A Leucine-Rich Motif Targets *Pseudomonas aeruginosa* ExoS within Mammalian Cells

Yue Zhang and Joseph T. Barbieri*

Medical College of Wisconsin, Department of Microbiology and Molecular Genetics,
8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

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Type III cytotoxins contribute to the ability of bacterial pathogens to subvert the host innate immune system. ExoS (453 amino acids) is a bifunctional type III cytotoxin produced by *Pseudomonas aeruginosa*. Residues 96 to 232 comprise a Rho GTPase activating protein domain, while residues 233 to 453 comprise a 14-3-3-dependent ADP-ribosyltransferase domain. An N-terminal domain (termed the membrane localization domain [MLD]) targets ExoS to the Golgi-endoplasmic reticulum (Golgi-ER) of mammalian cells. This study identifies an amino acid motif that is responsible for the membrane binding properties of the MLD. Deletion mapping showed that the MLD included a symmetrical leucine-rich motif within residues 51 to 77 of ExoS. The terminal dileucines and internal leucines and an isoleucine within the MLD, but not charged or other hydrophobic residues, targeted a reporter protein to the Golgi-ER region of HeLa cells. Mutations of the leucines within the MLD did not affect type III secretion or translocation into HeLa cells but limited the ability of ExoS to ADP-ribosylate Ras GTPases. Mutations of charged residues within the MLD did not affect type III secretion, delivery into HeLa cells, or the ability of ExoS to ADP-ribosylate Ras GTPases. The organization of the leucines within the MLD of ExoS is different from that of previously described leucine-rich motifs but is present in several other bacterial proteins. This implies a role for intracellular targeting in the efficient targeting of mammalian cells by type III cytotoxins.

Pseudomonas aeruginosa is a ubiquitous gram-negative opportunistic pathogen that infects compromised patients (7), including those with bone marrow transplants, burn wounds, AIDS, and cystic fibrosis and those who have undergone surgical procedures. Four type III cytotoxins contribute to *P. aeruginosa* cytotoxicity, ExoS, ExoT, ExoU, and ExoY (13). ExoS is a bifunctional cytotoxin that has a Rho GTPase-activating protein (RhoGAP) activity (residues 96 to 219) and a 14-3-3-dependent ADP-ribosyltransferase activity (residues 234 to 453) (2). Iglewski and coworkers identified exoenzyme S as an ADP-ribosyltransferase (3) that ADP-ribosylated Ras and several related GTPases (10, 18). ExoS RhoGAP activity was identified for Rho, Rac, and Cdc42 (16, 21).

Differential intracellular localization of several *Yersinia* type III cytotoxins within mammalian cells has been reported. YopM localizes to the nucleus and stimulates the activity of PRK2 and RSK1 kinases (27, 34). YopH localizes to the focal adhesion complexes, which is essential for antiphagocytosis and virulence (17). YopE is a type III cytotoxin and a RhoGAP for Rho, Rac, and Cdc42 (5, 36). Rho and Rac appear to be preferred intracellular targets, since transfection of cells with the dominant active RhoV14 inhibited actin reorganization which leads to cell rounding, while transfection with the dominant active RacV12 inhibited antiphagocytic activity of YopE. YopE localizes within both the cytosol (12) and the perinuclear region of cultured cells (33).

Type III-delivered ExoS localizes to intracellular membranes within cultured cells (29) through the action of the first

107 amino acids of ExoS. When a green fluorescent protein (GFP) fusion reporter system was used to measure targeting to the perinuclear region of cultured cells, residues 51 to 72 of ExoS were observed to constitute a membrane localization domain (MLD), which was necessary and sufficient to localize the reporter within cultured cells (28). Deletion of the MLD did not inhibit type III secretion from *P. aeruginosa*. However, MLD-deleted ExoS (ExoSΔMLD) localized in the cytosol, rather than being membrane associated. The type III secreted ExoSΔMLD stimulated cell rounding and expressed ADP-ribosyltransferase activity but had a limited ability to ADP-ribosylate intracellular Ras GTPases (28). This indicated that membrane localization influenced the intracellular host proteins that were ADP-ribosylated by ExoS. The present study describes a leucine-rich motif within the MLD that targets ExoS to the Golgi-endoplasmic reticulum region of mammalian cells.

