from BD Transduction Laboratories (catalog number 610888). AlexaFluor goat α-mouse or α-rabbit IgG was from Molecular Probes. Sheep α-human TGN46 was from Serotec (catalog number AHP500). Rabbit α-calnexin was from Stress-Gen (catalog number SPA-865).

**Immunoblot Analysis**—The PNS was subjected to SDS-PAGE (11–12%) and transferred to a polyvinylidene fluoride membrane. Membranes were incubated with the indicated primary antibody for 1 h at room temperature, washed with PBS, and then incubated for 1 h with goat α-mouse—HRP IgG or goat α-rabbit—HRP IgG (1:40,000; Pierce). HRP was developed with SuperSignal (Pierce) and exposed to x-ray film, which was developed in a film processor or digitized with an Alpha-Inatech 8900 imaging system. Mouse α-H-Ras antibody was from BD Transduction Laboratories (catalog number R02120-150). Mouse α-GFP antibody was from Covance (catalog number MMS-118P).

**Co-immunoprecipitation (Co-IP) of Host Protein Bound to Type III-delivered ExoS**—HeLa cells were plated in 150-mm dishes and grown to 90% confluence. Cells were infected with *P. aeruginosa* expressing a vector control (pUCP), ExoSΔMLD-KDD, or ExoS-KDD at an m.o.i. 8:1 (bacteria:HeLa) for 4 h. Cells were harvested, and the postnuclear supernatants were collected. The PNS was fractionated to cytosol (Cyto) and membrane (Mem) fraction by ultracentrifugation and resolved on SDS-PAGE. The percentage of ExoS in the membrane fraction (right panel) was calculated based on densitometry of the Western blot using mouse α-HA as the primary antibody (left panel). Fractionation of the cytosolic GFP was used as an internal control for fractionation efficiency. C. HeLa cells were transiently transfected with pEGFP (Clontech) and then infected with the indicated strain of *P. aeruginosa*. 4 h postinfection, cells were harvested, and the postnuclear supernatants were collected. The PNS was separated to cytosol (Cyto) and membrane (Mem) fraction by ultracentrifugation and resolved on SDS-PAGE. The percentage of ExoS in the membrane fraction (right panel) was calculated based on densitometry of the Western blot using mouse α-HA as the primary antibody (left panel). Fractionation of the cytosolic GFP was used as an internal control for fractionation efficiency.

**FIGURE 1.** Schematic view of ExoS constructs generated in this work. Wild type ExoS (ExoS-WT) and the ExoS derivatives (ExoS-KDD and ExoS-ΔMLD-KDD) were tagged with HA epitope (YPYDVPDYASL) for immunostaining and Western blotting experiments. ExoS-KDD and ExoS-ΔMLD-KDD were tagged with 3-flag (3FLAG) epitope (DYKDDDDK) for co-immunoprecipitation experiments. CFP fusion proteins of ExoS-KDD and ExoS-ΔMLD-KDD were used in FRET determinations.

