Title: Intracellular membrane localization of *Pseudomonas* ExoS and *Yersinia* YopE in mammalian cells.

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Running Title: Membrane localization of ExoS & YopE in mammalian cells.
Abstract

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 peri-nuclear vesicles within mammalian cells. YopE (219 amino acids) is a type-III cytotoxin of *Yersinia* that is also a Rho GAP. Residues 96 to 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 punctuate-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 molecular weight 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.
**Introduction**

*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 Rho, 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 peri-nuclear 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 ExoSΔMLD 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 molecular weight...
membrane bound proteins. Type-III delivered ExoS\textsuperscript{AML}D was cytotoxic for eukaryotic cells, uncoupling the ADP-ribosylation of Ras with ExoS-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 cytotoxin, YopE, has been subjected to considerable investigation, yielding a wealth of knowledge concerning its molecular and cellular properties. Straley and coworkers initially determined that YopE contributed to the pathogenesis of *Yersinia* (9), while Wolf-Watz and coworkers observed that YopE mediated a cytotoxic response on HeLa cells and macrophages that required the binding of the *Yersinia* to the host cell surface (10,11). 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 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 (12). Recently, Schneewind and coworkers implicated a role for an RNA intermediate in the secretion of YopE by the type-III apparatus (13), 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 $\alpha$-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 $\alpha$-helix. A reporter system which comprises
YopE fused to the Bordetella adenylate cyclase has been useful to measure YopE
translocation 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.
Materials and Methods

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 GIBCO (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, 7.5% 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 transfer system. Experiments described in this study were performed with both HeLa and CHO cells with similar results, except figure 7 where only CHO cells were analyzed. This was due to the limited ability of tetanolysin to allow sufficient $^{32}$P-NAD to diffuse into HeLa cells to allow detection of ADP-ribosylated host proteins (Riese and Barbieri, unpublished results).

Construction of ExoS and YopE expression vectors. pYopE-HA: Forward and reverse primers 5’- GAGATCGAATTTCATGAAAATATCATCATTT-3’ and 5’-GAGATCGGATCCTCACAAGCTGGCGTAGTCGTCGGGCTCGTCGTAGGGGTAGTCAATGACAGTAA-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 Yersinia pseudotuberculosis PCD-1 plasmid (obtained from Robert Perry, University of Kentucky) as template. The amplified product, which encoded a 5’ EcoR1 and a BamH1 site immediately 3’ to the
stop codon, was subcloned into pEGFP-N1. **pYopE1-84/GFP and pYopE1-84/HcRed:**

Forward and reverse primers 5'-GAGATCGAATTCTGATGAAAATATCATCATT-3' and 5'-GAGATCACCAGTTTTATGGCTCCCCTCCGA-3' were designed from the amino acid sequence of YopE. DNA encoding YopE1-84 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 (Clonetech) to express the N-terminal 84 amino acids of YopE fused in frame with green fluorescent protein (GFP) or red fluorescent protein (HcRed). **pYopE76-219-HA:** Forward and reverse primers 5'-

GATCGAGAATTCGCCACCATGCATCGCATGTTCTCGGAG-3' and 5'-

GAGATCGGATCCTCACAAGCTGGCGTAGTC-3' were designed from the amino acid sequence of YopE-HA and used to amplify YopE76-219-HA using pYopE-HA as template. The amplified product, which encoded a 5’ EcoRI and a BamHI site immediately 3’ of the stop codon, was subcloned into pEGFP-N1. **YopE54-75/GFP and YopE64-75:** The fusion constructs 54-75/GFP and 64-75/GFP were engineered by subcloning double stranded DNA encoding an ATG codon followed by DNA encoding residues 54-75 or 64-75 of YopE (GenBank accession number Y00543), respectively, in frame into the 5’EcoR1 and 3’ BamHI sites of pEGFP-N1. **ExoSYopMLD:**