MATERIALS AND METHODS

Materials. HeLa cells (CCL-2) were from the American Type Culture Collection. Tissue culture media and sera were from Invitrogen-Gibco. Reagents for molecular and cell biological techniques were from New England Biolabs or Invitrogen and chemicals were from Sigma, unless noted. DNA primers were purchased from Operon Technologies.

Bacterial strains and reagents. *P. aeruginosa* strain PA103 (Δ exoU *exoT*::Tc) with a pUCP derivative to express the indicated form of ExoS was cultured as described previously (2, 21, 26).

Construction of expression vectors. Mammalian expression plasmids encoding the indicated regions of ExoS fused to GFP [ExoS(51–66)/GFP, ExoS(51–72)/GFP, and ExoS(1–107)/GFP] were engineered as previously described (21, 22). Mammalian expression plasmids encoding ExoS(62–77)/GFP, ExoS(51–77)/GFP, and ExoS(51–77)DiL→4N were engineered by amplification, using pExoS-HA as a template. Products were digested with EcoRI and BamHI, and the digested products were subcloned into pEGFP-N1 (Clontech, Palo Alto, CA). EcoRI-BamHI fragments in pEGFP-N1 were sequenced to confirm the

* Corresponding author. Mailing address: Medical College of Wisconsin, Microbiology and Molecular Genetics, 8701 Watertown Plank Rd., Milwaukee, WI 53226. Phone: (414) 456-8412. Fax: (414) 456-6535. E-mail: jtb01@mcw.edu.

open reading frame. Mammalian expression plasmids for ExoS(57–77), ExoS(51–72)2RD→3N, ExoS(51–72)4L→4N, ExoS(51–72)4L→4S, ExoS(51–66)L53Q, ExoS(51–66)L54Q, ExoS(51–66)L57Q, and ExoS(51–66)L61Q were engineered by annealing the complementary oligonucleotides with the indicated restriction sites and subcloned into the pEGFP-N1 vector. ExoS(MLD 4L→4N) (BglII/KpnI) and ExoS(MLD 2RD→3N) (BglII/KpnI) were subcloned into pUCPEXoS(ΔMLD) (Table 1).

Cell culture growth. HeLa cells were cultured in complete medium (minimum essential medium plus 10% fetal calf serum, 0.1% sodium bicarbonate, 50 U penicillin-streptomycin/ml, 1 mM sodium pyruvate, and 0.1 mM minimal essential medium nonessential amino acids). Cells were maintained at 37°C humidified in a 10% CO₂ (vol/vol) incubator.

Transfection and cellular fractionation of HeLa cells. HeLa cells (85-mm dishes) were grown to ~70% confluence and transfected with Lipofectamine Plus using 1 µg of the indicated DNA (16). After 18 to 24 h, transfected cells were washed twice with phosphate-buffered saline (PBS), harvested in 10 ml homogenization buffer (HB1) (250 mM sucrose, 3 mM imidazole, pH 7.4), pelleted at 1,000 rpm for 5 min, washed in 300 µl of HB1, suspended in 300 µl of HB2 (HB1 plus 1% mammalian protease inhibitor mixture set III [Sigma, St. Louis, MO], 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 2,000 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled, and stored at –20°C.

***P. aeruginosa* secretion analysis.** *P. aeruginosa* PA103 (Δ*exoU* *exoT*::Tc) containing the indicated plasmid was cultured for 4 h to reach an optical density of approximately 4 to 5 in TBSD containing nitrilotriacetic acid (NTA) (23). Bacteria were pelleted in a refrigerated microcentrifuge for 30 min, and secreted material (supernatant fluid) was precipitated with ammonium sulfate (65%) overnight at 4°C. The precipitate was collected by centrifugation, suspended in SDS-PAGE sample buffer, and subjected to SDS-PAGE.

