Intracellular Membrane Localization of Pseudomonas ExoS and Yersinia YopE in Mammalian Cells*

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ExoS (453 amino acids) is a bi-functional type-III cytotoxin of Pseudomonas aeruginosa. Residues 96–233 comprise the Rho GTPase-activating protein (Rho GAP) domain, while residues 234–453 comprise the 14-3-3-dependent ADP-ribosyltransferase domain. Residues 51–72 represent a membrane localization domain (MLD), which targets ExoS to perinuclear vesicles within mammalian cells. YopE (219 amino acids) is a type-III cytotoxin of Yersinia that is also a Rho GAP. Residues 96–219 comprise the YopE Rho GAP domain. While the Rho GAP domains of ExoS and YopE share structural homology, unlike ExoS, the intracellular localization of YopE within mammalian cells has not been resolved and is the subject of this investigation. Deletion mapping showed that the N terminus of YopE was required for intracellular membrane localization of YopE in CHO cells. A fusion protein containing the N-terminal 84 amino acids of YopE localized to a punctate-perinuclear region in mammalian cells and co-localized with a fusion protein containing the MLD of ExoS. Residues 54–75 of YopE (termed YopE-MLD) were necessary and sufficient for intracellular localization in mammalian cells. The YopE-MLD localized ExoS to intracellular membranes and targeted ExoS to ADP-ribosylate small mammalian cell membrane proteins as observed for native type-III delivered ExoS. These data indicate that the YopE MLD functionally complements the ExoS MLD for intracellular targeting in mammalian cells.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes life-threatening infections in cystic fibrosis patients, individuals with burn wounds, and the immune compromised (1). The pathogenicity of P. aeruginosa involves both cell-associated and secreted virulence factors. P. aeruginosa produces four type-III cytotoxins (ExoS, ExoT, ExoU, and ExoY) that are delivered by bacteria directly into the eukaryotic cell (2). ExoS is a bi-functional cytotoxin that encodes a 14-3-3-dependent ADP-ribosyltransferase domain in the C terminus and a Rho GTPase-activating protein (Rho GAP) domain in the N terminus. In cultured cells, the Rho GAP domain stimulates actin reorganization (3) while the ADP-ribosyltransferase domain causes cell death (4). ExoS is a Rho GAP for Rac, and Cdc42 both in vitro and in vivo (5, 6).

Type-III-delivered ExoS localizes to intracellular membranes within eukaryotic cells (7). Fusion of the first 107 amino acids of ExoS to the green fluorescent protein (GFP) directed this reporter protein to the perinuclear region of mammalian cells. Residues 51–72 of ExoS encode a membrane localization domain (MLD), which is both necessary and sufficient for localization within mammalian cells (6). Deletion of the MLD did not inhibit type-III secretion of ExoS from P. aeruginosa or type-III delivery into mammalian cells. Type-III delivered ExoSAMLID was located in the cytosol of mammalian cells and expressed ADP-ribosyltransferase activity, but did not ADP-ribosylate Ras. This indicated that membrane localization of ExoS was required for the efficient ADP-ribosylation of Ras as well as a subset of small mammalian cell membrane bound proteins. Type-III delivered ExoSAMLID was cytotoxic for eukaryotic cells, uncoupling the ADP-ribosylation of Ras with YopE-induced cell death.