**FIGURE 2.** Steady state localization of type III-delivered ExoS on the plasma membrane and perinuclear region of HeLa cells. A. HeLa cells were infected with *P. aeruginosa* PA103 ΔexoUe, exoT::Tc (pUCP vector control), pUCP-ExoS WT, pUCP-ExoSΔMLD-KDD, or pUCP-ExoS-KDD at an m.o.i. 8:1 (bacteria:HeLa) for 4 h. Cells were stained by immunofluorescence procedures as described under “Experimental Procedures.” Images were generated with MetaMorph series 6.0 software exposed for the same time. B. HeLa cells were transfected with pEGFP (Clontech) and then infected with the indicated strain of *P. aeruginosa*. 4 h postinfection, cells were harvested, and the postnuclear supernatants were collected. The PNS was separated to cytosol (Cyto) and membrane (Mem) fraction by ultracentrifugation and resolved on SDS-PAGE. The percentage of ExoS in the membrane fraction (right panel) was calculated based on densitometry of the Western blot using mouse α-HA as the primary antibody (left panel). Fractionation of the cytosolic GFP was used as an internal control for fractionation efficiency. C. HeLa cells were transiently transfected with plasmids encoding ExoS-KDD or ExoSΔMLD-KDD. After 18–20 h, ExoS was immunostained with mouse α-HA-IgG as described in A. The amount of Alexa-546 staining was quantified as pixels by ImageJ, Research Services Branch, NIMH (Bethesda, MD). N, nucleus; P, plasma membrane; y axis = gray value (arbitrary units); x = axis distance (pixels).
ExoS-KDD at an m.o.i. of 8:1 (bacteria:HeLa). Note, both ExoS/H9004 MLD-KDD and ExoS-KDD used in this co-IP had a 3xFLAG (Sigma) epitope engineered onto the C terminus (Fig. 1). After 4–5 h of infection, HeLa cells were harvested, and 600 μl of PNS were collected as described in the cell fractionation protocol. NaCl was added to a final concentration of 150 mM. One hundred and fifty μl of 3x-FLAG antibody-conjugated agarose beads (50% slurry, Sigma) were aliquoted into a microcentrifuge tube. The beads were washed twice with HB2 buffer to remove glycerol. PNS (500 μl) were added to the beads, and the tubes were incubated for 2 h at 4°C on a wheel rotator. The beads were pelleted with a pulse spin; the supernatant was discarded, and the beads were washed three times with TBS buffer (50 mM Tris-HCl, 150 mM NaCl, 1% mammalian protease inhibitor mixture set III (Sigma), and 0.5 mM EDTA) for 10 min each. The beads were incubated overnight in 150 μg of 3xFLAG peptide in 100 μl of TBS buffer. The suspension was pulse-spun, and the supernatant was discarded. After extraction, beads were boiled to determine ExoS that remained bound to beads following extraction with the 3xFLAG peptide. Mouse α-FLAG M2 affinity gel was from Sigma (catalog number A2220). 3xFLAG peptide was from Sigma (catalog number F4799).

MALDI-TOF Analysis—Bands from five gels were excised and subjected to trypsin digestion (1 μg; Promega, Madison, WI) in 50 μl of 100 mM NH₄HCO₃, pH 8, at 37°C for 24 h. After digestion, gel slices were sonicated twice in 200 μl of 80% acetonitrile and 1% formic acid (in H₂O) for 10 min. Eluted material was combined and evaporated, and the pellet was dissolved in 15 μl of 0.1% trifluoroacetic acid (in H₂O). Peptide solutions were desalted with C₁₈ Zip Tips (Millipore, Bedford, MA) that had been equilibrated successively in 15 μl of acetonitrile, 15 μl of 50% acetonitrile (H₂O), and 15 μl of 0.1% trifluoroacetic acid (H₂O). Resin was washed twice with 0.1% trifluoroacetic acid (H₂O). Peptides were eluted in 2 μl of 60% acetonitrile and 0.1% trifluoroacetic acid (H₂O saturated with α-cyano-4-hydroxycinnamic acid) and applied to a sample plate to air-dry. Samples were ionized by an N₂ UV laser using a PE-pro mass spectrometer (Applied Biosystems). Two hundred laser shots were conducted at an accelerating voltage of 25,000 and laser intensity of 2075 (repetition rate 3 Hz). Scans were processed using Biosystems Voyager 6004 software. Peptide fingerprinting was used to identify proteins present in the band, using Protein Prospector (University of California, San Francisco).

Fluorescence Resonance Energy Transfer (FRET) Determinations—DNA encoding 14-3-3-γ, 14-3-3-ε, or 14-3-3-β was subcloned into pYFP-C1 (Clontech), and DNA encoding...
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ExoS-KDD, ExoS-ΔMLD-KDD, or ExoS-(1–416)-KDD was subcloned into pCFP-N1 (Clontech). HeLa cells cultured on cover slips were co-transfected with 150 ng of pYFP-14-3-3-γ and 150 ng of either p-ExoS-KDD-CFP or p-ExoS-(1–416)-KDD-CFP. Control experiments showed that this ratio of the acceptor to donor yielded optimal FRET measurements (data not shown). 18 h post-transfection, cells were fixed and mounted onto microscope slides. Acceptor photobleach experiments were carried out with a Leica TCS SP2 laser scanning confocal microscope according to manufacturer’s instructions. Briefly, the YFP signal in a selected region of interest was bleached with a 514 nm laser until fluorescence was ~20% of the original intensity; Fig. 7A shows the bleached area as a box. Average fluorescence intensity of donor (D, CFP) and acceptor (A, YFP) before (pre) and after photobleach (post) was measured. FRET efficiency was calculated with Equation 1, 

\[
FRET_{\text{eff}} = \frac{D_{\text{pre}} - D_{\text{post}}}{D_{\text{pre}}} \quad \text{for} \quad D_{\text{post}} > D_{\text{pre}} \quad \text{(Eq. 1)}
\]

Control experiments were performed with one frame of photobleach with 0% laser power (35).