Tetanolysin analysis of intracellular proteins that are ADP-ribosylated by type III secreted ExoS. Stock cultures of *P. aeruginosa* were cultured overnight on plates with 400 µg of carbenicillin/ml, to select for plasmid maintenance. The next morning bacteria were suspended in tissue culture media and normalized spectrophotometrically, using 1 A₅₄₀ unit to equal 8 × 10⁶ bacteria/ml. HeLa cells were infected at a multiplicity of infection (MOI) of 8:1 (bacteria to cells) and at the first indication of cell rounding (typically 3 to 3.30 h) were permeabilized with tetanolysin (List Biologicals, Campbell, CA) using a procedure adapted from the work of Ahnert-Hilger et al. (1). *P. aeruginosa*-infected HeLa cells (six-well plate) were washed with PBS at room temperature and incubated in 2 ml total of cold HG1 buffer [20 mM PIPES, 2 mM Na⁺-ATP, 4.8 mM Mg(CH₃COO)₂, 150 mM potassium glutamate, 2 mM EGTA, 1 mM dithiothreitol, and KOH to obtain pH 7.0] with 0.8 µg tetanolysin for 15 min on ice and washed with cold HG1 buffer. Next, 2 ml of HG1 buffer containing 20 nM [³²P]adenylate phosphate-NAD (10 µCi) was added, and cells were incubated for 40 min at 37°C in 5% CO₂. Cells were washed in PBS, lysed with the addition of 100 µl of SDS-PAGE sample buffer, and subjected to SDS-PAGE. In estimating the amount of cell-associated ExoS, bacterial contamination can contribute to experimental error. Using auto-ADP-ribosylation of ExoS as a measurement of internalization, essentially all cell-associated ExoSΔMLD and ExoS(MLD 4L→4N) were determined to be internalized. Previous studies showed that ~20 to 30% of wild-type ExoS is auto-ADP-ribosylated and is completely ADP-ribosylated within a 45-min chase (31). Thus, the determination of the amount of wild-type ExoS and ExoS(MLD 2RD→3N) internalized may be a low estimation, which does not compromise interpretation of the data.

RESULTS

Mapping the membrane localization domain of ExoS. The percentage of ExoS(51–72)/GFP that was associated with membranes was dependent upon the ionic strength of the extraction buffer. In a low-salt extraction buffer (250 mM sucrose, 3 mM imidazole, and 1 mM EDTA), ~50% of ExoS(51–72)/GFP was membrane bound, while in a high-salt extraction buffer (≥150 mM NaCl), ~90% of the fusion protein was associated with the membrane fraction (data not shown).

Characterization of several regions surrounding the MLD showed that extending the membrane localization domain to residues 51 to 77, which added dileucine residues, made membrane association independent of the ionic strength of the extraction buffer, with ~90% of ExoS(51–77)/GFP being membrane associated (Fig. 1). Fractionation in low-ionic-strength buffer showed that deletion at either end of the MLD reduced membrane association (Fig. 1). Previous studies observed that deletion of dileucines and adjacent regions [ExoS(57–72)/GFP] abolished membrane localization (22). This indicated that residues at both proximal and distal points within 51 to 77 of ExoS contribute to membrane association.

Leucine residues of the MLD contribute to intracellular perinuclear localization. Enhanced membrane association with increased ionic strength suggested a hydrophobic interaction between the MLD of ExoS and host membranes. Inspection of the primary amino acid sequence between residues 51 and 77 identified a symmetrical leucine-rich organization that included dileucines at each end of the MLD and several internal leucines or an isoleucine within the internal residues of the MLD (Fig. 2A). The roles of leucines and other residues within the MLD in membrane association were determined by engineering several combinations of mutations within the DNA encoding ExoS(51–72)/GFP. Using ExoS(51–72)/GFP as a platform for mutagenesis provided greater resolution of localization effects than using longer forms of the MLD. Physical and visual analysis showed that a four-leucine substitution [L53N, L54N, L57N, L61N: ExoS(MLD 4L→4N)] disrupted perinuclear localization, while the charge substitution [R56N, R63N, D70N: ExoS(MLD 2RD→3N)] did not affect the intracellular localization of the MLD (Fig. 2B and C). Asparagine was chosen as a point mutation substitution to maintain the overall bulk of the R group, with loss of hydrophobicity. To address potential structural disruption due to the asparagine mutation, leucine-to-serine mutations [L53S, L54S, L57S, L61S: ExoS(MLD 4L→4S)] were also tested and was observed to disrupt perinuclear localization, indicating that a conservative substitution also changed the binding properties of the MLD and that gross changes to protein structure were not responsible for the change in localization (data not shown). Additional analysis showed that individual leucines contributed to membrane localization, since single leucine-to-glutamine mutations disrupted the membrane localization when introduced into ExoS(51–66)/GFP. Valine- or isoleucine-to-glutamine mutations were examined in ExoS(62–77)/GFP: the protein with the Val66→Gln substitution retained ~50% perinuclear localization, while the Iso68→Gln substitution abolished this localization (Fig. 3A and B). Again, a short version of the MLD was used to enhance the sensitivity of the analysis. Since the Leu61Asn mutation disrupted localization of ExoS(51–66)/GFP as determined both visually and by examination of subcellular fractionation, this mutation along with 2diL→4N was characterized in ExoS(51–77), the full-length MLD. The Leu61Asn and 2diL→4N mutations had partial effects on localization, which supports an additive role for leucines in intracellular localization. Together, these data indicated that membrane association was mediated by multiple hydrophobic interactions of leucines and an isoleucine that were situated throughout the MLD.