Yersinia pestis, the causative agent of plague, shares numerous features with Y. enterocolitica and Y. pseudotuberculosis, including a tropism for lymphoid tissues and resistance to the host innate immune system. In addition to chromosomally encoded virulence factors, these three pathogens possess a common large virulence plasmid, which encodes a type-III secretion apparatus and several type-III cytotoxins (termed Yops (Yersinia outer membrane proteins)) (8). One type-III cytoxin, YopE, has been subjected to considerable investigation, yielding a wealth of knowledge concerning its molecular and cellular properties. Straley and Cibull (9) initially determined that YopE contributed to the pathogenesis of Yersinia, while Wolf-Watz and co-workers (10, 11) observed that YopE mediated a cytotoxic response on HeLa cells and macrophages that required the binding of the Yersinia to the host cell surface. YopE is synthesized in the cytoplasm of the Yersinia and the chaperone protein, YerA, binds and stabilizes YopE in a secretion-competent conformation. Wolf-Watz and co-workers (12) showed that the first 11 amino acids of YopE were required for secretion out of the bacterium and that the N-terminal 49 amino acids were required for translocation across the eukaryotic cell membrane. Recently, Anderson and Schneewind (13) implicated a role for an RNA intermediate in the secretion of YopE by the type-III apparatus, which has been controversial (14, 15). Similar to ExoS, YopE is a GAP for Rho, Rac, and Cdc42 and utilizes an arginine finger to stimulate the GAP activity of the Rho GTPases (16–18). The structure of the GAP domain of YopE is primarily α-helical and similar to ExoS, but does not have obvious structural similarity with the eukaryotic Rho GAPs (19, 20). Unlike the catalytic arginine of the eukaryotic GAPs, which is contained within a loop, the active site arginine of YopE and ExoS are located within an α-helix. A reporter system, which comprises YopE fused to the Bordetella adenylyl cyclase, has been useful to measure YopE transloca-

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1 The abbreviations used are: Rho GAP, Rho GTPase-activating Protein; YopS, Yersinia Outer membrane Proteing; MLD, membrane localization domain; MOI, multiplicity of infection; CHO, Chinese Hamster Ovary-K1; GFP, green fluorescent protein; HA, hemagglutinin; HRP, horseradish peroxidase.

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tion into eukaryotic cells (21). There is limited characterization of the intracellular localization of YopE (22, 23), which prompted this study to define its intracellular location in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Chinese Hamster Ovary-K1 (CHO) cells (CCL-61) and HeLa cells (CCL-2) were from the ATCC. Tissue culture media and sera were from Invitrogen. Reagents for molecular and cell biological techniques were from New England Biolabs or Invitrogen, and chemicals were from Sigma, unless noted. CHO cells were cultured in F-12 complete medium containing 10% newborn calf serum, 7.75% sodium bicarbonate, and 2.5% penicillin/streptomycin. HeLa cells were cultured in Minimal Essential Medium with Earle’s salts containing 10% fetal bovine serum, non-essential amino acids, sodium pyruvate, 7.5% sodium bicarbonate, and 2.5% penicillin/streptomycin. Transfections of DNA into mammalian cells used the LipofectAMINE PLUS transfection system. Experiments described in this study were performed with both HeLa and CHO cells with similar results, except Fig. 7, where only CHO cells were analyzed. This was due to the limited ability of tetanolysin to allow sufficient 32P-NAD to diffuse into HeLa cells to allow detection of ADP-ribosylated host proteins.

Construction of ExoS and YopE Expression Vectors—pYopE-HA: forward and reverse primers, 5'-GAGATCGAATTCATGAAATTATCTACATTT3'- and 5'-GAGATCGGAGCCTCTAGAAGCAGGATGACCGG-3' were designed from the amino acid sequence of YopE (GenBank™ accession number Y00543) and the hemagglutinin (HA) epitope tag (underlined) and used to amplify YopE-HA, using the Versini in pseudotuberculosis CVD-1 plasmid (obtained from Robert Perry, University of Kentucky) as template. The amplified product, which encoded a 5'-EcoRI site and a BamHI site immediately 3' to the stop codon, was subcloned into pEGFP-N1. pYopE1-84/GFP and pYopE1-84/HcRed: forward and reverse primers, 5'-GAGATCGAATTCATGAAATTATCTACATTT3'- and 5'-GAGATCGCCGTTTTATTGCTCCTCCCGA-3' were designed from the amino acid sequence of YopE DNA encoding YopE1-84. The amplified product was amplified by PCR, using pYopE-HA as template. The amplified product encoded a 5'-EcoRI and a 3'-AgeI site was subcloned into pEGFP-N1 and pHCRed-N1 (Clontech) to express the N-terminal 84 amino acids of YopE fused in-frame with GFP or red fluorescent protein (HcRed).

pYopE76–219-HA: forward and reverse primers 5'-GATCGGAGAATTCGGACCCCATGATCACGCTGCTGG-3' and 5'-GAGATCGGAGCCTCTAGAAGCAGGATGACCGG-3' were designed from the amino acid sequence of YopE-HA and used to amplify pYopE76–219-HA using pYopE-HA as template. The amplified product, which encoded a 5'-EcoRI and a BamHI site immediately 3' to the stop codon, was subcloned into pEGFP-N1. pYopE54–75/GFP and pYopE64–75/GFP (Data not shown). The fusion proteins were expressed at levels comparable to GFP. Deletion of either half or the first 5 amino acids of YopE fused in-frame with GFP or red fluorescent protein (HcRed).