ExoSYopMLD was constructed by subcloning double stranded DNA encoding residues 54-75 of YopE (GenBank accession number Y00543) into unique KpnI, BglIII restriction sites in pUCP/ExoSΔMLD. **ExoSExoMLD:** ExoSExoMLD was constructed by subcloning DNA encoding residues 51-72 of ExoS into unique KpnI, BglIII restriction sites in pUCP/ExoSΔMLD. PCR derived constructs were sequenced to assure the fidelity of the amplified DNA fragment. **ExoSMLD/GFP:** DNA encoding deletion MLD/GFP
fusion proteins were engineered by subcloning DNA encoding an ATG codon followed
by the indicated sequence of the MLD fragment into the EcoRI – BamHI restriction sites
of pEGFP-N1 in-frame with DNA encoding GFP. DNA was sequenced to assure the
correct DNA sequence in the subclone.

Mammalian cell fractionation. Cells were fractionated as earlier described (5). Briefly,
cells were seeded in 85 mm plates and transiently transfected at 60-80% density with the
indicated amount of effector DNA and 0.5 µg of reporter DNA (pEGFP-N1). 18-24
hours after transfection, cells were harvested for 5 min at 1000 rpm in a clinical
centrifuge (4°C). Cells were washed in 10 ml of PBS, pelleted, washed in 10 ml of ice-
cold buffer HB2 and suspended in buffer HB1. HB1 contains 250mM sucrose, 0.5M
NaCl, 3mM imidazole (pH 7.4); HB2 is HB1 plus Protease Inhibitor Cocktail Set III
(Calbiochem) and 0.5mM EDTA. Cells were broken by passage 20 times through a 25 g
needle. An aliquot of the whole-cell lysate was boiled with sample buffer and frozen at -
80°C, and the remainder was centrifuged for 10 min at 2,000 rpm in a microfuge (4°C).
The pellet (nuclei and unbroken cells) contained about 10 % of the total HA reactive
material and was not further analyzed. Post-nuclear supernatants were fractionated by
centrifugation (68,000 x g in TLA 100.3 Beckman rotor for 30 min.) into soluble
(cytosol) and pellet (membrane) fractions. The pellet was suspended in an equal volume
of HB2 + 1% Triton, suspended in a volume equivalent of SDS-PAGE sample buffer,
boiled, and stored at -80°C.
**CHO cell permeabilization and detection of ADP-ribosyltransferase activity.** CHO cells were permeabilized with tetanolysin (List Biologicals, CA) using a procedure adapted from (24). At the first indication of rounding, *P. aeruginosa* infected CHO cells (85 mm dishes) were washed with 10 ml of PBS (RT) and incubated in 6 ml ice-cold HG1 buffer (20 mM PIPES, 2 mM Na\(^+\)ATP, 4.8 mM Mg(CH\(_3\)COO)\(_2\), 150 mM potassium glutamate, 2 mM EGTA, 1 mM DTT, and KOH to obtain pH 7.0). Cells were incubated for 10 min (4°C) with 2.4 µg of tetanolysin and washed with ice-cold HG1 buffer. Next, 6 ml of HG1 buffer containing 20 nM \(^{32}\)P-(adenylate phosphate)-NAD (6 µCi) were added and cells were incubated for 25 min at 37°C in 5% CO\(_2\). Cells were harvested in 0.5 ml of HB2 buffer and broken by passage 20 times through a 25 g needle. Unbroken cells and nuclei were removed by centrifugation in clinical centrifuge at 2500 RPM for 5 min. Post-nuclear supernatants were fractionated as described above in the Mammalian cell fractionation section.

**Western blot analysis of mammalian cell lysates.** Fractionated or whole-cell lysates were subjected to SDS-PAGE (13.5% separating gels) and probed with monoclonal antibodies against the HA epitope or GFP (Covance) and then with the secondary antibody goat α-mouse-HRP-IgG (Pierce). The blot was developed with ECL (Pierce, Super Signal) and imaged with X-ray film, which was quantified by densitometry.