**FIGURE 4. Co-immunoprecipitation of host proteins with type III-delivered ExoS.** A, HeLa cells (150 mm dishes) were grown to 90% confluence and intoxicated with *P. aeruginosa* ΔexoU, exoT::Tc (pUCP), pUCP-ExoS-ΔMLD-KDD-3xFLAG, ΔMLD, or pUCP-ExoS-KDD-3xFLAG, KDD at an m.o.i. of 8:1. After 4 h, cells were lysed, and cell lysate (PNS) was prepared. Co-immunoprecipitation of host protein with ExoS was performed as described under “Experimental Procedures.” Immunoprecipitated material was acetone precipitated and subjected to 11% SDS-PAGE. Gels were silver-stained. The amount of ExoS precipitated was determined by Western blot (WB) (top). Arrows show the migration of ExoS and 14-3-3 proteins (factor-activating exoenzyme S (FAS)) as determined by Western blotting. B, immunoprecipitated material released with the 3xFLAG peptide was also acetone-precipitated and subjected to isoelectric focusing, using pH 4–7 strips with two-dimensional standards. The IEF strips were subjected to 11% SDS-PAGE and silver-stained. The two-dimensional gel electrophoresis of the IP material was performed five independent times. In each of the five independent experiments, bands 1–2 and 1–6 were observed in the co-immunoprecipitates with ExoSΔMLD and ExoS, respectively. Bands 1 and 2 were subjected to MALDI-TOF (Table 1), whereas bands 3–6 were subjected to MALDI-MS/post-source decay (Table 2). ExoSΔMLD and ExoS were detected by Western blotting of an identical sample and circled (ExoSΔMLD) or ExoS(KDD).

**RESULTS**

Type III-delivered ExoS Associates with the Plasma Membrane and Perinuclear Region—How type III cytotoxins localize and traffic within mammalian cells is not clear. To limit toxic effects on the host cell, several noncatalytic forms (ExoSΔMLD-
R146K, E379D, E381D) (ExoSΔMLD-KDD) and ExoS (R146K, E379D, E381D) (ExoS-KDD) were used to monitor the intracellular localization of type III-delivered ExoS (Fig. 1). In Fig. 2, the infection was stopped when wild type ExoS (ExoS-WT) fully round the cells. At this time, intoxication with P. aeruginosa expressing pUCP, ExoSΔMLD-KDD, and ExoS-KDD had minimal effect on cell morphology (Fig. 2A). ExoS-WT expression was detectable but less than the noncatalytic ExoS-KDD or ExoSΔMLD-KDD due presumably to the toxic nature of the protein. Low expression of the catalytically active ADP-riboseyltransferase domain in transiently transfected mammalian cells has also been observed (38). Type III-delivered ExoSΔMLD-KDD localized throughout HeLa cells, whereas ExoS-KDD localized on both the plasma membrane and the perinuclear region (Fig. 2A). Because both ExoSΔMLD-KDD and ExoS-KDD have an HA epitope, the HA epitope did not appear responsible for the observed intracellular localization. HeLa cells infected with ExoSΔMLD-KDD or ExoS-KDD were separated to soluble and membrane fractions by ultracentrifugation. Western blot analysis showed that ExoSΔMLD-KDD was present in the cytosol, whereas ExoS-KDD was distributed in both the membranes and cytosol (Fig. 2B). Cells were pretransfected with pGFP, and GFP was used as an internal control for efficiency of cell fractionation. To determine whether the plasma membrane localization was an intrinsic property of type III-delivered ExoS, localization of transiently transfected ExoSΔMLD-KDD and ExoS-KDD was examined. ExoSΔMLD-KDD localized throughout the cells, whereas ExoS-KDD accumulated near the perinuclear region without apparent plasma membrane association (Fig. 2C); quantification of the staining is also shown. This indicates that the MLD-dependent association of ExoS with the plasma membrane occurs during type III delivery. Plasma membrane association of type III-delivered ExoS may require a P. aeruginosa factor(s) or may represent a difference in the experimental protocol used for expression of ExoS in a bacterial intoxication versus expression during a transfection. This is currently under investigation.