Microscopy—Cells were seeded in 24-well plates or 8-well microscope slides and transfected with 200 or 100 ng of effector DNA, respectively, 18–24 h post-transfection. Cells were washed with phosphate-buffered saline, suspended in 4% paraformaldehyde in phosphate-buffered saline. Cells were visualized using a Nikon inverted microscope, using filter sets for either EGFP (HQ: F712, Nikon) or Hc-Red (DM575, Nikon). The final magnification prior to imaging was ×75, using a ×60 objective and a ×1.25 magnifier in the camera lens. Images were photographed with a Spot II CCD camera and cropped in Corel PhotoPaint 11. Cells transfected with identical amounts of DNA encoding EGFP or Hc-Red did not have detectable fluorescence using the reciprocal filter set when images were captured at identical exposures. Cell Cytotoxicity—CHO cells were seeded in 12-well plates and transfected with DNA that was co-transfected with P. aeruginosa PA103 ΔexoU, exoT::Tc containing (pUCP1), (pUCPExoSAMLD), (pUCPExoSoyMPD), or (pUCPExoSAML), at an MOI 8:1 (bacteria:CHO cell). Four hours post-infection, cells were washed with 1 ml of OPTI-MEM (Invitrogen, Life Technologies, Inc.), stained for 5 min with 0.4% Trypan Blue (Invitrogen, Life Technologies, Inc.), and visualized at a Nikon inverted microscope. The percentage of cells that did not exclude Trypan Blue was determined from three representative fields.

RESULTS

The Minimal Membrane Localization Domain of ExoS—Previous studies showed that residues 51–72 were necessary and sufficient for membrane localization of ExoS, which was termed the membrane localization domain (MLD) (6). A series of internal and terminal deletion mutations were engineered within the MLD and expressed as GFP fusion proteins in HeLa cells (Fig. 1) to determine if the MLD represented a minimal localization sequence. Western blot analysis of cell lysates confirmed the expression of the deleted forms of the MLD-GFP fusion protein and showed that each fusion protein had a slightly slower migration rate by SDS-PAGE relative to native GFP (Data not shown). The fusion proteins were expressed at levels comparable to GFP. Deletion of either half or the first 5 N-terminal amino acids eliminated the ability of the MLD to target GFP to the perinuclear region of cells. In contrast, deletion of the C-terminal 5 amino acids of the MLD retained a limited capacity to localize GFP to the perinuclear region, but not as efficiently as the complete MLD (Fig. 1). This indicated that while a dominant component for membrane localization

2 M. J. Riese and J. T. Barbieri, unpublished results.
The expression properties of ExoS-(1–234) and YopE-(76–219) were similar to that previously reported (7). Both full-length ExoS-(1–234) and YopE-(76–219) were primarily membrane associated, while ExoS-(78–234) and YopE-(76–219) were present in the cytosol (Table I). This indicated that membrane localization was not required to stimulate actin reorganization (cell rounding) and that the N-terminal region of YopE, like ExoS, contained a membrane localization domain.

Upon transient expression, the N-terminal 1–84 amino acids of YopE localized GFP (YopE1-84/GFP) to the perinuclear region of mammalian cells (Fig. 3). Subcellular fractionation of cell lysates followed by Western blot analysis with α-HA antibody showed that YopE1-84/GFP localized to cell membranes and migrated as a ~34 kDa protein, similar to the predicted molecular mass of 36 kDa (YopE-(1–84): 8.8 kDa + GFP: 27 kDa, data not shown). Direct fluorescence was used to determine if the N termini of ExoS and YopE co-localized upon expression in mammalian cells, using fusion proteins comprising the N-terminal 84 amino acids of YopE fused to a red fluorescent protein (YopE1–84/HcRed) and the N-terminal 107 amino acids of ExoS fused to GFP (ExoS1-107/GFP). Filter sets did not show overlap of fluorescence between the probes at the level of protein expression used in the analysis. The steady state expression and location of both fusion proteins were similar (Fig. 4). The merged image showed yellow fluorescent vesicles, indicating co-localization of the two probes, and vesicles with primarily green or red fluorescence. This indicated