TABLE 1. ExoS constructs generated in this study^a

Construct	Forward primer	Reverse primer
ExoS(51-77)	5' GATCGATC GAATTC ATG GAG GGG CTG CTC GCT CG 3'	5' GATCGATC GGATCC CG GCC CAA CAG TTT GCC CAG 3'
ExoS(57-77)	5' AATTC ATG CTG GGC GCG GCG CTC GTG CGT CCG TTC GTG GCG ATC ATG GAC TGG CTG GGC AAA CTG TTG GGC CGG 3'	5' GATCC CG GCC CAA CAG TTT GCC CAG CCA GTC CAT GAT CGC CAC GAA CGG ACG CAC GAG CGC CGC GGC CAG CAT G 3'
ExoS(51-72) 2RD→3N	5' AATTC ATG GAG GGG CTG CTC GCT AAC CTG GGC GCG GCG CTC GTG AAC CCG TTC GTG GCG ATC ATG AAC TGG CTG CCG 3'	5' GATCC CG CAG CCA GTT CAT GAT CGC CAC GAA CGG GTT CAC GAG CGC CGC GGC CAG GTT AGC GAG CAG CCC CTC CAT G 3'
ExoS(51-72) 4L→4N	5' AATTC ATG GAG GGG AAC AAC GCT CGC AAC GGC GCG GCG AAC GTG CGT CCG TTC GTG GCG ATC ATG GAC TGG CTG CG G 3'	5' GATCC CG CAG CCA GTT CAT GAT CGC CAC GAA CGG AGC CAC GTT CGC CGC GGC GGC GTT GCG AGC GTT GTC CCC CTC CAT G 3'
ExoS(51-72) 4L→4S	5' GATCT ATG GAG GGG TCC TCC GCT CGC TCC GGC GCG GCG TCC GTG CGT CCG TTC GTG GCG ATC ATG GAC TGG CTG GTA C 3'	5' CAG CCA GTT CAT GAT CGC CAC GAA CGG ACG CAC GGA CGC CGC GGC GGA GCG AGC GGA GGC CCC CTC CAT A 3'
ExoS(51-77) 2DiL→4N	5' GATCGATC GAATTC ATG GAG GGG AAC AAC GCT CGC CTG GCG G 3'	5' GATCGATC GGATCC CG GCC GTT GTT TTT GCC CAG CCA GTC CAT G 3'
ExoS(51-66) L53Q	5' AATTC ATG GAG GGG CAG CTC GCT CGC CTG GGC GCG GCG CTC GTG CGT CCG TTC GTG CCG 3'	5' GATCC CG CAC GAA CGG ACG CAC GAG CGC CGC GGC CAG GCG AGC GAG CTG CCC CTC CAT G 3'
ExoS(51-66) L54Q	5' AATTC ATG GAG GGG CTG CAG GCT CGC CTG GGC GCG GCG CTC GTG CGT CCG TTC GTG CCG 3'	5' GATCC CG CAC GAA CGG ACG CAC GAG CGC CGC GGC CAG GCG AGC CTG CAG CCC CTC CAT G 3'
ExoS(51-66) L57Q	5' AATTC ATG GAG GGG CTG CTC GCT CGC CAG GGC GCG GCG CTC GTG CGT CCG TTC GTG CCG 3'	5' GATCC CG CAC GAA CGG ACG CAC GAG CGC CGC GGC CTG GCG AGC GAG CAG CCC CTC CAT G 3'
ExoS(51-66) L61Q	5' AATTC ATG GAG GGG CTG CTC GCT CGC CTG GGC GCG GCG CAG GTG CGT CCG TTC GTG CCG 3'	5' GATCC CG CAC GAA CGG ACG CAC CTG CGC CGC GGC CAG GCG AGC GAG CAG CCC CTC CAT G 3'
ExoS(62-77)	5' GATCGATC GAATTC ATG GTG CGT CCG TTC GTG GCG 3'	5' GATCGATC GGATCC CCG CCC AAC AGT TTG CCC AGC 3'
ExoS(62-77) V66Q	5' TC ATG GTG CGT CCG TTC GAG GCG ATC ATG GAC TGG CT 3'	5' AG CCA GTC CAT GAT CGC CTC GAA CGG ACG CAC CAT GA 3'
ExoS(62-77) I68Q	5' ATG GTG CGT CCG TTC GTG GCG GAG ATG GAC TGG CTG GGC AAA CTG 3'	5' TTT GCC CAG CCA GTC CAT CGT CGC CAC GAA CGG ACG CAC CAT 3'
ExoS(51-77) L61N	5' TC GCT CGC CTG GGC GCG GCG CAG GTG CGT TTC GTG GCG ATC 3'	5' GAT CGC CAC GAA CGG ACG CAC CTG CGC CGC GGC CAG GCG AGC GA 3'
ExoS(MLD 4L→4N) (BglII/KpnI)	5' GATCT GAG GGG AAC AAC GCT CGC AAC GGC GCG AAC GTG CGT CCG TTC GTG GCG ATC ATG GAC TGG CTG GGTAC 3'	5' C CAG CCA GTC CAT GAT CGC CAC GAA CGG ACG CAC GTT CGC CGC GGC GTT GCG AGC GTT GTT CCC CTC A 3'
ExoS(MLD 2RD→3N) (BglII/KpnI)	5' GATCT GAG GGG CTG CTC GCT AAC CTG GGC GCG CTC GTG AAC CCG TTC GTG GCG ATC ATG AAC TGG CTG GGT AC 3'	5' C CAG CCA GTT CAT GAT CGC CAC GAA CGG GTT CAC GAG CGC CGC GGC CAG GTT AGC GAG CAG CCC CTC A 3'