**Fig. 5. Rab9, Rab6, and Rab5 co-immunoprecipitate with type III-delivered ExoS but not ExoSΔMLD.** Proteins that co-immunoprecipitated with a vector control (pUCP), ExoSΔMLD-KDD-3FLAG (ΔMLD), or ExoS-KDD-3FLAG (KDD) were subjected to 12% SDS-PAGE in a nonreducing gel. Proteins were transferred to a polyvinylidene fluoride membrane and probed sequentially with mouse α-Rab9 IgG, rabbit α-Rab6 IgG, mouse α-Rab5 IgG, and rabbit α-Rab4. Between probes the blot was stripped and tested for stripping efficiency using x-ray film. (Note, the migration of Rab9, Rab6, Rab5, and Rab4 are unique and do not overlap in this SDS-PAGE system.) Secondary antibodies used were goat-α-mouse or rabbit-HRP IgG and membranes were developed with SuperSignal (Pierce). The reactive bands on the right are probed for total Rab in the indicated lysates prior to immunoprecipitation (input). These experiments were performed two times independently with similar results. 

**Table 1**

| IP       | Proteina | ID | MOWSE scoreb | Peptide match | Coverage | kDa, pl (observed)c | kDa, pl (predicted)c |
|----------|----------|----|--------------|---------------|----------|---------------------|----------------------|
| ΔMLD     | 1 14-3-3e | 6.6 × 10^7 | 10/24 | 35 | 30 (4.8) | 29 (4.6) |
|          | 2 14-3-3e | 3.4 × 10^5 | 9/24 | 23 | 28 (4.6) | 28 (4.8) |
| KDD      | 1 14-3-3e | 1.1 × 10^5 | 8/24 | 21 | 28 (4.6) | 28 (4.7) |
|          | 2 14-3-3e | 2.4 × 10^6 | 6/24 | 25 | 30 (4.8) | 29 (4.6) |

a Co-immunoprecipitated proteins from a cell lysate of infected HeLa cells immunoprecipitated with ExoSΔMLD-KDD (ΔMLD) or ExoS-KDD (KDD) were subjected to two-dimensional electrophoresis (see Fig. 4). Spots 1 and 2 were excised from the gel and subjected to trypsin digestion followed by MALDI-TOF analysis.

b MALDI-TOF analysis was performed by Protein-Nucleic Acid Shared Facility at the Medical College of Wisconsin. MOWSE score was calculated by ProteinProspector (University of California, San Francisco) and represents the fidelity of the identification of the peptides with a data base of peptides for individual proteins. In this analysis, scores of >74 indicate identity or homology at p < 0.05.

c Values were determined by comparing with protein standards of known molecular masses and pl values.

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Type III-delivered ExoS Is Temporally Localized on the Plasma Membrane —To investigate the stability of plasma membrane-associated ExoS, a pulse-chase type experiment was performed to measure the stability of type III-delivered ExoS on the host cell membrane. Between 3 and 4 h postinfection, type III-delivered ExoS-KDD was detected on both the plasma membrane and in the perinuclear region (Fig. 3A). At 4 h postinfection, the infection was stopped by washing cells and adding gentamicin and ciprofloxacin to the culture media. During the chase, plasma membrane association of ExoS-KDD decreased by 50% in ∼20 min, whereas the overall amount of cell associated-ExoS-KDD remained constant with the majority of ExoS-KDD now located in the perinuclear region (Fig. 3, A and B). Plasma membrane-associated ExoS-KDD was observed at the earliest time points, when total ExoS-KDD within the cell was low. Type III-delivered ExoSΔMLD-KDD did not associate with membranes in a similar time course experiment; the 4-h time point of HeLa cells infected with P. aeruginosa expressing ExoSΔMLD-KDD and stained for ExoS is shown in Fig. 3A (4.00, ΔMLD). These data suggest a sequential movement of type III-delivered ExoS from the plasma membrane to the perinuclear region.