TABLE I

| Protein         | % Pellet (membrane) | % Supernatant (cytosol) |
|-----------------|---------------------|-------------------------|
| ExoS1–234       | 93 ± 6              | 7 ± 8                   |
| YopE            | 91 ± 4              | 9 ± 3                   |
| YopE76–219      | 16 ± 5              | 84 ± 2                  |
| GFP             | 13 ± 6              | 87 ± 7                  |

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The results are the average of two independent experiments. Each fusion protein was calculated by densitometry of the x-ray film. Localized ExoS-(1–84) into Hela cells. After 16–18 h, cells were imaged by fluorescent (Fluo) or phase contrast (Phase) microscopy. C, fractionation of transfected Hela cells. After transfection, cells were fractionated by 20 passages through a 25-gauge needle. Nuclei and unbroken cells were removed by 3000 rpm for 5 min (clinical centrifuge). The post-nuclear supernatant was centrifuged at 68,000 × g for 30 min. The cytosol and membranes were subjected to SDS-PAGE and EGFP fusion proteins were detected by Western blot with α-GFP antibody followed by ECL. The amount of each fusion protein was calculated by densitometry of the x-ray film. The results are the average of two independent experiments.

the presence of two populations of vesicle, vesicles that co-localized ExoS-(1–107) and YopE-(1–84) and vesicles that were enriched for one of the proteins. Similar co-localization of the N-terminal fusion proteins of ExoS and YopE was also observed in CHO cells (data not shown).

Residues 54–75 Constitute a Membrane Localization Domain (MLD) of YopE—The N-terminal 84 amino acids of YopE include two short hydrophobic stretches, comprising residues 1–15 and 54–75 (Kyte-Doolittle analysis (GCG), data not shown). The N-terminal hydrophobic amino acids are involved in protein secretion through the type-III apparatus, while residues 54–75 were tested for the ability to function as a membrane localization domain. Residues 54–75 of YopE were fused to GFP (YopE54–75/GFP) and analyzed for sufficiency of intracellular localization. The amount of YopE54–75/GFP that was membrane localized following subcellular fractionation was less than YopE1–84/GFP, but similar to ExoS51–72/GFP. This indicated that residues 54–75 constituted a minimal membrane localization domain of (MLD) YopE in HeLa cells (Fig. 3) and CHO cells (data not shown). Subcellular fractionation of cell lysates followed by Western blot analysis using α-GFP antibody showed that YopE54–75/GFP localized to cell membranes and migrated as a ~31 kDa protein, similar to the predicted molecular mass of 29 kDa (YopE-(54–75): 2.4 kDa + GFP: 27 kDa, data not shown). YopE-(54–75) appeared to constitute a minimal MLD, as a fusion protein that contained YopE-(64–75) fused to GFP (YopE64–75/GFP) localized to the cytosol when transiently expressed in the pEGFP mammalian expression system (Fig. 3). Together, these studies define residues 54–75 as the YopE MLD.

Functional Complementation of the YopEMLD and ExoSMLDs—Experiments were designed to test the functional complementation between the MLDs of YopE and ExoS by measuring the ability of the YopEMLD to substitute for the ExoSMLD in targeting type-III delivered ExoS to ADP-ribosylate host proteins. Earlier studies showed that the ExoSMLD allowed type-III delivered ExoS to efficiently ADP-ribosylate Ras and other low molecular weight membrane bound mammalian proteins (6). Thus, functional complementation between the two MLDs could be tested by measuring the ADP-ribosylation profiles of ExoS upon the substitution of the ExoSMLD with the YopEMLD. The ExoSMLD was replaced with the YopEMLD by subcloning DNA encoding the MLD of YopE into pExoSΔMLD, yielding ExoS-YopMLD. As a control, the ExoSMLD was also subcloned into pExoSΔMLD (ExoS-ExoMLD) and assayed for the ability to recover membrane localization and in vivo ADP-ribosylation profiles similar to wild type ExoS. Analysis of the secreted forms of the ExoS derivatives showed that the introduction of either the YopEMLD or ExoSMLD into ExoSΔMLD yielded proteins which migrated more slowly by SDS-PAGE than ExoSΔMLD (Fig. 5). These data indicated that YopE MLD did not disrupt type-III secretion of ExoS by P. aeruginosa. Other control experiments showed that introducing the YopEMLD into ExoS also did not interfere with the ability of type-III delivered ExoS to elicit a cytotoxic effect on CHO cells (Fig. 6). In this experiment, CHO cells were infected with the indicated strain of P. aeruginosa at an MOI of 8:1 (bacteria:CHO cell). Four hours post-infection, the cells were stained with 0.4% Trypan Blue and examined by light microscopy.