^a Underlining indicates the sequence encoding the restriction endonuclease site or amino acid substitution.

FIG. 1. Membrane localization of deletion peptides of the MLD of ExoS. (A) Schematic of ExoS/GFP fusion proteins that were expressed by transient transfection in HeLa cells. Residues of ExoS fused to the N terminus of GFP are indicated on the left. (B) Physical location of ExoS/GFP proteins. HeLa cells (85-mm dishes) were transfected with plasmids (1 μ g) encoding the indicated ExoS/GFP fusion protein for ~18 h. Cells were harvested, lysed (Total), and separated into nuclear fraction (Pellet) and postnuclear supernatant (PNS), which was centrifuged to generate a cytosol (Cyto) and a membrane (Mem) fraction. Samples were normalized by volume and subjected to SDS-PAGE. ExoS/GFP fusion proteins were detected by Western blotting using mouse anti-GFP immunoglobulin G followed by goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase. GFP-reactive bands were detected by enhanced chemiluminescence; the exposed X-ray film is shown. Ctrl, 5 ng of a GFP standard. (C) Percent of ExoS/GFP fusion protein localized within the membrane. The ratio of ExoS/GFP fusion protein in the membrane fraction to that in the PNS times 100 was calculated by densitometry and is reported as the mean plus standard error of two to five independent experiments. For ExoS(51-77) and ExoS(62-77), $P = 0.01$, and for ExoS(51-77) and ExoS(57-77), $P = 0.008$ (significantly different).