Intracellular Type III-delivered ExoS Is Associated with 14-3-3 Proteins, Tip47, and Rab GTPases —Co-IP was used to gain insight into the cellular basis for intracellular localization of ExoS. Cell lysates were prepared from HeLa cells intoxicated for 4 h with P. aeruginosa possessing a vector control (pUCP), ExoSΔMLD-KDD, or ExoS-KDD. To facilitate the IP of ExoS from the cell lysate, a 3xFLAG epitope was
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**TABLE 2**

| Band | ID      | kDa (observed) | kDa (predicted) | CID(MS/MS) sequence | MOWSE score |
|------|---------|----------------|-----------------|---------------------|-------------|
| 3    | Tip47   | 49 (5.1)       | 47.0 (5.3)      | 1. TATLSGEVAVQVR    | 63          |
|      |         |                |                 | 1. MOSAQQDGNSPHX    | 145         |
| 4    | TPDS2   | 27 (4.9)       | 22.2 (5.26)     | 2. TPVEQNLTRAERRL   |             |
|      |         |                |                 | 3. GLLSDMTFDVTDGAAR |             |

* Co-immunoprecipitated proteins from a cell lysate of infected HeLa cells immunoprecipitated with ExoSΔMLD-KDD (ΔMLD) or ExoS-KDD (KDD) were subjected to two-dimensional electrophoresis (see Fig. 4). Protein spots 3 and 4 were excised from the gel and subjected to tryptic digestion followed by MALDI-tandem mass spectrometry analysis. Peptide(s) from each protein spot were subjected to post-source decay analysis. Protein spots 5 and 6 were also analyzed but did not yield peptides.

* Values were determined by comparing to protein standards of known molecular masses and pl values.

* MALDI-mass spectrometry /post-source decay was performed by John Leszyk (University of Massachusetts Medical School, Laboratory for Proteomic Mass Spectrometry).

* In this analysis, MOWSE scores of >41 indicate identity or homology at p < 0.05.

FIGURE 6. Co-localization of Rab9, Rab6, and Rab5 with type III-delivered ExoS. A, HeLa cells were infected with *P. aeruginosa* PA103 ΔexoU, exoT::Tc (pUCP-ExoS-KDD) at an m.o.i. of 8:1 (bacterial:HeLa). At 4 h, cells were immunostained with mouse α-HA IgG-zenon-Alexa-568 (1:500). Rab9 was detected with mouse α-Rab6 IgG followed by goat α-rabbit IgG-Alexa-488. Rab6 was detected with rabbit α-Rab6 IgG followed by goat α-mouse IgG-Alexa-488, whereas Rab5 is detected with mouse α-Rab5 IgG followed by goat α-mouse IgG-Alexa-488. Fluorescent images were captured with MetaMorph series 6.0 software. B, HeLa cells were infected with *P. aeruginosa* PA103 ΔexoU, exoT::Tc (pUCP-ExoS-KDD) or pUCP-ExoSΔMLD-KDD at an m.o.i. of 8:1 (bacterial:HeLa) and double-stained for Rab9 and HA as described in A. Images were obtained with a Leica confocal scanning microscope. Color correlation (indicated by ICQ) of boxed regions was calculated using ImageJ intensity correlation analysis (National Institutes of Health). Arrows denote examples of vesicles that co-stained positive for ExoS and Rab9.