**Microscopy.** Cells were seeded in 24 well plates or 8 well microscope slides and transfected with 200 ng or 100 ng of effector DNA, respectively. Eighteen to 24 hours post-transfection, cells were washed with PBS, suspended in 4% paraformaldehyde in
PBS. 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 75X, using a 60 X objective and a 1.25 X magnifier in the camera lens. Images were photographed with a Spot II CCD camera and cropped in Corel Photo-Paint 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 infected with *P. aeruginosa* PA103 ΔexoU,exoT::Tc containing (pUCP), (pUCPExoSΔMLD), (pUCPExoSYopMLD), (pUCPExoSExoMLD) or (pUCPExoS), at an MOI 8:1 (bacteria:CHO cell). Four hours post-infection, cells were washed with 1 ml of OPTI-MEM (Gibco), stained for 5 min with 0.4% Trypan Blue (Gibco), and visualized at 20X with 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 (Figure 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 peri-nuclear 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 peri-nuclear region, but not as efficiently as the complete MLD (Figure 1). This indicated that while a dominant component for membrane localization existed within the N terminus of the MLD, the complete MLD was necessary for optimal intracellular targeting of a reported protein.

The N terminus of YopE comprises a membrane localization domain in mammalian cells. To address the steady state expression of YopE in mammalian cells and determine if YopE had the propensity to localize to intracellular vesicles, YopE and YopE(76-219) were subcloned with C-terminal HA epitopes into a mammalian expression vector and expressed in HeLa or CHO cells. Transfection of the equivalent amounts of DNA
yielded steady state expressions of YopE and YopE(76-219) that were about 50-75% of ExoS and ExoS(78-234) (Figure 2, insert). YopE was expressed as a doublet with an apparent molecular mass of ~28 kDa, while YopE(76-219) migrated as a single protein with an apparent molecular mass of ~20 kDa. The expression properties of ExoS(1-234) and ExoS(78-234) were similar to that previously reported (7). Both full length and N-terminal forms of ExoS and YopE stimulated the rounding of both CHO cells (Figure 2) and HeLa cells (data not shown). Subcellular fractionation showed that both ExoS(1-234) and YopE were primarily membrane associated, while ExoS(78-234) and YopE(76-219) were present in the cytosol (Table 1). 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 peri-nuclear region of mammalian cells (Figure 3). Subcellular fractionation of cell lysates followed by Western blot analysis using α-GFP 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 (Figure 4). The merged
time showed yellow fluorescent vesicles, indicating co-localization of the two probes,
and vesicles with primarily green or red fluorescence. This indicated the presence of two
populations of vesicle, vesicles that co-localized ExoS1-107 and YopE1-84 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 (Figure 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 (YopE(64-75)/GFP) localized to the cytosol when transiently expressed in
the pEGFP mammalian expression system (Figure 3). Together, these studies define residues 54-75 as the YopE MLD.