Leucines within the MLD are responsible for the ability of ExoS to ADP-ribosylate host proteins. The role of leucines within the MLD in the intracellular targeting of type III secreted ExoS was examined by measuring the ability of type III secreted ExoS(MLD 4L→4N) and ExoS(MLD 2RD→3N) to ADP-ribosylate host proteins (Fig. 4A). ExoS(MLD 4L→4N) and ExoS(MLD 2RD→3N) were secreted from *P. aeruginosa* into NTA medium with efficiency similar to that of ExoS and ExoSΔMLD, and type III secreted ExoS(MLD 4L→4N) and ExoS(MLD 2RD→3N) elicited a rounding phenotype, which indicated that the mutated proteins were delivered into HeLa cells (Fig. 4). A time course study showed that ExoSΔMLD rounding of HeLa cells was delayed ~15 to 30 min relative to that elicited by wild-type ExoS. Figure 4C shows an early time point in the infection (3 to 3.5 h); extending the infection by 30 min yielded complete cell rounding by all four ExoS derivatives (data not shown).

A tetanolysin assay (31) was used to assess the ability of type III secreted ExoS to ADP-ribosylate intracellular host proteins. Overall, the host proteins that were ADP-ribosylated by wild-type ExoS and ExoS(MLD 2RD→3N) were similar, while ExoSΔMLD and ExoS(MLD 4L→4N) ADP-ribosylated a different set of host proteins. The ability to ADP-ribosylate

the Ras GTPases was used as a measurement of the ability of ExoS to traffic to cell membranes. ExoS wild type and ExoS(MLD 2RD→3N) ADP-ribosylated the family of Ras GTPases (proteins marked as -RAS in the autoradiogram [Fig. 5B]) with similar efficiencies, while neither ExoSΔMLD nor ExoS(MLD 4L→4N) efficiently ADP-ribosylated the Ras GTPases. Normalizing the amount of the Ras GTPases ADP-ribosylated to the amount of cell-associated ExoS showed that ExoS(MLD 2RD→3N) was ~70% as active in the ADP-ribosylation of the Ras GTPases as wild-type ExoS, while ExoSΔMLD was only 6% as active and ExoS(MLD 4L→4N) only 12% as active as wild-type ExoS in the ADP-ribosylation of the Ras GTPases (Fig. 5C). This implies that the leucine-rich motif within the MLD contributes to membrane localization and efficient ADP-ribosylation of the Ras GTPase family of proteins by type III secreted ExoS. In vitro, ExoS and ExoSΔMLD have similar capacities to ADP-ribosylate Ras (31).

Leucine-rich motifs within the N terminus of type III cytotoxins. BLAST alignment identified several bacterial proteins that possessed MLD-like regions, including ExoT, another type III exotoxin of *P. aeruginosa*; AexT, a type III exotoxin from *Aeromonas salmonicida*; YopE, a type III toxin from

FIG. 2. Leucines, but not charged residues, are critical for the membrane localization of the MLD of ExoS. (A) Schematic of ExoS/GFP fusion proteins that were expressed by transient transfection in HeLa cells; mutated amino acids are indicated in black. (B) Physical location of ExoS/GFP proteins. HeLa cells (85-mm dishes) were transfected with plasmids (1 μ g) encoding the indicated ExoS/GFP fusion protein for ~18 h. Cells were harvested, fixed in 1% paraformaldehyde-PBS, and imaged with a Nikon inverted microscope for either phase contrast or fluorescence. (C) The percentage of the fusion protein present in the membrane was determined as the ratio of the amount in the membrane to the amount in the cytosol plus the membrane times 100. Samples were normalized by the volume and subjected to SDS-PAGE, and the amount of protein present was calculated by densitometry. The results are reported as the means plus standard errors calculated by densitometry and are the averages of two to five independent experiments.

Yersinia; and a putative methyl-accepting chemotaxis protein from *P. aeruginosa* strain PA14 (Table 2).