engineered at the C terminus of ExoSΔMLD-KDD or ExoS-KDD (Fig. 1). Control experiments showed that fusion of the 3xFLAG epitope to the C terminus of ExoSΔMLD-KDD or ExoS-KDD did not interfere with toxin secretion and translocation (data not shown). To facilitate the isolation of proteins associated with ExoS, the 3xFLAG peptide was added to the IP to release antibody-bound ExoS and ExoS-associated proteins (Fig. 4, A and B). The immunoprecipitation efficiency was ~60% of the total ExoS in the cell lysate (data not shown). The two prominent bands in the IP were ExoSΔMLD-KDD and ExoS-KDD (Fig. 4A). A 28- and 30-kDa protein co-immunoprecipitated with ExoSΔMLD-KDD and ExoS-KDD and was identified by MALDI-TOF as various isoforms of the 14-3-3 proteins (Fig. 4A and Table 1). Differences in the intracellular localization may be responsible for the association of ExoS and ExoSΔMLD with different 14-3-3 isoforms. To enhance the resolution of ExoS-associated proteins, proteins in the co-IP were subjected to two-dimensional gel electrophoresis. IEF-SDS-PAGE analysis detected the two bands representing the 14-3-3 proteins that co-immunoprecipitated with ExoSΔMLD-KDD and ExoS-KDD and four proteins that co-immunoprecipitated specifically with ExoS-KDD (Fig. 5). Mass spectroscopy identified two of the four proteins that co-immunoprecipitated with ExoS-KDD as Tip47 and TPDS2 (Table 2). Subsequent Western blotting identified Rab9, Rab6, and Rab5 as proteins that co-immunoprecipitated with ExoS-KDD (Fig. 5). Rab9 and Rab6 were chosen for the analysis because they are associated with late endosomes where Tip47 is found. The co-immunoprecipitation of Rabs with ExoS, but not with ExoSΔMLD, supports the presence of ExoS on intracellular vesicles and that ExoS is associated with several endosomal compartments. The inability to detect Rab4 in the IP may reflect the limited sensitivity of the antibody, the relative abundance of Rab4, or that ExoS is not associated with recycling endosomes. Tip47/Rab9-associated vesicles have been implicated as a novel, directional vesicle transport system from the late endosome to the Golgi/ER (39, 40). Together, this indicated the intracellular localization of ExoS with early and late endosomes.
Intracellular Localization of ExoS

Co-localization of Type III-delivered ExoS and Rab Proteins on Intracellular Vesicles—Co-localization of ExoS with Rab proteins in cultured cells was next measured to support or refute the observed association of ExoS with Rab proteins. HeLa cells were infected with *P. aeruginosa* expressing ExoS-KDD and subjected to immunofluorescence. ExoS was stained with Alexa-594 and endogenous Rab5, Rab6, and Rab9 were stained with Alexa-488, respectively (Fig. 6). Endogenous Rab5, Rab6, and Rab9 localized in unique vesicular patterns. Rab5 was located in the periphery and the perinuclear region; Rab6 was located in the perinuclear region and was condensed within Golgi stacks (41), and Rab9 was located in the peri-nuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the perinuclear region and was condensed within the TGN region with an extension toward the periphery. ExoS partially co-localized with Rab5 and Rab9 localized in unique vesicular patterns. Rab5 was located in the periphery and the perinuclear region; Rab6 was located in the perinuclear region and was condensed within Golgi stacks (41), and Rab9 was located in the peri-nuclear region with an extension toward the periphery. ExoS partially co-localized with each of the Rab proteins. Partial co-localization of Rab5 and ExoS was observed in the periphery. Rab6 and ExoS co-localized on the crescent where Rab6 was enriched, presumably in the TGN region, and co-localization was not observed in the periphery (Fig. 6A, merge). Confocal microscopy also showed co-localization of Rab9 and ExoS and vesicles that contained either Rab9 or ExoS, which was consistent with the partial co-localization of ExoS on Rab9-associated vesicles (Fig. 6B). Color correlation analysis of the staining pattern indicated partial coincidence of staining between ExoS-KDD and Rab9 but not between ExoSΔMLD-KDD and Rab9.

**FRET Occurs between ExoS and 14-3-3 Proteins but Not Rab Proteins—**Acceptor photobleaching FRET determinations tested the physical association between ExoS and 14-3-3 proteins and Rab proteins. Co-transfected ExoS-CFP and YFP-14-3-3-γ showed FRET efficiency of about 20% (Fig. 7, A and B), whereas a truncated form of ExoS-(1–416), which lacked the 14-3-3 binding domain (42), did not show FRET with YFP-14-3-3-γ (Fig. 7, A and B). The FRET efficiency of ExoSΔMLD-CFP and YFP-14-3-3-γ was intermediate to ExoS and ExoS-(1–416) at ∼12% FRET efficiency (Fig. 7B), which was not statistically different from ExoS-CFP and YFP-14-3-3-γ FRET efficiency. There were no statistically significant differences in the FRET efficiency of ExoS with the three 14-3-3 isoforms (Fig. 7B). In control experiments, in the absence of photobleaching FRET was not observed (data not shown). Sensitized emission FRET, performed with the same constructs, also gave similar FRET efficiencies (data not shown). Using acceptor quenching, FRET was not detected between ExoS-CFP and YFP-Rab5, YFP-Rab6, or YFP-Rab9 above the FRET observed for ExoS-(1–416)-CFP and YFP-14-3-3-γ (data not shown). This indicates that ExoS physically associates with 14-3-3 proteins but not Rab5, Rab6, and Rab9 in HeLa cells.