Analysis of the tetanolysin permeabilization experiment (Fig. 7) defined the effect of substituting the YopEMLD for the
residues 51–72. In addition, ExoS-YopEMLD ADP-ribosylated that substitution of the YopEMLD did not affect the intrinsic derivates of ExoS were auto-ADP-ribosylated, which indicated the membrane and cytosolic fractions showed that the various associated type-III-delivered ExoS. Radiographic analysis of CHO cells or the ratio of membrane-bound versus cytosol-amount of type-III delivered ExoS that was associated with the ExoSMLD with the YopEMLD did not influence the cytosolic proteins that were ADP-ribosylated. Substitution of then subjected to cell fractionation to identify membrane and residues 51–72. Lower panel, Western blot analysis of secreted proteins. P. aeruginosa PA013 (pUCPExoS, WT), (pUCPExoSΔMLD, ΔMLD), (pUCPExoSExoMLD, Exo), or (pUCPExoSYopMLD, Yop) were cultured under conditions to induce type-III secretion. The culture medium was concentrated (20×), and cellular equivalents were subjected to SDS-PAGE followed by Western blot using, mouse α-HA IgG as the primary antibody and goat α-mouse IgG-HRP as secondary antibody, and developed by ECL (arrow denotes the migration of proteins with reactivity to the HA probe).

Fig. 5. Construction and expression of ExoS-YopE chimeras. Schematic, upper panel, indicated constructs of ExoS and YopE were engineered and subcloned into pUCP for expression in P. aeruginosa or pEGFP-N1 under the control of the CMV promoter for constitutive expression in eukaryotic cells. The horizontal striped box indicates YopE residues 54–75 whereas the vertical striped box indicates ExoS residues 51–72. Lower panel, Western blot analysis of secreted proteins. P. aeruginosa PA013 (pUCPExoS, WT), (pUCPExoSΔMLD, ΔMLD), (pUCPExoSExoMLD, Exo), or (pUCPExoSYopMLD, Yop) were cultured under conditions to induce type-III secretion. The culture medium was concentrated (20×), and cellular equivalents were subjected to SDS-PAGE followed by Western blot using, mouse α-HA IgG as the primary antibody and goat α-mouse IgG-HRP as secondary antibody, and developed by ECL (arrow denotes the migration of proteins with reactivity to the HA probe).

Fig. 6. Type-III-delivered ExoSYopMLD is cytotoxic to CHO cells. CHO cells were infected with P. aeruginosa ΔexoU, exoT::Tc (pUCP), (pUCPExoS), (pUCPExoSΔMLD), (pUCPExoSExoMLD), or (pUCPExoSYopMLD) at an MOI of 8:1 (bacteria::cultured cells). Four hours post-infection, the cells were stained with 0.4% Trypan Blue and examined by light microscopy. The percentage of cells that were stained with Trypan Blue was determined in three representative fields. Error bars represent S.D. from duplicate experiments.