DISCUSSION

The perinuclear localization of type III secreted ExoS is mediated by a novel leucine-rich motif where multiple leucines and an isoleucine, including two dileucines, contribute to membrane association. Thus, membrane vesicle association may be through AP-like proteins, which bind leucine-rich sequences (32). Leucines are well-known hydrophobic interactions in leucine zipper motifs, leucine-rich regions (LRR), or dileucine

motifs. Leucine zipper motifs are often associated with transcriptional activators, including Jun and Fos (9, 19). Transcriptional activators can form homodimers or heterodimers through the leucine zippers, where basic regions of the dimers bind DNA in a site-specific manner (15, 19). LRRs are generally 20 to 29 residues and contain the conserved 11-residue segment LXXLXLXX(N/C)XL (X can be any amino acid and L is a valine, isoleucine, or phenylalanine) (20). LRRs form a concave face where an adjacent loop is responsible for protein-protein interactions typically with an α -helix on an adjacent protein (25). LRRs are widely distributed among proteins, including GTPase-activating proteins, spliceosomal protein

FIG. 3. Flanking dileucines and internal leucine and isoleucine play an important role in localization. (A) Schematic of ExoS/GFP fusion proteins that were expressed by transient transfection in HeLa cells. Mutated amino acids are indicated in black. (B) The percentage of the fusion protein present in the membrane was determined as the ratio of the amount in the membrane to the amount in the cytosol plus the membrane times 100. Samples were normalized by the volume and subjected to SDS-PAGE, and the amount of protein present was calculated by densitometry. The results are reported as the means plus standard errors calculated by densitometry and are the averages of two to five independent experiments.

U2A', Rab geranylgeranyl-transferase, internalin B, dynein light chain 1, nuclear export protein TAP, and the type III cytotoxin YopM of *Yersinia* (20). Identification of a role for the dileucine motif in mediating protein-protein interactions is relatively recent. Dileucine motifs play roles in endocytosis, targeting proteins to endosomes and lysosomes (11, 24, 35). Although similar to previously described motifs, the MLD within ExoS is unique: since leucines are not divided evenly, as observed for the leucine zipper motif, there are fewer leucines relative to the LRR motifs, and the organization of the ExoS MLD is unique to the dileucine motifs.

Relative to bacterial exotoxins, steps in the internalization and trafficking of the type III cytotoxins are less clear. Current models propose that these toxins are injected directly into the cytosol by the type III secretion system and intracellular targeting is related to toxin function. The *Yersinia* type III cytotoxins are among the most studied with respect to intracellular localization. YopM is a protein scaffold in the nucleus that recruits and stimulates the activity of PRK2 and RSK1 kinases (27). YopM travels to the nucleus via a vesicle-associated pathway that is inhibited by brefeldin A, monensin, and bafilomycin

A1 and dependent upon microtubules, which are inhibited by colchicine and nocodazole (34). YopH localizes to the focal complexes and is essential for antiphagocytosis and virulence (17, 30). The N-terminal domain of YopH was shown to interact with p130Cas in vitro (6). YopH dephosphorylates the focal complex proteins p130Cas and focal adhesion kinase (FAK) (4, 30). p130Cas and FAK are therefore important for the uptake of *Yersinia* (30). YopE is a GTPase activating protein for Rho, Rac, and Cdc42 that localizes to cytoplasmic granules and the perinuclear region (14, 22). The YopE membrane localization domain (residues 54 to 75) can target ExoS to efficiently ADP-ribosylate Ras (22). Previous studies showed that the membrane localization regions of YopE and ExoS MLD are interchangeable, which suggests the importance of membrane localization-involved expression of RhoGAP activity. In addition to the membrane localization function, Cornelis and coworkers found that residues 50 to 77 are inhibitory to YopE release and the binding of chaperone SycE overcomes this inhibitory effect, which suggests that YopE MLD may have multiple functions (8). The molecular basis for the trafficking of these type III toxins remains to be determined.