**Type III-delivered ExoS ADP-ribosylates Golgi/ER-localized Ras—**To determine whether type III-delivered ExoS can ADP-ribosylate a perinuclear localized host protein, HeLa cells were transfected with GFP-H-Ras(C181S,C184S), which is retained in Golgi/ER, in contrast to GFP-RasWT, which is localized at the plasma membrane (Fig. 8A). Partial co-localization of Ras-(C181S,C184S) with ER and Golgi markers was observed. The Golgi marker, TGN46, showed tighter localization than Ras-(C181S,C184S), whereas the ER marker, calnexin, expression extended beyond Ras-(C181S,C184S) (Fig. 8B) (43, 44). Next, HeLa cells were transfected with either RasWT or Ras(C181S,C184S) and intoxicated with *P. aeruginosa* expressing ExoS. Using a tautomycin-based assay (29), where 3²P-NAD was incorporated into host cells, type III-delivered ExoS ADP-ribosylated both RasWT and Ras-(C181S,C184S) with similar efficiency (Fig. 8C). Because RasWT and Ras-(C181S,C184S) have preferred localizations (Fig. 8A) and Ras(C181S,C184S) has been shown previously not to traffic to the plasma membrane (45), one can conclude that...
Ras(C181S,C184S) is localized in the Golgi/ER and is ADP-ribosylated by ExoS. In contrast, ExoSΔMLD did not ADP-ribosylate Ras or Ras(C181S,C184S), consistent with previous observations (11, 46), and was used as a control for specificity. Tetanolysin did not appear to influence ExoS localization because ExoS has similar localization in tetanolysin-treated and nontreated cells (data not shown). This supported the functional trafficking of type III-delivered ExoS to the Golgi/ER.

**Partial Inhibition of the ADP-ribosylation of Golgi/ER-localized Ras by Dominant Negative Rab9**—A gel shift assay was used to measure the influence of dominant negative Rabs and siRNA of the Rabs on the ADP-ribosylation of Ras(C181S,C184S) by type III-delivered ExoS. Rab6A/A’ siRNA knocked down ∼70% of total endogenous Rab6A/A’, whereas Rab9 siRNA knocked down >90% of total endogenous Rab9. However, knockdown of either Rab6 or Rab9 had a minimal effect on the ability of type III-delivered ExoS to ADP-ribosylate Golgi/ER-localized Ras (data not shown). Rab9DN had a partial inhibition (∼20%) on the ability of type III-delivered ExoS to ADP-ribosylate Golgi/ER-localized Ras, whereas Rab6DN did not inhibit (<10%) the ADP-ribosylation of Golgi/ER-localized Ras (data not shown).

**DISCUSSION**

The data in this study support a model where type III-delivered ExoS initially localizes on the plasma membrane and traffics to the Golgi/ER through a vesicle transport system (Fig. 9). Plasma membrane association of ExoS requires the MLD and type III delivery. The ADP-ribosylation of Ras−WT supports the association of ExoS on the cytoplasmic leaflet of the plasma membrane (Fig. 9, step 1). The ability to chase ExoS from the plasma membrane suggests a temporal status for plasma membrane association. However, this does not rule out the possibility that ExoS may enter by two pathways, one locating ExoS directly on plasma membrane and another delivering ExoS directly to the perinuclear region. Current understanding of Tip47-vesicle transport does not resolve how ExoS associates with the plasma membrane for transport to late endosome, but ExoS may travel through Rab5-dependent early endosome vesicles (Fig. 9, step 2). Association with Tip47/Rab9 vesicles provides a directional trafficking of ExoS from the late endosome to the Golgi (Fig. 9, step 4), whereas association with Rab6A/A’/6A provides a mechanism for direct trafficking from early endosome to the TGN (step 3). These pathways may overlap and may compensate each others’ function.