ExoS-YopMLD. In this experiment, CHO cells were infected with the indicated strain of P. aeruginosa at an MOI of 8:1 (bacteria: CHO cell). Upon first detection of cell intoxication (cell rounding at −3.5–4 h post-infection), cells were permeabilized with tetanolysin and incubated with [32P]NAD. The cell lysate was then subjected to cell fractionation to identify membrane and cytosolic proteins that were ADP-ribosylated. Substitution of the ExoSMLD with the YopEMLD did not influence the amount of type-III delivered ExoS that was associated with CHO cells or the ratio of membrane-bound versus cytosol-associated type-III-delivered ExoS. Radiographic analysis of the membrane and cytosolic fractions showed that the various derivatives of ExoS were auto-ADP-ribosylated, which indicated that substitution of the YopEMLD did not affect the intrinsic ADP-ribosyltransferase activity of ExoS and that the toxins were internalized. In addition, ExoS-YopEMLD ADP-ribosylated the same subset of small membrane bound proteins as ExoS and at an efficiency that was comparable to ExoS. The efficiency of ADP-ribosylation was determined by measuring the amount of radiolabel in a subset of small membrane-bound proteins divided by the amount of ExoS in the membrane fraction determined from the autoradiogram (bracketed area) divided by the amount of ExoS in the membrane fraction determined from the autoradiogram and the Western blot, respectively, and are reported in arbitrary units (<sup>1</sup>). This experiment was performed four independent times with similar results; results from two independent experiments are shown.

![Sequence alignment of MLD of ExoS, ExoT, and YopE.](image)

Fig. 7. Functional complementation of the YopE and ExoS MLDs. CHO cells were infected for 3.5 h with P. aeruginosa ΔexoU, exoT::Tc (pUCPExoS), (pUCPExoSΔMLD), (pUCPExoSExoMLD), or (pUCPExoSYopMLD) at an MOI of 8:1. Cells were washed, permeabilized with tetanolysin, and incubated with [32P]NAD before harvesting in SDS-PAGE loading buffer as described under “Experimental Procedures.” Cell lysates were fractionated into membranes and cytosol by centrifugation at 68,000 × g in TLA 100.3 Beckman rotor for 30 min. They were then subjected to SDS-PAGE, and proteins were transferred to a polyvinylidene difluoride membrane for autoradiography (x-ray film shown, upper panel) or Western blot with α-HA antibody followed by ECL, using goat α-mouse-HRP-conjugated IgG (x-ray film shown, middle panel). Asterisks are located above auto-ADP-ribosylated forms of ExoS. Bottom panel, radiolabel/ExoS represents the ADP-ribosylation of small molecular weight proteins in the membrane fraction determined from the autoradiogram (bracketed area) divided by the amount of ExoS in the membrane fraction determined from the ECL signal (HA reactive material). Values were determined by densitometry of scans from the autoradiogram and the Western blot, respectively, and are reported in arbitrary units (<sup>1</sup>). This experiment was performed four independent times with similar results; results from two independent experiments are shown.

![Sequence alignment of MLD of ExoS, ExoT, and YopE.](image)

Fig. 8. Sequence alignment of MLD of ExoS, ExoT, and YopE. Residues 51–72 of ExoS and ExoT, and residues 54–75 YopE from Y. pseudotuberculosis (ps), Y. pestis (pe), and Y. enterocolitica (en) are aligned based on sequence homology. The secondary structure of YopE (54–75), resolved by Ref. 30 is shown above the sequences.

DISCUSSION

Early models did not address intracellular sites of delivery for the type-III cytotoxins. However, as detection systems became more sensitive, these toxins were found associated with intracellular membranes or organelles of mammalian and
lower eukaryotic cells. YopM of *Yersinia pestis* was found to be localized to both the cytosol and nucleus by a brefeldin A sensitive mechanism dependent on microtubules (25). YpKA of *Y. pseudotuberculosis*, a Ser/Thr protein kinase, was observed to be targeted to the plasma membrane (26). The intracellular localization of YopE, a Rho GAP of *Y. pestis*, has been observed in the cytosol (22) and associated with intracellular membranes (23). Recent studies by Haraga and Miller (27) showed that SspH1, a member of the bacterial Lpx repeat protein family, localized to the mammalian nucleus and inhibited NF-kB-dependent gene expression. In addition, the utility of using yeast to characterize the function of type-III effector proteins has recently been reported (28). *P. aeruginosa* ExoS is targeted to a perinuclear intracellular membrane compartment within mammalian cells (7) through the action of residues 51–72 (MLD) (6). Deletion of the MLD did not interfere with intracellular targeting of the Rho GAP domain, but did inhibit the ability of ExoS to ADP-ribosylate Ras and other small molecular weight membrane-bound proteins. In this study, residues 54–75 of YopE have been identified as the YopE MLD, which co-localized with the ExoS MLD and can functionally complement for the ExoS MLD in mammalian cells. Although there is little primary amino acid homology between the MLDs of ExoS and YopE (Fig. 8), the regions share common hydrophobic moments, which may represent the intracellular targeting determinant for each respective cytotoxin. Fractionation experiments, defined both 51–72 of ExoS and 54–75 of YopE as minimal MLD, but with less membrane-association relative to the full-length form of the respective toxin. This may indicate that the MLD within the fusion protein may not have the same orientation as the MLD in the native protein or that other regions of the toxin may also contribute to localization. This is currently being addressed.