BLAST alignment identified several bacterial proteins that

FIG. 4. Secretion and cell rounding by type III secreted ExoS. (A) The indicated constructs of ExoS-MLD mutations were engineered and subcloned into pUCP for expression in *P. aeruginosa*. The vertically striped box indicates ExoS with leucine mutations (L53N, L54N, L57N, L61N) (4L→4N), and the horizontally striped box indicates ExoS with charged-residue mutations (R56N, R63N, D70N). (B) *P. aeruginosa* PA103(pUCPExoS), PA103(pUCPExoSΔMLD), PA103(pUCPExoS, MLD 4L→4N), or PA103(pUCPExoS, MLD 2RD→3N) was cultured under conditions to induce type III secretion. The culture medium was concentrated and was subjected to SDS-PAGE followed by Coomassie staining. Protein expression was further confirmed by the Western blotting in which a C-terminal hemagglutinin tag of ExoS was detected by anti-hemagglutinin antibody followed by enhanced chemiluminescence, using a goat anti-mouse immunoglobulin horseradish peroxidase-conjugated immunoglobulin G. (C) HeLa cells were infected with *P. aeruginosa* with the ExoS MLD mutants with an MOI of 8:1. After 3 h, cells were scored for rounding. The percent rounded cells was determined in five representative fields (50 cells/field). Error bars represent standard deviations from five fields.

possessed MLD-like regions, including ExoT, another type III exotoxin of *P. aeruginosa*; AexT, a type III exotoxin from *Aeromonas salmonicida*; YopE, a type III toxin from *Yersinia*; and a putative methyl-accepting chemotaxis protein from *P. aeruginosa* strain PA14 (Table 1). For each protein, while the leucines are conserved, the primary amino acid sequences adjacent to the leucines vary, suggesting specificity for each membrane interaction. The methyl-accepting chemotaxis protein from *P. aeruginosa* strain PA14 shares considerable sequence homology with ExoT but lacks an N-terminal secretion signal and part of the C-terminal ADP-ribosyltransferase domain. While the MLD targets ExoS, ExoT, and YopE, a role for intracellular targeting of SptP, another bacterial RhoGAP,

FIG. 5. Leucine mutations within the MLD disrupt the ADP-ribosylation of Ras by type III secreted ExoS. (A) Schematic of wild-type ExoS (WT), ExoS(ΔMLD), ExoS(MLD 4L→4N), and ExoS(MLD 2RD→3N). (B) HeLa cells were infected for 3.5 h with *P. aeruginosa* Δ*exoU* *exoT*::Tc pUCP (pUCP), pUCPExoS (WT), pUCPExoS (ΔMLD), pUCPExoS(MLD 4L→4N), or pUCPExoS(MLD 2RD→3N) (2RD→3N) at an MOI of 8:1 or left uninfected (Ctrl). Cells were washed, permeabilized with tetanolysin, and incubated with [³²P]NAD before harvesting in SDS-PAGE loading buffer as described in Materials and Methods. Cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes for autoradiography (X-ray film is shown [lower panel]) or immunoblotting with anti-hemagglutinin antibody followed by enhanced chemiluminescence (X-ray film is shown [upper panel]). To the right of the gel is indicated the ADP-ribosylation of members of the family of Ras GTPases (–Ras). (In Western blotting, 4L→4N runs slightly higher than wild type and 2RD→3N. This is due to a complete auto-ADP-ribosylation.) (C) Efficiency of ADP-ribosylation of Ras GTPases was calculated as the amount of ADP-ribosylation of the members of the family of Ras GTPases (Ras) relative to the amount of ExoS in the lysate (hemagglutinin-reactive material). The activity of the wild-type ExoS was normalized to 100%, and values are the averages of three independent experiments.

remains to be defined. SptP does not contain a leucine-rich motif that resembles the MLD of ExoS. The presence of MLDs within bacterial toxins implies a role for membrane trafficking in the translocation and substrate targeting of bacterial type III cytotoxins within mammalian cells.

TABLE 2. Bacterial proteins with homology to the MLD of ExoS

Protein	Sequence ^a	Accession no.
ExoS	E GL LARLGAA L VRPFVA I MD W L G K L L G	A53735
ExoT	E GL LSRLGA A LARPFVA I IEW L G K L L G	JC6026
Mac	E GL LSRLGA A LARPFVA I IEW L G K L L G	ZP_00140447
AexT	Q G LLSRLGA Q LARPFVA I KE W I S N L L G	AF288366
YopE	S S LA S R I IERLSSVA S V I GF I Q R M F S	Y00543

^a Boldface indicates residues conserved among aligned proteins.

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