Functional complementation of the YopE MLD and ExoS MLDs. Experiments were designed to test the functional complementation between the MLDs of YopE and ExoS by measuring the ability of the YopE MLD to substitute for the ExoS MLD in targeting type-III delivered ExoS to ADP-ribosylate host proteins. Earlier studies showed that the ExoS MLD 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 ExoS MLD with the YopE MLD. The ExoS MLD was replaced with the YopE MLD by subcloning DNA encoding the MLD of YopE into pExoSΔMLD, yielding ExoS-YopMLD. As a control, the ExoS MLD 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 had showed that the introduction of either the YopE MLD or ExoS MLD into ExoSΔMLD yielded proteins which migrated more slowly by SDS-PAGE than ExoSΔMLD (Figure 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 YopE MLD into ExoS also did not interfere with the ability of type-III delivered ExoS to elicit a cytotoxic effect on CHO cells (Figure 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 (Figure 7) defined the effect of substituting the YopEMLD for the ExoSMLD. 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 and 1/2 hr to 4 hr post infection), cells were permeabilized with tetanolysin and incubated with $^{32}$P-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 derivates 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 that was determined to be membrane bound by measurement of HA reactivity as shown in Figure 7 (two independent determinations are shown). This calculation allowed the normalization of the amount of ExoS that was cell associated, which varied among infection experiments. This indicated that the YopEMLD
functionally complemented the ExoS MLD. Together, these studies showed that the
YopE MLD expressed both physical co-localization and functional complementation in
the targeting of ExoS in mammalian cells, despite the limited primary amino acid
homology between the MLDs (Figure 8).
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 Miller and coworkers (27) showed that SspH1, a member of the bacterial LPX repeat protein family, localized to the mammalian nucleus and inhibited NF-kappa B-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 (Figure 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 associated 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 multi-mutant bacterium (ΔHOPEM) (29). Boyd *et al.* 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 monomeric 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 *et al.* (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 (Krall and Barbieri, unpublished data). 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 that latter model since 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 observation that 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 the three dimensional studies that showed these Rho GAP domains were similar and that catalytic residues are conserved (19,20). The MLD's 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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Literature cited.


Figure Legends.

Figure 1. Intracellular localization of ExoS-MLD/GFP fusion proteins in HeLa cells. (A) The schematic of ExoS-MLD/GFP fusion proteins that were subcloned into PEGFPN-1 to produce N-terminal fusions with GFP. Black bar indicates a perinuclear localization phenotype, while an open bar indicates a cytosolic (non-localized) phenotype upon transfection into mammalian cells. (B) Plasmids encoding (200 ng) of the indicated ExoS-MLD/GFP fusion protein were transfected into Hela cells in 12 well dishes. After 16-18 hr, cells were fixed in 1% paraformaldehyde-PBS and imaged by phase contrast (phase) or for fluorescence (Fluo) or (C) cells were fractionated into membrane and cytosolic fractions. After cells were lysed and the nuclear fraction removed, the post nuclear supernatant was centrifuged at 68,000 x g for 30 min. Fusion proteins were detected by Western blot with α-GFP antibody followed by ECL, using a goat α-mouse-HRP conjugated IgG. The % of fusion protein in each fraction was calculated by densitometry and reflects the average of 2-4 independent experiments.

Figure 2. YopE and YopE(76-219) induce mammalian cell rounding. (Upper panel) CHO cells were transfected with 50ng of pEGFP-N1 and the indicated amount of pExoS1-234-HA (closed circle), pExoS78-234-HA (open circle), pYopE-HA (closed triangle), or pYopE76-219-HA (open triangle). 16 hours post-transfection the cells were scored for rounding. The % of rounded cells was determined in three representative fields (50 cells/field). Error bars represent standard deviations from duplicate experiments. (Lower panel) Lyates from cells transfected with 50ng of pEGFP-N1 and 300 ng of pExoS1-234-HA, pExoS78-234-HA, pYopE-HA, or pYopE76-219-HA were subjected to
SDS-PAGE, transferred to membranes, which were probed with $\alpha$-HA antibody and followed by ECL. An image of the x-ray film is shown.

**Figure 3. Residues 54-75 of YopE target GFP to intracellular vesicles in mammalian cells.** A) The schematic of YopE-MLD/GFP fusion proteins that were subcloned into pEGFP N-1 vector to produce N-terminal fusions with GFP. Black bar indicates a perinuclear localization phenotype, while an open bar indicates a cytosolic (non-localized) phenotype upon transfection within HeLa or CHO cells. B) Plasmids (200 ng) that encode YopE-MLD-GFP fusion proteins were transfected into Hela cells. After 16-18 hours, 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 5min (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 $\alpha$-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.