The determination that type III-delivered ExoS is temporally associated with the plasma membrane and associates with Tip47 implicates a role for vesicle trafficking in the movement of ExoS to the Golgi/ER. The biological significance for this trafficking pathway follows the observation that ExoSΔMLD does not associate with Tip47/Rab9 or ADP-ribosylate the Ras
GTases. Tip47 defines a novel class of vesicles that function independent of the TGN38/shiga toxin pathway (39). Although another type III cytoxins localize within host cells, the transient plasma membrane localization of ExoS appears to be novel. 

**Pseudomonas** ExoU (47, 48) and YopO/YpkA (49) localize stably to the plasma membrane, and *Yersinia* YopH (50) localizes to focal adhesion complexes, and *Yersinia* YopM (23, 51) localizes to the nucleus via a vesicle-associated pathway. Toxin localization may correlate to efficient targeting of host proteins within the cell.

Another protein present in the co-immunoprecipitate with ExoS was TPD52. TPD52-like proteins are coiled-coil motif-bearing proteins first identified through expression in human breast carcinoma, which have been proposed to represent signaling intermediates and regulators of vesicle trafficking (52). TPD52 displays granular cytoplasmic distribution in breast carcinoma cells (53) and is localized on the ER of human PLC hepatoma cells (54). TPD52 may function as an adaptor protein that works in parallel or in series with Tip47 to target trafficking of ExoS on intracellular vesicles. TPD52 has limited homology (32% similarity and 25% identity within ~200 amino acids) with the region of Tip47 that interacts with the cytoplasmic tail of the mannose 6-phosphate receptor. This region of homology may represent a common binding domain between the two proteins. Future studies will address the function of TPD52/Tip47 family proteins as protein adaptors for intracellular trafficking of ExoS.

There was a preferred association of ExoS with Rabs, supporting the association of ExoS with intracellular vesicles. Rab9 was initially chosen for the analysis because they are associated with late endosomes where Tip47 is found. The data indicate that ExoS is associated with several endosomal compartments. The inability to detect Rab4 in the IP may reflect the limited sensitivity of the antibody, low abundance of the protein, or that ExoS is not associated with recycling endosomes. Rab proteins play an essential role in protein trafficking pathways, regulating vesicle budding, movement, and fusion. Approximately 70 Rabs are found in the human genome (55), and at least 12 Rabs localize to the endocytic pathway of mammalian cells (56). Rab5 is important for sequestering ligands into clathrin-coated pits and subsequent fusion of vesicles with early endosomes (57). Molecules exit early endosomes along several different pathways. A direct pathway for recycling receptors to the plasma membrane depends on Rab4 (58), which recycles transferrin receptors and membrane lipids back to plasma membrane (59). Molecules transported to the **trans-Golgi** network from endosomes follow multiple routes. One pathway that has been defined by internalized TGN38 and several bacterial toxins involves the transport from early or recycling endosomes to the **trans-Golgi** network (17, 60). This transport is controlled by specific members of protein families as follows: the t-SNARE proteins, syntaxin 6, syntaxin 16, Vti1a; the early endosomal v-SNARE proteins, VAMP4 and VAMP3/cellubrevin; and Rab6A’ (18). Although Rab6A and Rab6A’ play nonoverlapping roles in membrane trafficking (37), knockdown of Rab6A/A provided only a partial inhibition of trafficking. Recently shiga toxin (37), exotoxin A (22), and ricin toxin (61) were observed to utilize this pathway. Another pathway from early or recycling endosomes to the **trans-Golgi** network is used by the cation-independent mannose 6-phosphate receptor and furin, which occurs via Rab9-late endosomes (62–64). Association with multiple endosome vesicles may explain the limited ability of DN-Rab9 and DN-Rab6 and si-Rab9 and si-Rab6 to inhibit the ADP-ribosylation of a Golgi/ER substrate by ExoS. Multiple endocytosis pathways allow cholera toxin access to the Golgi and ER (19) where inhibition of endocytosis by clathrin-, caveolin-, or ARF6-dependent mechanisms does not block cholera toxin movement into the cell or attenuate toxicity (20, 21).

How ExoS moves within the host cell via the MLD is an intriguing question. The MLD is composed of a redundant multiple leucine motif (12) where leucines are clustered on one side of the helical face and charge residues clustered on the opposite helical face of a helical wheel model. The physical basis for the association of ExoS with endosomes is not known, but it is a topic of future investigations.

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