In the absence of the chaperone binding domain (residues 15–50), YopE is not translocated into cells by wild type *Y. enterocolitica*, but is delivered by the type-III apparatus into mammalian cells by a Yop effector multimeric bacterium (ΔHOPEM) (29). Boyd et al. (29) proposed that the binding of SycE to YopE introduces a hierarchy of effector translocation by competition with other Yops. Although YopE residues 15–50 are sufficient for chaperone binding, crystallographic studies of SycE complexed to YopE showed that the chaperone spans residues 15–77 (30). Crystallographic studies of YopE bound to its chaperone, SycE, indicate that the secondary structure in this region is a β-strand followed by an α-helix (30). The β-strand of YopE 54–75 interacts with SycE through hydrophobic interactions with a hydrophobic patch of an amphipathic α-helix of SycE. In the absence of residues 50–77 of YopE, SycE is not required to promote translocation of YopE (29) suggesting that this domain establishes the need for chaperone binding and that this domain maintains YopE in a secretion competent state prior to type-III delivery within *P. aeruginosa*. Previous studies identified residues 50–77 of YopE as a secretion inhibition domain that prevented translocation of YopE in the absence of its cognate chaperone (29). This inhibition was overcome by the binding of SycE, although the mechanism for this release has not been elucidated. In the absence of the secretion inhibition domain, SycE still binds to YopE and assists in YopE translocation, suggesting that residues 50–77 are not essential for chaperone binding or translocation (29). Similar to the ExoSMLD, the YopEMLD constitutes a hydrophobic region that may be responsible for aggregative properties of YopE. One explanation for the secretion inhibition behavior of residues 50–77 is that in the absence of SycE, the MLD is exposed and promotes intracellular aggregation of the toxin, which hampers YopE translocation by the type-III apparatus. The binding of SycE might prevent aggregation and allow for YopE to be secreted from the bacteria in an extended monomer conformation.

YopE and ExoS are biochemically indistinguishable with respect to their *in vitro* Rho GAP activity. Previous studies indicated that RhoA, Rac1, and Cdc42 were in *vivo* targets of the Rho GAP domain of ExoS (6). In contrast, dominant active (DA)-Rac reversed the reorganization of the actin cytoskeleton elicited by YopE and DA-Rho reformed the stress fibers in YopE-treated cells, which suggested that RhoA and Rac1 were in *vivo* targets of YopE. Studies by Andor et al. (18) and Black and Bliska (17) also indicated that Rac and Rho were preferred targets of YopE based upon the ability of YopE to inhibit Rho GTPase signaling by ligand stimulation and the inhibition of cell rounding, respectively. These results have been corroborated in our laboratory. Several possibilities may account for differences between *in vitro* and *in vivo* activities of ExoS and YopE. ExoS and YopE could localize to distinct intracellular fractions providing unique Rho GTPases as targets or the catalytic domains of ExoS and YopE could dictate *in vivo* substrate specificity. We favor the latter model because ExoS and YopE localize to similar perinuclear regions of eukaryotic cells, suggesting the disparity between *in vitro* and *in vivo* activity is not due to a difference in intracellular targeting by the MLD. This was supported by the observed functional complementation of the YopE and ExoS MLDs.

Bacterially-encoded Rho GAPs perform similar functions despite the lack of primary amino acid sequence similarity, which was explained by three-dimensional studies that showed these Rho GAP domains were similar and that catalytic residues are conserved (19, 20). The MLDs of ExoS and YopE also demonstrate low amino acid homology, but conserved functional secondary structure; a theme that is becoming more prominent as the molecular architecture and *in vivo* activities of bacterial toxins are resolved.

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