**Figure 4. Intracellular membrane localization of YopE and ExoS in mammalian cells.** HeLa cells were co-transfected with 100 ng of pExoS1-107/GFP (upper left) and pYopE1-84/HcRed (upper right). Twenty four hours post-transfection, cells were examined by fluorescent microscopy, using a HQ:F 712 filter (Nikon) to detect EGFP
and a DM 575 filter (Nikon) to detect Hc-Red. The two images were merged (lower left). A phase contrast image of the cells is shown (lower right).

**Figure 5. Construction and expression of ExoS-YopE chimeras.** (Schematic, upper panel) The 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. (Western blot analysis of secreted proteins, lower panel). *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 (20X) 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).

**Figure 6. Type-III delivered ExoSYopMLD is cytotoxic to CHO cells.** CHO cells were infected with *P. aeruginosa ΔexoU, exoT::Tc* (pUCP), (pUCPExoS), (pUCPExoSΔMLD), (pUCPExoSYopMLD), or (pUCPExoSExoMLD 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 standard deviations from duplicate experiments.
Figure 7. **Functional complementation of the YopE and ExoS MLDs.** CHO cells were infected for 3.5 hours with *P. aeruginosa* ∆*exoU, exoT::Tc* (pUCPExoS), (pUCPExoSΔMLD), (pUCPExoSYopMLD), or (pUCPExoSExoMLD) 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 in the methods section. Cell lysates were fractionated into (membranes) and (cytosol) by centrifugation at 68,000 x g in TLA 100.3 Beckman rotor for 30 min, which were then subjected to SDS-PAGE and proteins were transferred to a PVDF 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). * 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). Value were determined by densitometry of scans from the autoradiogram and the Western blot, respectively, and are reported in arbitrary units (a). This experiment was performed four independent times with similar results; results from two independent experiments are shown.

Figure 8. **Sequence Alignment of MLD of ExoS, ExoT, and YopE.** Residues 51-72 of ExoS and ExoT, and residues of 54-75 of YopE from *Y. pseudotuberculosis* (ps), *Y. pestis* (pe), and *Y. enterocolitica* (en) are aligned based on sequence homology. The secondary structure of YopE54-75, resolved by (30) is shown above the sequences.
Table 1. Fractionation of ExoS and YopE in CHO cells<sup>a</sup>.

<table>
<thead>
<tr>
<th>Protein</th>
<th>% pellet (membrane)</th>
<th>% supernatant (cytosol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExoS1-234</td>
<td>93 ± 6</td>
<td>7 ± 8</td>
</tr>
<tr>
<td>YopE</td>
<td>91 ± 4</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>YopE76-219</td>
<td>16 ± 5</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>GFP</td>
<td>13 ± 6</td>
<td>87 ± 7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Post nuclear supernatants of CHO cells transfected with DNA encoding the indicated protein were fractionated by ultracentrifugation. Volume equivalents of the high speed pellet (membranes, cytoskeleton) and high-speed supernatant (cytosol) were resolved on 13.5% gels, transferred to nitrocellulose and subjected to Western blot analysis with an antibody against the carboxyl-terminal HA epitope or GFP. Spot densitometry was used to calculate the amount of each protein in each fraction. Values are the average of two independent experiments.
Figure 1 Krall, et al.
Figure 2. Krall, et al.
Figure 3. Krall, et al
Figure 4. Krall, et al.
Figure 5. Krall, et al
Figure 6. Krall, et al.
<table>
<thead>
<tr>
<th>Exp</th>
<th>radiolabel/ExoS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT  ΔMLD  Yop  ExoS</td>
</tr>
<tr>
<td>1</td>
<td>3.8  0.9    4.3   3.8</td>
</tr>
<tr>
<td>2</td>
<td>1.9  0.2    1.7   2.2</td>
</tr>
</tbody>
</table>

Figure 7. Krall, et al
Figure 8. Krall, et al.
Intracellular membrane localization of Pseudomonas ExoS and Yersinia YopE in mammalian cells
Rebecca Krall, Yue Zhang and Joseph T. Barbieri

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